Design and Evaluation of Floating Gastroretentive Multi Unit Particulate Systems for Levodopa and Carbidopa

THESIS

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by

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CERTIFICATE

This is to certify that the thesis entitled **"Design and Evaluation of Floating Gastroretentive Multi Unit Particulate Systems for Levodopa and Carbidopa"** submitted by **Raut Prashant Popatrao**, ID No. 2009PHXF006P for award of Ph.D. Degree of the Institute, embodies original work done by him under my supervision.

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Abbreviations and Symbols	
#	Mesh
%	Percentage
%, w/w	Percentage weight by weight
%, w/v	Percentage weight by volume
% RSD	Percentage relative standard deviation
% RTD	Percentage remaining to be degraded
λ_{max}	Wavelength of maximum absorbance
<	Less than
>	More than
≤	Less than equal to
≥	More than Equal to
=	Equal to
≈	Approximately equal to
σ	Standard deviation of y intercept of regression equation
˚C	Degree centigrade
ΔH	Enthalpy
ρb	Bulk density
ρt	Tapped density
3D	Three dimensional
˚C/min	Degree centigrade per minute
µm	Micrometer
µL	Micro liter
µg/mL	Micro gram per milliliter
µg/L	Microgram per liter
µg. h/L	Micro gram hour per liter
5-HT	5-hydroxy tryptamine
3-OMD	3-O-Methyl dopa
AADC	Aromatic L-amino acid decarboxylase
AIC	Akaike information criteria
ANOVA	Analysis of variance
ASD	Average of standard deviation
AT	Accelerated temperature
AUC	Area under curve
AUMC	Area under the first moment curve
$AUC_{(0-\infty)}$	Area under plasma concentration time curve

Avicel®	Microcrystalline cellulose
BBB	Blood brain barrier
BCS	Biopharmaceutical classifications system
BEN	Benserazide
CD	Carbidopa
D1	Dopamine receptor
D2	Dopamine receptor
d_g	Geometric mean diameter on weight basis
cm	Centimeter
cm^2	Centimeter square
C_{max}	Maximum concentration
CNS	Central nervous system
COMT	Catechol-O-methyl transferase
CO_2	Carbon dioxide
Conc.	Concentration
cps	Centipoises
CR	Controlled release
CR-GRDF	Controlled release gastroretentive dosage forms
CRT	Controlled room temperature (25 ± 2ºC/60 ± 5 % RH)
DA	Dopamine
DEP	Diethyl Phthalate
DBP	Dibutyl pthalate
DOE	Design of experimentation
DOPAC	3, 4-dihydroxy phenyl acetic acid
DSC	Differential scanning calorimetry
EC	Ethyl cellulose
EC_{50}	Concentration required to obtain 50% of E_{max}
E_{max}	Maximum therapeutic response
ERL	Eudragit® RL30D
ERL100	Eudragit® RL100
ERS	Eudragit® RS100
EST	Extrusion spheronization technique
ENE	Eudragit® NE30D
et al.	Co-workers
f_1	Dissimilarity factor
f_2	Similarity factor

FDA	Food and drug administration
F_r	Relative bioavailability
FT	Refrigerated temperature (5 ± 2^{o}C)
FTIR	Fourier transform infrared
g	Gram
g/L	Gram per liter
g/mL	Gram per milliliter
GI	Gastrointestinal
GIT	Gastrointestinal tract
GRDDS	Gastroretentive drug delivery system
h	Hour
HBS	Hydrodynamically balanced system
HCl	Hydrochloric acid
$HClO_4$	Perchloric acid
HETP	Height equivalent to theoretical plates
H_2O_2	Hydrogen peroxide
HPC	Hydroxy propyl cellulose
HPLC	High performance liquid chromatography
HPLC-UV	High performance liquid chromatography ultra violet
HPLC-ECD	High performance liquid chromatography-Electrochemical detector
HPMC E5	Hydroxy propyl methyl cellulose
HPTLC	High performance thin layer chromatography
HRS	Hypokinetic rigid syndrome
HSA	1-heptanesulphonic acid monohydrate
HQC	Higher quality control sample
IAEC	Institutional animal ethics committee
ICH	International conference on harmonization
IR	Immediate release
IRP	Immediate release pellets
IS	Internal standard
i.p.	Intraperitoneal
IVIVC	In vitro in vivo correlation
IUPAC	International union of pure and applied chemistry
J/g	Joule per gram
k_0	Zero order release rate constant
k_1	First order release rate constant

k_H	Release rate constant for ‘Higuchi’ model
k_{kp}	Release rate constant for 'Korsmeyer-Peppas’ model
K_d	Degradation rate constant
L	Liter
LCMS	Liquid chromatography coupled with mass spectrophotometer
LD	Levodopa
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantitation
LNAA	Large neutral amino acids
LOD	Limit of detection
Log % RTD	Log percentage remaining to be degraded
Log P	Log of oil water partition coefficient
LOQ	Limit of quantitation
LQC	Lower quality control
M	Molar
MAO	Monoamine oxidase
MCC	Microcrystalline cellulose or cellulose microcrystalline
MDT	Mean dissolution time
MFB	Medial forebrain bundle
MFT	Minimum film formation temperature
mg	Milligram
mg/day	Milligram per day
Mg/kg	Milligram per killogram
mg/mL	Milligram per milliliter
Mg stearate	Magnesium Stearate
min	Minute
mL	Milliliter
mL/min	Milliliter per minute
mM	Millimolar
mm	Millimeter
mm/s	Millimeter per second
MMC	Migrating motor complex
MQC	Medium quality control
MRFP	Modified release floating pellets
MRT	Mean residence time
MUPS	Multi unit particulate system

MW	Molecular weight
M_t/M_∞	Fraction of drug released at time t
MΩ.cm	Milliohm centimeter
N	Newton
N	Theoretical plates
N	Diffusional exponent indicative of release mechanism in krosmeyer-peppas model
$NaHCO_3$	Sodium bicarbonate
$Na_2S_2O_5$	Sodium metabisulfite
Na_2EDTA	Disodium ethylene diaminetetraacetic acid
NDDS	Novel drug delivery systems
ng/mL	Nanogram per milliliter
ng/spot	Nanogram per spot
ng h/mL	Nanogram hour per milliliter
nL/s	Nanoliter per second
nm	Nanometer
6-OHDA	6-hydroxy dopamine
PBS	Phosphate buffer saline
PD	Parkinson's disease
PEG	Poly ethylene glycol
pH	Negative log to the base 10 of hydrogen ion concentration
pKa	Acid dissociation constant
PK-PD	Pharmacokinetic and pharmacodynamic
P value	Significance level in statistical tests (probability of a type I error)
PVPK30	Povidone or Polyvinyl pyrolidone
Log P	Hydrophobicity
QC	Quality control
R	Regression coefficient
R_f	Retention factor
RH	Relative humidity
RPHPLC	Reverse phase-High performance liquid chromatography
rpm	Revolutions per minute
Rs	Resolution
RSD	Relative standard deviation
R_t	Retention time
RW	Resultant weight

S	Slope of the least square regression line
SD	Standard deviation
sec	Seconds
SEM	Scanning electron microscopy
SNpc	Substantia nigra pas compacta
SNRI	Serotonin and norepinephrine reuptake inhibitors
SPE	Solid phase extraction
SSRIs	Selective serotonin reuptake inhibitors
T	Temperature
T_f	Tailing factor
$t_{1/2}$	Half life
$T_{90\%}$	Time required to retain 90% of drug potency
$t_{80\%}$	Time taken for 80% of drug release from formulation
TA	Tartaric acid
TCA	Trichloroacetic acid
TEC	Triethyl citrate
TDW	Triple distilled water
T_m	Melting temperature
T_g	Glass transition temperature
TGA	Thermogravimetric analysis
TLC	Thin layer chromatography
T_{max}	Time taken to reach maximum concentration
USA	United States of America
USFDA	United States Food and Drug Administration
USP	United States Pharmacopoeia
UV	Ultra Violet
Vd	Apparent volume of distribution
Vis	Visible
Vss	Apparent volume of distribution at steady-state
v/v	Volume by volume
v/v/v	Volume by volume by volume
w/w	Weight by weight
w/v	Weight by volume

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Abstract

The principle objectives of the present research work was to design and develop modified release multi unit particulate floating drug delivery system based on gas generation technique for Levodopa and Carbidopa combination to improve the therapeutic efficacy by enhancing bioavailability. To achieve this broad objective, following specific studies were carried out in the present work.

Suitable analytical and bioanalytical methods based on thin layer and liquid chromatographic techniques were developed and validated for the determination of the drug in formulation and biological matrices. Prior to the formulation development, preformulation studies were performed to establish necessary physicochemical data of the Levodopa and Carbidopa. Studies were performed to address the product specific questions related to the solubility, stability, drug-excipient compatibility etc. Extrusion spheronization technique was used for the manufacture of Levodopa and Carbidopa loaded core pellet. Modified release floating pellets were manufactured by multiple coating of sustained release layer, gas generating layer and gas entrapped polymeric layer using fluidized bed coating technique. Formulation development was carried out by identifying and optimizing the critical formulation and process parameters. Further, designed modified release floating pellets were characterized for surface morphology and shape, particle size, drug content, in vitro floating and drug release behavior. In addition, optimized formulations were also studied for the drug product stability and reproducibility. Oral pharmacokinetic studies were performed using rat model to investigate the area under plasma concentration time curve following administration of pure drug, immediate release pellets and optimized formulation of modified release floating pellets. The plasma level of the 3-O- methyl dopa (metabolite of Levodopa) was also monitored as an index of turnover of the Levodopa in vivo. Further, pharmacodynamic studies were performed to investigate the therapeutic efficacy of pure drug and modified release floating pellets in diseased rats. Parkinson's disease was induced in rats by administration of 6-OH-dopamine in rat medial forebrain bundle.

Results indicated that the developed thin layer and liquid chromatographic methods were selective and sensitive in the determination of both Levodopa and Carbidopa. These validated analytical and bioanalytical methods were successfully used for various

preformulation, formulation development, pharmacokinetic and pharmacodynamic studies. Preformulation studies indicated that both Levodopa and Carbidopa demonstrates a charge dependent solubility profile with increasing solubility towards acidic pH. The liquid state stability studies demonstrated that both drugs undergo degradation with increasing pH of the buffer. The results of the drug-excipient compatibility studies indicated absence of significant interaction of both the drugs with various excipients used in the formulations.

Levodopa and Carbidopa loaded core pellet characteristics such as pellet shape, size distribution, usable yield, mechanical crushing strength and friability were found to be influenced by formulation parameters such as binder concentration, spheronization aid amount and particle size of spheronization aid. The particle shape and microscopic imaging confirmed that Levodopa and Carbidopa loaded core pellets manufactured using Avicel® PH101 and Avicel® PH105 were spherical in shape. The in vitro drug release and floating behavior of modified release floating pellets were found to be influenced by the coating levels of sustained release and effervescent layer/s and gas entrapped polymeric layer. The optimized batches of modified release floating pellets showed immediate floating and remained buoyant for more than 20 h. Furthermore, in vitro drug release from optimized formulations of modified release floating pellets was controlled over 10 to 12 h, which could be well explained by the first order release kinetics. All optimized formulations demonstrated good stability and reproducibility. Thus, set manufacturing conditions provided good quality modified release floating pellets with reproducible characteristics.

Increase in the area under the drug concentration curve in rat plasma with modified release floating pellets was observed. In addition, residence time of Levodopa was also found to be more in case of modified release floating pellets. In case of 6-OH dopamine treated rats, Levodopa and Carbidopa treatment using modified release floating pellets demonstrated significant reduction in abnormal involuntary movement and improvement motor activity compared to the oral solution and immediate release pellets.

Collectively, these results indicate that the designed modified release floating pellets have great potential as gastroretentive delivery system for Levodopa and Carbidopa

combination. Further, drug delivery using modified release floating pellets approach would be advantageous over the currently available commercial formulation.

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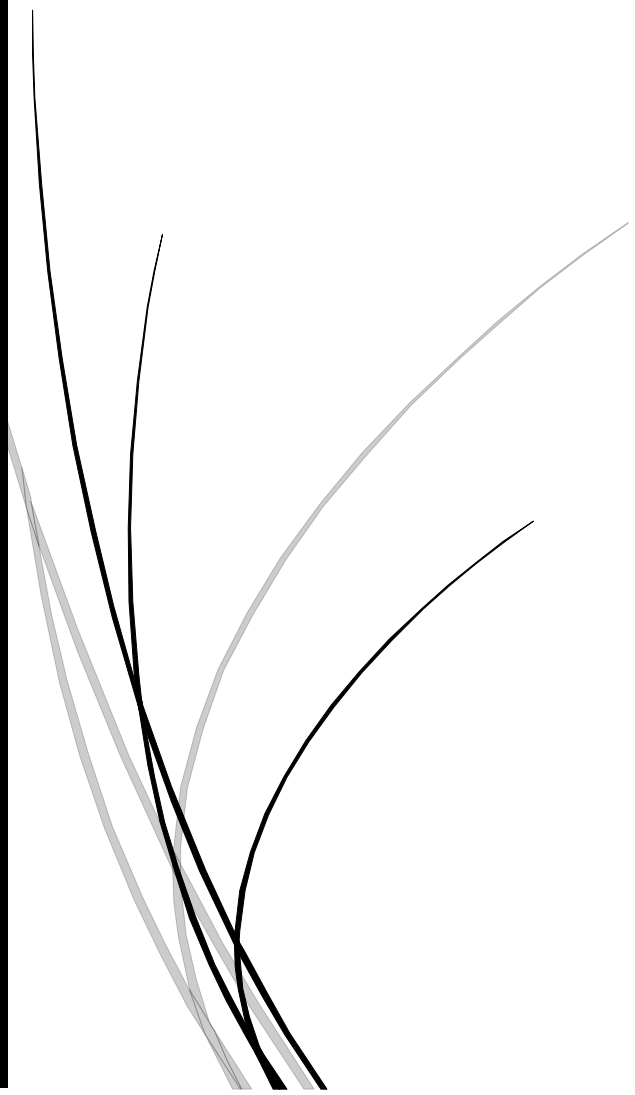
1 INTRODUCTION



1.1. Introduction

Among all the routes that have been employed for the systemic delivery of drugs, oral delivery remains the most preferred route of drug administration in spite of tremendous advancements in injectables, transdermal, nasal and other routes of administration. Oral drug delivery systems offers mighty advantages such as ease of administration, patient compliance and flexibility in formulation which makes it the most popular drug delivery route (Chien, 1992a). However, administration of some drugs through oral route for systemic delivery is unsuccessful since it suffers from certain disadvantages like unpredictable and erratic absorption, gastric emptying, gastrointestinal intolerance, incomplete absorption, degradation of drug in gastrointestinal contents and presystemic metabolism resulting in reduced bioavailability. To overcome such disadvantages associated with oral route, drugs can either be administered through other routes of administration or oral drug delivery has to be modified for successful delivery of orally less efficient drugs. Over the last decade, there has been a particular interest in investigating novel oral drug delivery systems for successful systemic delivery of such drugs to improve therapeutic benefits. Conventional oral immediate release dosage forms have poor control over drug release which may cause side effects due to fluctuating plasma drug concentration (Chawla and Bansal, 2003). In contrast, oral controlled release delivery systems provides drug release at predetermined and predictable rate to maintain constant plasma drug concentration thus it may increase therapeutic efficacy with less frequent dosing. However, achieving optimum drug bioavailability is a major obstacle in the development of oral controlled release drug delivery systems as various factors [gastrointestinal tract (GIT) physiology related factors and drug related factors] influences the oral bioavailability of dosage forms (Sungthongjeen et al., 2008).

1.2. Gastrointestinal Tract Physiology

Stomach is located in the left upper part of abdominal cavity immediately below the diaphragm. Stomach size varies according to the amount of distension: up to 1500 mL following a meal; after food has emptied, a collapsed state is obtained with resting volume of 25-50 mL (Waugh and Grant, 2001). GIT is in a state of continuous motility and on the basis of sate of feeding it can categorized into two modes of motility viz. interdigestive

motility mode (fasted state) and digestive motility mode (fed state). In fasted state, various cyclic events occur commonly termed as interdigestive motility mode or migrating motor complex (MMC). MMC regulates gastrointestinal motility patterns which is subdivided into four consecutive phases: basal (Phase I), pre-burst (Phase II), burst (Phase III), and Phase IV intervals (Read and Sugden, 1987; Hall, 2011). First phase known as basal phase lasts for 45-60 min where contraction occurs rarely. The period for second phase lasts for 30 - 50 min where contraction occurs and this phase is termed as pre-burst phase. The frequency and intensity of contractile motions increase gradually as phase progresses. Third or burst phase is characterized by intense large and regular contractions which last for 10 - 20 min. The contractions are also called as housekeeper waves as during this phase undigested food is swept out of the stomach. The transition period of 0 - 5 min between phases III and I for two consecutive cycles is termed as Phase IV. MMC is interrupted immediately after ingestion of food and digestive phase starts. Motor activity starts in 5 to 10 min after ingestion of food and persists till the time food is present in stomach (Coupe et al., 1991). GIT physiology influences the movement of oral dosage forms in GIT which leads to bioavailability problems. In addition, gastric retention time of formulations is influenced by various factors like fed state of stomach, frequency of food administration, quality of food, age, gender, posture, concomitant drug administration, pathological conditions etc.

1.3. Gastroretentive Drug Delivery System

Unlike conventional dosage forms, gastroretentive drug delivery system (GRDDS) is a delivery system in which dosage forms retain in stomach for longer period. Over the last two decades, various GRDDSs have been reported to prolong gastric residence time (Talukder and Fassihi, 2004a; Pawar et al., 2011).

1.3.1. Rationale for GRDDS

Drug administered in the form of oral controlled drug delivery systems are sometimes not absorbed uniformly throughout the GIT. Most of the drugs are absorbed from the particular regions of the GIT at different rate and extent. Therefore, such drug is said to have ‘absorption window’ which is the particular region of the GIT through which it gets

absorbed. In addition, physiological, physicochemical or biochemical factors are also responsible for uneven absorption of drug through GIT which leads to unpredictable bioavailability and low therapeutic benefits.

Drug related factors like pH dependent solubility and stability plays significant role in absorption through GIT (Chien, 1992b; Heigoldt et al., 2010; Mou et al., 2011). The variability in physiological conditions of the GIT like pH, intestinal fluids and transit time is also responsible for variation in drug absorption through GIT (Chawla and Bansal, 2003; Söderlind and Dressman, 2010; Bhattachar et al., 2011). Drugs absorbed by passive diffusion mechanism in unionized form show, non-uniform absorption due to variation in extent of ionization at different pH regions in the GIT e.g. weakly basic and acidic drugs. Furthermore, gastric residence time (GRT) of dosage form which depends on the gut motility and flow shows significant influences on absorption of the drug through GIT (Singh and Kim, 2000). Gastric emptying time in human is normally 2 to 3 h (Vasavid et al., 2014). The type and timings of food ingestion and nature of the formulation varies the gut motility and flow of GIT (Mou et al., 2011). A short gastric emptying time also results in an incomplete absorption of drug which exhibit solubility at acidic pH leading to reduced efficacy of the administered dose (Chavanpatil et al., 2006).

Drugs that are absorbed from a specific region of upper GIT are difficult to design as oral controlled drug delivery system because amount of drug released only in the gastrointestinal (GI) region above and in close vicinity to the absorption window is available for absorption. Drug released after absorption window goes waste with either negligible or no absorption which results in unpredictable bioavailability (Figure 1.1a).

For the successful performance of oral controlled drug delivery system in case of such drug, release of drug should be achieved within the vicinity of absorption site to ensure continuous absorption of the released drug in order to maximize the drug absorption and bioavailability. These considerations led to the designing and development of oral controlled release gastroretentive delivery systems (Figure 1.1b).

The controlled release GRDDS offers various advantages over conventional immediate release and controlled release drug delivery systems. The bioavailability of the drugs undergoing degradation in intestinal fluids can be increased by GRDDS (Kakumanu et al., 2008). Floating microsponges of curcumin have been reported for the site specific delivery

for management of gastric cancer. Significant increase in the bioavailability of curcumin and reduction of degradation in alkaline environment of intestine has been reported for floating microsponges compared to native curcumin (Arya and Pathak, 2014). GRDDS also increases bioavailability of the drug specifically absorbed from upper GIT may be because of high surface area provided by jejunum and ileum for absorption (Kakumanu et al., 2008). In addition to this, some drugs exhibit less oral bioavailability may be due to pH-dependent solubility e.g. weakly basic drugs. GRDDS improves the bioavailability of such drugs by retaining and releasing complete dose of drug in stomach (Chien, 1992b; Klausner et al., 2003b). Further, undesirable activities of drugs in colon can be prevented by delivering them through GRDDS. Beta lactam antibiotics are mostly absorbed from small intestine. Release of beta lactam antibiotics in colon leads to development of microorganism's resistance which can be prevented by designing GRDDS (Bhattachar et al., 2011). Pharmacotherapy of stomach has also been reported by designing controlled release GRDDS, since it releases drug specifically at its site of action (Arya and Pathak, 2014).

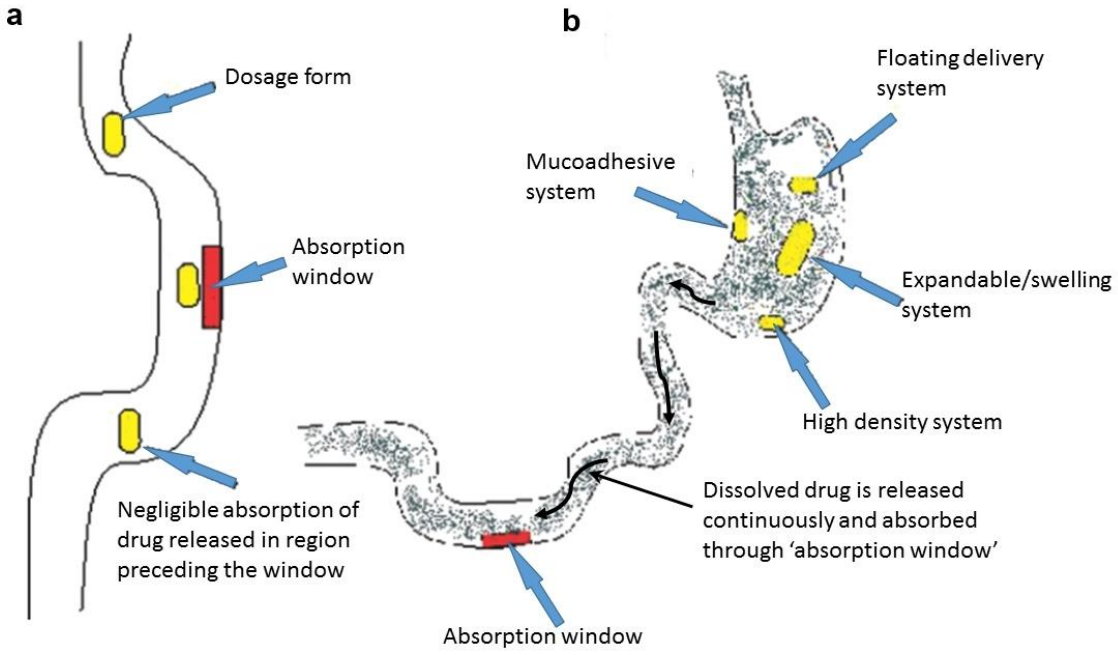


Figure 1.1. Drug absorption in (a) conventional dosage forms and (b) gastroretentive drug delivery systems (Pawar et al., 2011).

Controlled release GRDDS releases drug at predetermined rate for prolonged period and maintain stable plasma concentration within therapeutic window for longer duration. Thus, it helps in improvement of patient compliance due to less dosing frequency (Söderlind and Dressman, 2010). In addition, controlled release GRDDS reduces drug concentration dependent adverse effects associated with peak concentration especially in case of drugs with narrow therapeutic window (Bhattachar et al., 2011). Controlled release GRDDS have also been reported to increase bioavailability compared to conventional immediate or controlled release dosage forms for the drugs which are p-glycoprotein substrate and do not undergo oxidative metabolism eg. Digoxin (Bhattachar et al., 2011). Moreover, GRDDS not only provides pharmacokinetic advantages but it also offers pharmacodynamics benefits (Emara et al., 2013).

In conclusion, drugs that are stable in stomach, exhibit narrow therapeutic window and are locally active in stomach can be the ideal candidates for designing of GRDDS. Further, the release of drug from GRDDS can be customized depending on requirement of drug to improve therapeutic outcomes. Table 1.1 summarizes various types of drugs that have been explored using gastroretentive drug delivery systems.

1.3.2. Gastroretention Techniques

Various gastroretentive drug delivery systems based on different gastroretentive techniques have been designed by researchers for successful delivery of drugs to improve therapeutic efficacy (Figure 1.1b).

Bio/Mucoadhesive delivery system are designed to adhere to gastric mucosal lining and provide drug release at controlled rate for improved therapeutic outcome thus it serves as a potential means for gastroretention (Pund et al., 2011; Sarparanta et al., 2012). Although, some of the polymers show excellent bioadhesion, these system have limited retention time because of the rapid turnover of mucus in the GIT (Sinha et al., 2004). *Swelling and expanding dosage forms* that, after oral intake either swell or expand to the extent that prevents passage through the pyloric sphincter have also been reported as gastroretentive delivery system. For swelling system, dissolution of polymer is prevented due to presence of physicochemical crosslinks in polymer network which maintains physical integrity of dosage forms and achieves the controlled release of the drugs (Figure 1.1b).

Table 1.1. Categories of Drugs Ideal for Delivery Using GRDDS

Type	Example of drugs	Rational of gastroretention	References
pH dependent solubility	Verapamil, Diazepam, Chlorpheniramine, Propranolol HCl, Metaprolol, Chlordiazepoxide Ofloxacine, Ciprofloxacin HCL	Release of drug from dosage form due to solubility problem	(Sawicki, 2002; Streubel et al., 2003; Chavanpatil et al., 2006; Mostafavi et al., 2011)
Drugs locally acting in stomach	Antacids, Tetracycline Clarithromycin	Local treatment	(Hejazi and Amiji, 2002; Talukder and Fassihi, 2004b; Jain and Jangdey, 2008)
Drugs mainly absorbed in stomach	Levodopa, Salbutamol, Sotalol	To improve bioavailability	(Goole et al., 2008a; Goole et al., 2008b; Goole et al., 2008c)
Drugs mainly absorbed from proximal part of small intestine	Acyclovir, Riboflavin, Nitrofurantoin, allopurinol	To improve bioavailability	(Stops et al., 2008; El Gamal et al., 2011)
Drugs undergoing degradation in colon	Ranitidine, Metaprolol	To protect drug from degradation in colon and to improve bioavailability	(Adhikary and Vavia, 2008)
Drugs unstable in intestinal fluids	Captopril, Famotidine	To maintain stability of drug	(Gupta and Pathak, 2008; Meka et al., 2008)

In expanding system, the expansion can be achieved either by swelling or by unfolding in the stomach. Although swelling and expandable systems have interesting characteristics, these systems are difficult to industrialize and may not be cost-effective. Further, systems need to be designed in such a way that they should not cause a local damage and undergo easy biodegradation because permanent retention of such system may cause bowel obstruction, intestinal adhesion, and gastropathy (Klausner et al., 2003c).

High density systems exhibit density approximately 3 g/mL and are retained in the lower part of the stomach helping them to withstand peristaltic movement. The high density pellets are retained in rugae or folds of stomach near the pyloric region by sedimentation due to high density and small size. The pellets having density in the range of 2.6 - 2.8 g/mL can be retained in the lower parts of the stomach (Clarke et al., 1993). Although high density pellets show gastric retention, it is very difficult to manufacture it with maximum drug loading (> 50%) and to achieve required threshold density of 2.4 - 2.8 g/mL. Therefore, till today there is no such system in the market. Unlike conventional hydrogels, *superporous hydrogels* have average pore size more than 100 µm and swell to equilibrium within a minute, due to rapid water uptake by capillary wetting through numerous interconnected open pores. These gels swells to large size and exhibit sufficient mechanical strength to withstand pressure exerted by the gastric contraction (Park et al., 2006). It has been reported that the superporous hydrogel composites retains in the human stomach for more than 24 h (Chen and Park, 2000).

Ion exchange resins can also be used for gastroretention. The negatively charged drugs bound to the ion exchange resins in which bicarbonates are also loaded. Such beads are encapsulated into semipermeable membrane to prevent rapid loss of the carbon dioxide gas (Atyabi et al., 1996a, b). When theses beads get in contact with acidic environment of the stomach, exchange of chloride and bicarbonate ion takes place which leads to generation of carbon dioxide gas. The generated carbon dioxide gas gets entrapped into semipermeable membrane which leads to floating of ion exchange resin beads due to reduction in density (Burton et al., 1995; Umamaheshwari et al., 2003). Further, use of *Passage delaying agents* have also been reported for gastroretention. This approach is based on type of food which influences the gastric emptying. The passage delaying agents like triethanolamine myristate has been proposed to delay gastric emptying (Kumar and

Philip, 2007). However, use of passage delaying drugs (propantheline) are considered undesirable because of potential adverse effects (Kumar and Philip, 2007). In addition, *magnetic systems* in which extracorporal magnet controls the gastrointestinal transit of dosage forms containing a small internal magnet has also been reported (Gröning et al., 1996). *Raft system* comprises of the alginate gel solution (containing bicarbonates) that in contact with gastric fluid swells and form viscous gel containing entrapped carbon dioxide. Swelling and entrapment of carbon dioxide in gel results in floating of this delivery system which leads to gastroretention for prolonged time.

Floating systems are described as the systems that have sufficient buoyancy to float over gastric content in stomach due to density lower than the gastric fluids (Davis, 2005). During retention in stomach, these systems release the drug for prolonged period at a controlled rate which can be absorbed continuously through absorption window. Thus, it helps in reducing fluctuations in drug plasma concentration and associated side effects. Controlled release floating systems offers mighty advantages like reduction in dosing frequency, improvement in patient compliance etc. In addition, therapeutic outcome of the short half-life drugs can also be improved by designing controlled release floating drug delivery system. Furthermore, it enhances the absorption and bioavailability for the drugs which are soluble at gastric pH (Zhang et al., 2012). However, floating drug delivery systems have certain disadvantages like high level of fluid requirement in the stomach to maintain buoyancy and efficient working of dosage form. Moreover, floating drug delivery systems are not feasible for the drugs having solubility or stability problems in gastric fluid. Further, applicability of floating drug delivery is limited for the drugs which causes irritation of gastric mucosa. In addition to this single unit floating dosage forms sometimes fail to float probably due to simultaneous ingestion of food resulting in transit to intestine which leads to bioavailability problem.

An ideal floating drug delivery system should maintain an overall density lower than that of gastric contents (1.004 - 1.010 g/mL). Further, it should not interfere with normal gastric emptying process and must exhibit sufficient structural stability to form a cohesive gel barrier. Such drug delivery systems should release drug at a controlled rate to maintain constant plasma drug concentration.

Various techniques have been investigated for the floating of the dosage forms on the gastric content which are classified into different classes on the basis of floating mechanisms viz. *low density systems, hydrodynamically balanced systems and gas generating systems.*

Low density systems are manufactured using low density material and because of their lower density (< 1 g/mL) than gastric content they show immediate buoyancy (Streubel et al., 2002, 2003). Low density systems include hollow microspheres (microballoons), hollow beads, microparticles, emulgel beads or floating pellets (Sato et al., 2004a; Sato et al., 2004b; Mastiholimath et al., 2008; Wei and Zhao, 2008). Currently, hollow microsphere are considered to be the most promising buoyancy systems, since they combine the advantages of multiple unit systems and good floating properties (Kawashima et al., 1992; Jain et al., 2005; Liu et al., 2011; Svirskis et al., 2014). *Hydrodynamically balanced dosage forms* (HBS) contain one or more gel forming hydrophilic polymers which includes hydroxyethyl cellulose (HEC), hydroxy propyl cellulose (HPC), sodium carboxyl methyl cellulose (NaCMC), agar, carrageenans or alginic acid etc. Hydroxypropyl methyl cellulose (HPMC) is most commonly used excipient in HBS. In this type of dosage forms, drugs mixed with polymer is usually administered in a hard gelatin capsule. Upon contact with gastric fluid, capsules shell dissolves and outermost hydrophilic colloid hydrates and swells which leads to floating of mass. In addition, these dosage forms retains shape of capsule preventing disintegration (Chen and Hao, 1998; Ali et al., 2007; Nayak and Malakar, 2011). Swollen gel present at outer side is responsible for the controlled release of drug from such dosage forms. Use of fatty excipients in hydrodynamically balanced system has been also reported to reduce density of dosage form in order to extend floating time and control release of drug for longer time (Sheth and Tossounian, 1984; Gerogiannis et al., 1993).

Flowability of dosage form can also be achieved by *incorporation of gas generating agents* like carbonates, bicarbonates etc. Gas generating agents can react with either gastric content or co-formulated acids like tartaric acid or citric acid resulting in generation of carbon dioxide gas (CO_2) due to neutralization reaction. Both coated and matrix floating systems manufactured using gas generating agent have been reported. Matrix type of dosage forms are manufactured using hydrophilic polymer. The hydrophilic polymer in

presence of dissolution media hydrates and swells immediately and entraps generated CO_2 gas which leads to floating due to reduction of density of dosage form. Various floating tablets and capsules systems have been reported using gas generating agents (Jaimini et al., 2007; Sungthongjeen et al., 2008; Tadros, 2010). In addition, multiple unit systems like pellets and minitablets have also been reported. Gas generating agent can be incorporated either in matrix of the formulation (Goole et al., 2007) or layered over surface of the formulation with coating of gas entrapped layer (Hamdani et al., 2006; Qi et al., 2015). Multi-unit particulate floating drug delivery systems are superior to single unit floating dosage forms as it provides uniform drug release and has less risk of dose dumping.

1.4. Pellets: A Multi Unit Particulate Drug Delivery System

Oral drug delivery systems can be classified as monolithic or multiple unit particulate systems (MUPS). MUPS comprise of number of discrete particles with diameter < 3 mm that are combined into one dosage unit (Shaji et al., 2007). Further, MUPS may exist as pellets, granules, sugar seeds (non-pareil), minitablets, ion-exchange resin particles, powders, and crystals with drugs being entrapped in or layered around cores (Porter and Bruno, 1990; Collett and Moreton, 2002).

Pellets are considered as spherical, free-flowing granules with a narrow size distribution, with particle size in the range of 500 and 1500 µm for pharmaceutical applications (Ghebre-Selassie, 1989). Pellets because of its multi unit particulate nature offers some important pharmacological as well as technological advantages over conventional single-unit solid dosage forms (Bechgaard and Nielsen, 1978). Therefore, recently compared to single unit systems much attention is being paid on the development of pellets because of their potential benefits like avoidance of dose dumping, less variation in gastric transit time, reduction of food effects and a more uniform distribution along the intestinal tract (Ghebre-Selassie, 1994). In addition to this, pellets disperse uniformly in stomach because of its small size thereby reducing potentially irritating effect on gastric mucosa. Moreover, it improves bioavailability with reduced drug plasma concentration fluctuations (Ghebre-Sellassie, 1989; Asghar and Chandran, 2006). After oral administration, the subunits of multiple unit preparations distribute readily over a large surface area in the GIT that could improve bioavailability. In addition, pellets are able to leave the stomach continuously like

liquids even if the pylorus is closed (Follonier and Doelker, 1992) thus reducing intra and inter subject variability in plasma levels and bioavailability (Krämer and Blume, 1994). Degradation of drug or irritation of gastric mucosa caused due to premature drug release from enteric coated or delayed release dosage forms in stomach, can also be reduced by delivery of drug in the form of coated pellets. This is because of its short gastric emptying time compared to enteric-coated tablets (Dechesne and Delattre, 1987). In pellets, the total drug is divided into small subunits and each coated pellet acts as a single drug reservoir releasing drug by following its own release mechanism. Coating imperfection would therefore only affect the release of a small drug portion present in pellet, unlike complete dose dumping from a single unit drug reservoir system (Bechgaard and Nielsen, 1978). Moreover, pellets offers dose flexibility (ease of adjustment of the strength of a dosage unit) possibility for administration of incompatible drugs in a single dosage unit by separating them in different MUPS and combination of MUPS with modified drug release properties (Ghebre-Sellassie and Knoch, 2002). Because of smooth surface morphology, narrow size distribution, spherical shape and low friability, high coating efficiency can be achieved for pellets compared to granules and minitablets. Further, pellets exhibit very good flow properties due to spherical nature and smooth surface which ensure reproducible die or capsule filling and consequently good content uniformity (Erkoboni, 2003). However, in order to ensure patient compliance and dosing accuracy, monolithic systems are preferred. Thus, pellets can be presented in the form of monolithic system either by compaction of multiple units into tablets (Marvola et al., 1983) or filling them into capsules (Aulton et al., 1994). Furthermore, MUPS sometimes tend to adhere to oesophagus during swallowing; such risk associated with administration can also be decreased by administration of them in the form of tablets or capsules (Marvola et al., 1983).

These advantages make pellets a promising drug delivery system. Therefore recently it has been used to design controlled release systems (Kramar et al., 2003; Young et al., 2005; Hamdani et al., 2006), enteric coated/colon targeted systems (Debunne et al., 2002; Debunne et al., 2004; Türkoğlu et al., 2004), gastroretentive systems (Sawicki and Łunio, 2005; Sungthongjeen et al., 2006; Cui et al., 2008), immediate release systems (Dukić-Ott et al., 2007; Wang et al., 2012), dual release systems (Zhao et al., 2010) etc.

1.5. Manufacture of Pellets

Among the various methods employed most popular methods used for the manufacture of pellets are layering processes such as solution/suspension layering, powder laying, direct pelletization using shear mixers and conventional or rotary fluid bed granulators and extrusion spheronization. Recently, freeze pelletization, cryopelletization and hot melt extrusion technique have also been used to produce spherical pellets.

1.5.1. Extrusion Spheronization Technique

Extrusion spheronization technique (EST) is most popular method for manufacture of pellets originally invented in 1964 (Nakahara, 1964) and first published in 1970 (Conine and Hadley, 1970; Reynolds, 1970). Pellets manufacturing by EST is now well established technique as it offers various advantages compared to the other techniques. EST provides high throughput with low wastage, narrow particle size distribution of pellets with low friability, smooth surface for film coating with easy manufacturing process. Furthermore, sustained and controlled release profile can be achieved from pellets manufactured by EST in comparison with other technique. EST is multi-stage mechanical process which involves four steps: mixing of ingredients and manufacturing of the wet mass (granulation), shaping the wet mass into cylinders (extrusion), breaking up the extrudate and rounding of the particles into spheres (spheronization) and finally drying of the pellets (Figure 1.2).

Initially dry mixing of ingredients is necessary for the uniform powder distribution. Various mixers can be used to achieve homogenous powder mixing like planetary mixer (Sandler et al., 2005) twin shell blender (Diane Bruce et al., 2003), high speed mixer (Kojima and Nakagami, 2002) and tumbler mixer (Koo and Heng, 2001). Plastic mass for extrusion can be prepared by wetting uniformly mixed dry powder mass using granulating liquid similar to the process employed during wet granulation while tableting. Various granulators are used for homogenous mixing of the powder blend and granulation liquid. Planetary or Hobart mixers (Pinto et al., 1992; Tapia et al., 1993; Chopra et al., 2002; Mezreb et al., 2004; Mehta et al., 2012) are most commonly used granulators for uniform mixing of the powder blend and the granulation liquid. Other mixers like high shear mixer (Franceschinis et al., 2005), sigma blade (Woodruff and Nuessle, 1972) have also been reported for this purpose. The amount of granulation liquid used for preparation of plastic

mass for EST is relatively more than the amount used for wet granulation in tablet manufacturing. Granulation step is followed by extrusion where rod shaped particles of uniform diameter are manufactured from wet mass.

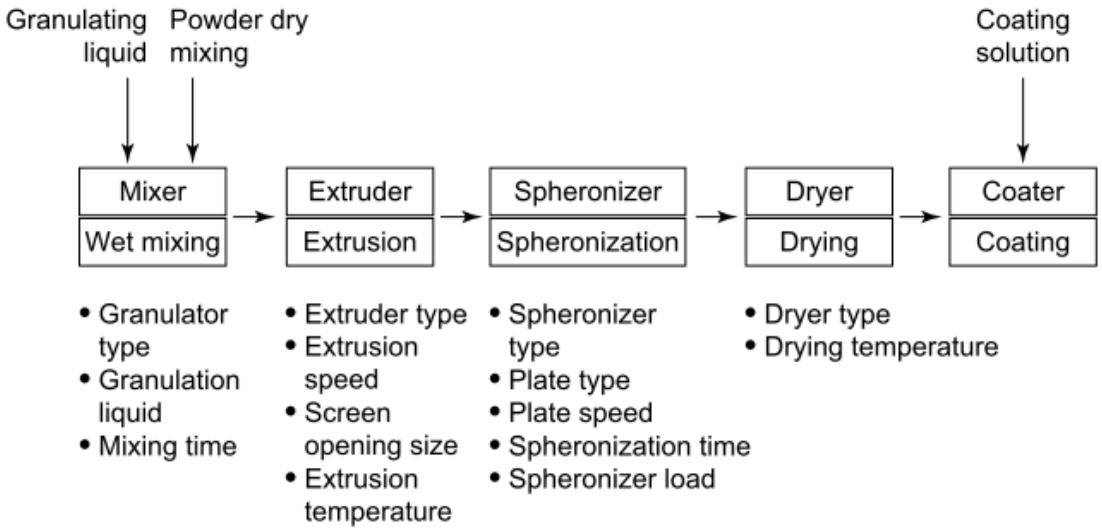


Figure 1.2. Flow diagram showing different steps, process parameters and equipment involved in extrusion and spheronization to produce spherical pellets (Gandhi et al., 1999)

Extrusion is process where a wet mass is forced through a die/screen to form small cylindrical particles (rods) with uniform diameter which are commonly termed as "extrudate". During extrusion of wet mass, extrudates break at similar length due to their own weight. Such extrudates must exhibit sufficient plasticity to deform easily under applied pressure. In addition, during manufacture of extrudates moisture level should be optimized in such way that extrudates should not adhere to each other during spheronization process. Various extruders used for production of extrudates are screw feed extruder (axial or end plate, dome and radial), sieve and basket extruders, gravity feed extruder (cylinder roll or gear roll) and piston feed extruder (ram) etc. In extrusion spheronization process, once production of cylindrical extrudates is over these extrudates are dumped onto the spinning plate of the spheronizer for preparation of spherical particles called pellets (Nakahara, 1964). The spinning plate of the spheronizer is called as friction plate. Due to friction between loaded extrudates and friction plate, extrudate is broken up into smaller cylinders with a length equal to their diameter (Conine and Hadley, 1970).

Further, these cylindrical extrudates are rounded in to spherical pellets due to frictional forces. Two mechanisms have been suggested for pellets formation (Rowe, 1985). According to Rowe (1985), during spheronization process, initially extrudates convert into round edged cylinders. Round edged cylinders then convert into dumbbells and elliptical shaped particles which finally convert into perfect spheres (Figure 1.3a). Another mechanism of pellet formation was reported in which a twisting of the cylinder occurs after the formation of cylinders with rounded edges, finally twisted cylinder breaks up into two distinct parts which eventually convert into perfect spheres (Baert and Remon, 1993) (Figure 1.3b). Usual time required for the spheronization of product is 2 - 10 min (Gamlen, 1985).

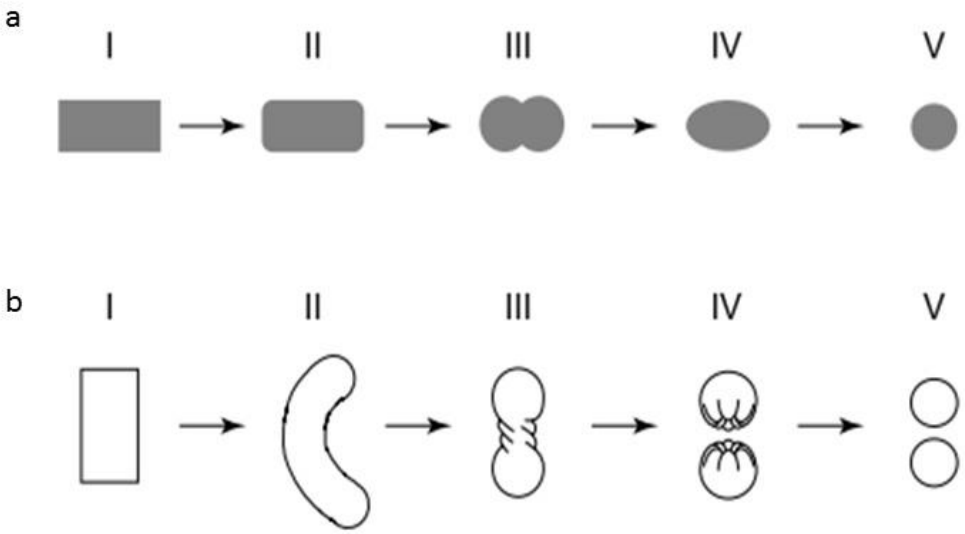


Figure 1.3. Pellet formation mechanism according to (a) Rowe- I. Cylinder, II. Cylinder with rounded edges, III. Dumb-bell, IV. Ellipse, V. Sphere and (b) Baert- I. Cylinder, II. Rope, III. Dumb-bell, IV. Sphere with a cavity outside V. Sphere (Vervaet et al., 1995).

A rotational speed of the friction plate in the range between 200 and 400 rpm is satisfactory to obtain a highly spherical pellets (Conine and Hadley, 1970; Vervaet et al., 1995). However, it has been found in literature that the rotational speed of the friction plate varies

from 100 - 2000 rpm. Thus, not only the absolute speed is important but the speed in combination with the diameter of friction plate has to be considered. Instead of comparing the absolute speed of the friction plate, friction plate peripheral velocity has to be compared (Lövgren and Lundberg, 1989). Friction plate peripheral velocity can be easily calculated from friction plate speed and diameter. Further, friction plates exhibit the grooved surface to increase the frictional forces. These grooves are geometrically placed on surface of friction plate in two arrangements viz. cross-hatch geometry and radial geometry. A cross-hatch geometry is the one where grooves form right angles while in case of radial geometry a radial pattern is used (Rowe, 1985).

The final stage of pellet formation is drying. After spheronization, pellets are dried in order to remove excessive moisture. Pellets can be dried at room temperature (Hasznos et al., 1992) or at elevated temperature either by using tray dryer/oven (Wan et al., 1993; Shettigar and Damle, 1996; Souto et al., 2005; Hu et al., 2006). In addition, use of fluidized bed dryer has also been reported for the drying of the pellets (Pinto et al., 1992; Baert and Remon, 1993; Ku et al., 1993). Furthermore, to avoid pellets having high size polydispersity index, screening is required for pellets manufactured by EST (Husson et al., 1992). Screening can be done by using sieves. Further, various process parameter influences the quality of final pellets manufactured by extrusion spheronization process which includes moisture content in wet mass, type of granulation liquid, physical properties of starting material, extruder type, extruder speed, extrusion screen properties, extrusion temperature, spheronization time, spheronization speed, spheronizer load and drying method employed etc.

1.5.2. Dry Powder Layering

In this technique, powder of drug or excipient or both is deposited in the form of layers over the surface of preformed nuclei or core with the help of the binding liquid (Nastruzzi et al., 2000; Rashid et al., 2001). Conventional coating pans are used for this purpose. Initially, the nonpareils or starter seeds are charged into rotating pan and adhesive solution is sprayed over the charged starter seeds. Once seeds are wetted properly powder is added in vortex, due to adhesive solution powder adhere to the surface of seeds. An accurate selection of both the layering powder and the aqueous binder formulations is important in

this technique. Process variables such as size of the starting cores, type of micronized powder with particular attention to its flowing properties and wettability, binder type and concentration, application rate of the sprayed binder solution and powder, presence of wetting, flowing, and anti-sticking agents, pan speed, spray gun position and atomization degree, inlet air and bed temperatures and size of the pellets have to be optimized for successful powder layering (Nastruzzi et al., 2000). A multiple unit floating drug delivery system based on gas formation technique has been designed which consists of drug-containing cores coated with effervescent layer and polymeric membrane (Sungthongjeen et al., 2006). Extended release drug delivery system have also been designed by application of powder layering technique (Pearnchob and Bodmeier, 2003a, b)

1.5.3. Hot Melt Extrusion

Hot melt extrusion is a popular technique for production of pellets in pharmaceutical industries (Follonier et al., 1994; Repka et al., 1999; Deshmukh and Amin, 2013). Melt extrusion process consists: melting or plasticizing a solid material, shaping the molten material and solidification of the material into the desired shape. In hot melt extruder, hopper feeds material continuously in heated barrel containing extruder where extrudate are manufactured and later shaped into spherical particles using spheronizer. Spheronizer temperature is optimized so that it softens the extrudate to facilitate deformation and then the material is spheronized. In this process active ingredient is dispersed in carrier material during mixing and melting before extrusion. Both active pharmaceutical ingredient and carrier material must exhibit thermal stability during processing. The material in which drug is dispersed is usually polymer or low melting point wax and it is called as thermal carrier. The drug release characteristics are mainly influenced by the physicochemical properties of thermal carrier. The mechanism of drug release is either diffusion controlled (Sato et al., 1997; Crowley et al., 2004) or by both diffusion as well as erosion depending on the water solubility of polymers/waxes used in formulation (Zhang and McGinity, 1999). In addition, plasticizers are incorporated in pharmaceutical polymers for easy thermal processing, to modify the drug release properties and to improve surface appearance of dosage form. Plasticizers reduce tensile strength and glass transition temperature improving flexibility of polymers. Although, wet mass extrusion is most

frequently used method for manufacture of pellets hot melt extrusion is best choice for drugs undergoing degradation in presence of moisture. In addition to this pellets manufactured by wet mass extrusion process usually require coating in order to modify the drug release. Since drug release is diffusion controlled from matrix of pellets, the pellets produced by melt extrusion do not require additional film coating.

1.5.4. Freeze Pelletization

Freeze pelletization is a new technique used for producing spherical matrix pellets (Cheboyina and Wyandt, 2008b). In this technique drug is dispersed in molten solid carrier and this mixture is added drop wise into an inert and immiscible column of liquid where droplets solidifies into spherical pellets. Pellets manufactured with this technique are spherical with narrow size distribution (Cheboyina and Wyandt, 2008b). Cryopelletization is used to produce drug loaded pellets for immediate as well as controlled release formulations. In this technique, by using liquid nitrogen as the solidifying medium droplets of liquid formulations such as aqueous organic solutions, suspensions or emulsions are converted into solid spherical pellets. Manufactured pellets are then freeze dried or lyophilized to remove water or organic solvent (Weyermanns, 1997). Hydrophilic carriers like polyvinyl alcohol, polyethylene glycol, low melting point sugars (dextrose, maltose) and hydrophobic carriers such as glyceryl palmitostearate, glyceryl behenate and glyceryl monstearate are used as solid carriers (Cheboyina and O'haver, 2008; Cheboyina and Wyandt, 2008b; Shukla et al., 2011). Liquid polyethylene glycol, ethyl alcohol, glycerin, water, oils such as Mineral oil, vegetable oil, silicon oil etc. are used as liquid for column.

1.6. Coating of Pellets

Controlled release pellets can be designed by direct coating with release retarding polymer film. Thus, matrix coated system and reservoir coated system are designed for controlled release (Figure 1.4a and Figure 1.4b). Drug solution and polymer dispersion can be coated on the inert core (sugar pellet) to design matrix coated pellet. In this case, drug is homogenously dispersed in polymer coat. Reservoirs system consist of drug layered core surrounded by a polymer membrane. In this case high drug loading and desired drug release profile can be achieved. (Figure 1.4 a, Figure 1.4b and Figure 1.4c).

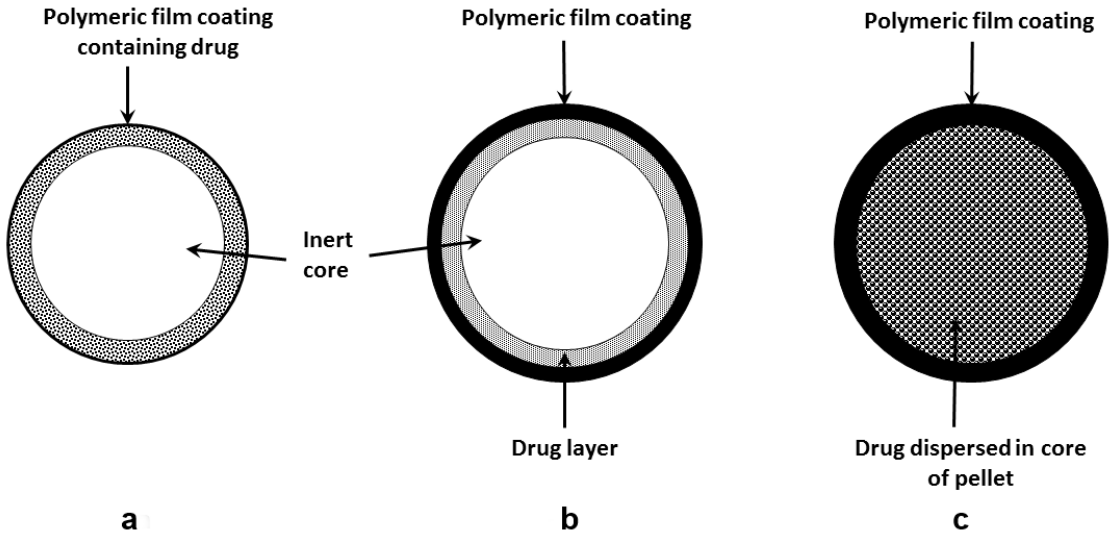


Figure 1.4. Schematic presentation of (a) matrix coated pellet; (b) reservoir coated pellets and (c) coated pellets in which drug is dispersed in core**.**

Apart from controlled release, coating of the pellets can be done to design other drug delivery systems like colon targeted system, floating sustained release system, pulsatile release system etc. Different coating technologies have been used for the coating of the pellets like fluidized bed coating (Sungthongjeen et al., 2006; Pauli-Bruns et al., 2010; Lei et al., 2011; Amrutkar et al., 2012), pan coating (Gupta et al., 2001; Gendre et al., 2011) etc. In case of fluidized bed coating process, pellets are fluidized in hot air stream and coating solution is sprayed on to the pellets where as in case of pan coating process pellets are rolled in pan and coating solution is sprayed on the pellets. All coating processes have common coating steps: a) formation of suitable sized droplet of coating solution; b) contact and adhesion of the droplet on the surface of the pellet and subsequently; c) spreading and coalescence to form uniform film (Sungthongjeen et al., 2006; Pauli-Bruns et al., 2010; Lei et al., 2011; Amrutkar et al., 2012). Pellets can be coated with aqueous polymeric dispersion or organic solution as per the requirement of drug delivery system. Organic coating has disadvantages like dependence of viscosity on molecular weight and the concentration of polymer used. On the other hand, aqueous dispersions exhibit low viscosity even at high solid contents (Wheatley and Steuernagel, 1997). In addition, organic

solvents has other disadvantages like residual solvents in the coating that can create changes in the film properties, environmental pollution and explosion hazards. Therefore, use of aqueous polymeric dispersions is mostly preferred in pharmaceutical coatings. Film formation mechanism vary on the basis of type of coating (aqueous or organic).

In case of organic polymer solutions, polymeric macromolecules dissolve and gives high viscosity solution. During solvent evaporation, it forms an intermediate gel like phase. Uniform polymeric film forms after complete solvent evaporation (Figure 1.5a). In contrast, film formation from aqueous dispersion is more complex process (Figure 1.5b). During drying polymeric particles comes into contact with each other in a closed packed order. High interfacial tension between air and water results in the formation of the polymer sphere filled with water. Particle fusion or coalescence occurs when capillary forces (air-water interfacial tension) are strong enough (Wheatley and Steuernagel, 1997).

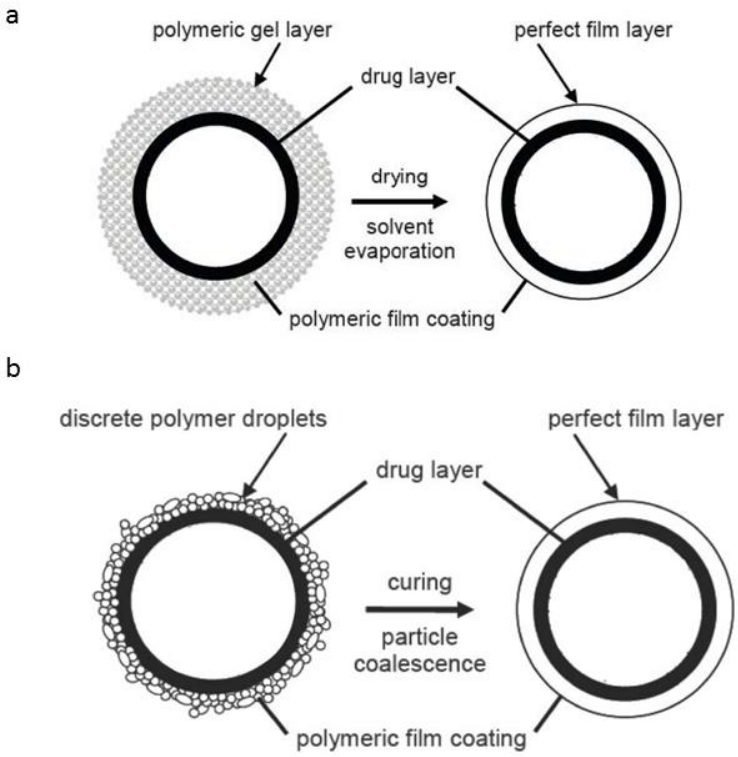


Figure 1.5. Schematic presentation of the film forming mechanism from (a) organic polymer solution (b) aqueous polymer dispersions (Clarke et al., 1995).

Usually coating process is carried out at high temperatures to ensure the softness of the discrete polymer particles. Softening of polymeric particles depends on the temperature applied and glass transition temperature (Tg) of the polymer. A curing step (post coating thermal treatment) is carried out after coating process to ensure complete film formation and to avoid further gradual coalescence (Harris and Ghebre-Sellassie, 1997). Aqueous dispersion mostly contain additional ingredient like surfactant which acts as stabilizer during coating process. Ingredients like plasticizers and anti-tacking agents are used to enhance film properties and for efficient coating. Plasticizers are added to promote the polymer particle coalescence, softening the particles and reducing minimum film formation temperature (MFT) (Wheatley and Steuernagel, 1997). Film formation depends on the glass transition temperature (Tg) of the polymer or MFT of aqueous dispersion. The MFT is the temperature above which polymer particles are soft in nature and can easily coalesce to form a continuous film (Ho and Khew, 2000). Below this temperature, dry latex is opaque and powdery. Water can decrease the Tg of some polymers (due to plasticizing effect) and thus for aqueous dispersion MFT is lower than the Tg of the polymer. Use of pore formers in coating solution have also been reported to modify the release properties of formulation by creating pores in polymer film. Low viscosity grade hydroxy propyl methyl cellulose (HPMC E5), polyvinyl pyrrolidone (PVPK30) are used as pore former in ethyl cellulose coating to modify the release. Various polymers and polymer dispersion have been used for the controlled release coating of pellets viz. ethyl cellulose (Hosseini et al., 2013; Rosiaux et al., 2014), Eudragit® RL100 (Gaber et al., 2015), Eudragit® RS100 (Pearnchob and Bodmeier, 2003b), Eudragit® RL30D (Moustafine et al., 2012), Eudragit® RS30D (Piao et al., 2013), Eudragit® NE30D (Amrutkar et al., 2012; Thakral et al., 2013), Kollicoat® SR30D (Dashevsky et al., 2005; Haaser et al., 2013).

1.7. Evaluation of Pellets

The quality of final pellets can be evaluated by estimating various parameters which includes particle size, particle shape, surface area, surface morphology, pellets shape, porosity, density, flow properties, hardness and friability, tensile strength, disintegration time, in vitro drug release behavior and in vivo pharmacokinetic and pharmacodynamic performance (Table 1.2).

Table 1.2. Summary of Evaluation Parameters and Factors Influencing the Quality of Pellets

Parameter	Significance	Method used for evaluation	Factors influencing quality of pellets	References
Particle size distribution	Drug content in pellets and drug release behavior of formulation	Sieve analysis using standard sieve computer aided image analysis, vernier calipers	extrusion screen, spheronization time and speed, drying technique and temperature, moisture content, binder type and its concentration, type of excipients	(Wan et al., 1993; Koo and Heng, 2001; Bashaiwoldu et al., 2004; Mezreb et al., 2004; Wiwattanapatapee et al., 2004)
Particle shape	Flow properties	Image analysis software	Extrusion screen, spheronization time and speed, type of excipients	(Koo and Heng, 2001)
Porosity	Influences the disintegration, dissolution, adsorption and diffusion of the drug from pellets	Gas adsorption (pore diameter < 2000 Å) and mercury intrusion porosimetry (MIP) techniques(pore diameter > 2000 Å)	Drying temperature and drying method, drug loading, amount of granulating liquid, spheronization time	(Lowell and Shields, 1981; Mehta et al., 2000; Perez and Rabišková, 2002)
Density	Flow properties and compressibility	Bulk density, tapped density, Hausner ratio, Carr's index	Amount of granulating agent, Amount of granulating liquid, Drying temperatures and drying methods employed	(Carr, 1965; Hausner, 1967)
Hardness, friability and tensile Strength	Mechanical strength needed for packaging and compression into tablet or for further coating	Friabilator, texture analyzer to estimate crushing strength	Amount and type of binder used in the granulation, moisture content excipient used, drying temperature, drying method	(Heng and Koo, 2001)

Table 1.2. Summary of Evaluation Parameters and Factors Influencing the Quality of Pellets (Cont.)

Parameter	Significance	Method used for evaluation	Factors influencing quality of pellets	References
Specific surface area	Drug release behaviour of formulation	Gas adsorption technique	Factors influences pellets size and shape	(Bashaiwoldu et al., 2004)
Surface morphology	Friability, flowability, wettability, adhesion to various substrates during coating and drug delivery behavior	Scanning electron microscopy, Mercury intrusion porosimetry	Drug composition and volume of the granulating liquid, additives, extrusion and spheronization variables and drying conditions	(Zhang et al., 2012)
Flow properties	Packaging and tableting/capsule filling	Angle of repose, shear strength determinations, and flow-rate measurements	Particle size, particle size distribution, particle shape, surface texture, surface energy, surface area influences flow properties	(Amrutkar et al., 2012)
Disintegration time	Immediate release pellet	Reciprocating cylinder method (USP Apparatus 3), Specially modified tablet disintegration tester	Porosity of pellets, amount of binder used, additive, method of drying employed	(Huyghebaert et al., 2005)
In vitro drug release studies	Assessment of the quality of modified release formulations	USP apparatus type1/ USP apparatus type 2	Nature of the carrier solid, aqueous solubility of the drug, physical state of the drug in the matrix, drug load and the presence of additives such as surfactants	(Thommes and Kleinebudde, 2006)
In vivo studies	Assessment of in vivo pharmacokinetic parameters	Suitable animal model (e.g. rat, rabbits, humans etc.)	Quality of pellets designed	(Sawicki, 2002; Setthacheewakul et al., 2010)

1.7.1. Physicochemical Properties

Mean particle size of pellets is important parameter which mainly influencing the drug content in pellets and drug release behavior of formulation (Husson et al., 1992). Pellets should have narrow particle size distribution. Shape of the pellets can be represented by calculation of aspect ratio and pellet circulatory (Bornhöft et al., 2005). Aspect ratio and pellet circulatory can be determined easily by using software from digitalized image. Porosity of pellets influences the disintegration, dissolution, adsorption and diffusion of the drug from pellets. It can be measured by gas adsorption method or mercury intrusion porosimetry technique. Further, packing behaviour of particles can be assessed by estimating bulk density which is greatly influenced by the diameter of spherical granules. Tapped and bulk densities depends on the pellets size, shape, surface texture and the material used for the manufacture of pellet. Further, cohesion between particles causes increase in tapped tensity and decrease in bulk density resulting into higher value of Hausner ratio (Chien, 1992a). Higher values of Hausner ratio represent poorer flow (Rowe, 1985). In addition, irregular pellets exhibit larger difference in tapped density and bulk density than more regularly shaped spheres which leads to higher Carr's index values (Carr, 1965; Neumann, 1967) and thus poor flow properties (Chien, 1992a). The pellets must exhibit sufficient hardness/tensile strength as far as packaging and further processing (coating, compression etc.) is concerned. Mechanical strength of pellets can be evaluated using texture analyzer and friabilator (Bashaiwoldu et al., 2004; Pund et al., 2011). In addition use of particle hardness tester for estimation of granule strength has also been reported in literature. Different types of equipment (friabilators or tumblers) were used by various researchers for different time intervals and at different speed in order to evaluate friability of pellets (Bianchini et al., 1992; Alvarez et al., 2002). Use of glass and steel balls in friabilator have also been reported as attrition agent to determine abrasion and friability of pellets (Lövgren and Lundberg, 1989; Pund et al., 2011). The compression behavior of pellets into tablet indicates stress holding capability of pellet. Deforming pellets are more suited than fragmenting pellets for successful compression into tablet. Further, surface texture and internal structure of pellets are the critical factors which influences pellets properties such as friability, flowability, wettability and adhesion to various substrates during coating and drug delivery behavior. Thus, morphology of the surface and internal

structure of dried pellets can be evaluated using scanning electron microscope (SEM) (Gómez-Carracedo et al., 2008). SEM images and mercury intrusion porosimetry (MIP) data gives information about the surface texture and inner structure of pellets. Pellets should show desired flow properties as it is presented as tablet or capsule in final form. Flow properties of the pellets can be determined by using various methods such as angle of repose, shear strength determinations and flow-rate measurements etc. Particle size, particle size distribution, particle shape, surface texture, surface area etc. influences the flow properties of the pellets (Podczeck and Newton, 1994). Disintegration time is one of the important evaluation parameter in case of immediate release pellet formulations. Furthermore, disintegration test for pellets was reported by using the reciprocating cylinder method (USP Apparatus 3)(Huyghebaert et al., 2005). Some researchers also performed disintegration of pellets in specially modified tablet disintegration tester (Thommes and Kleinebudde, 2006).

1.7.2. In Vitro Floating Study

In vitro floating studies of pellets in USP dissolution testing apparatus type 2 and beaker has been reported by various researchers (Sungthongjeen et al., 2006; Amrutkar et al., 2012). Pellets can be charged to dissolution medium maintained at 37 ± 0.2°C with appropriate agitation. The time required for pellets to starts floating can be considered as floating lag time whereas percentage pellets floating can be easily estimated by counting pellets (Ichikawa et al., 1991).

1.7.3. In Vitro Release Study

In designing of the drug delivery system, in vitro drug dissolution studies are one of the important parameter for the assessment of the quality of formulation especially in modified release formulations. In vivo bioavailability of the drug can be predicted from the data obtained from the in vitro dissolution studies of formulation since in vivo absorption of the drug depends on the release of the drug from the dosage form. The release of the drug from the pellets is mainly influenced by the composition, hardness, size and polymer used for coating of pellets. In vitro release of drug from pellets is determined by using USP apparatus type 1 (Flament et al., 2004; Cheboyina and Wyandt, 2008a) and by USP

apparatus type 2 (Bruce et al., 2003; Costa et al., 2003; Dashevsky et al., 2004; Souto et al., 2005; Hu et al., 2006; Thommes and Kleinebudde, 2006). The nature of the carrier solid, aqueous solubility of the drug, physical state of the drug in the matrix, drug load and the presence of additives such as surfactants influence the drug release profiles from pellets.

1.7.4. In Vivo Evaluation

In vivo pharmacokinetic evaluation of GRDDS have been reported in various animal models which includes rats (Elmowafy et al., 2009), rabbits (Joseph et al., 2002; Ali et al., 2007; Zhang et al., 2012), dogs (Klausner et al., 2003a; Patel et al., 2009) and humans (Sato et al., 2004b; Xu et al., 2006; Goole et al., 2008c; Shakya et al., 2013). In case of floating drug delivery system, gamma scintigraphy technique has already been reported for the evaluation of gastroretention in vivo in rabbits (Zhang et al., 2012) as well as in humans (Goole et al., 2008c). Good correlation between in vitro and in vivo study results have been reported for floating gastroretentive drug delivery system (Zhang et al., 2012).

1.8. GRDDS: Industrial Perspective and Regulatory Considerations

Although various GRDDSs have been designed in past only few technologies are successful in the market (Table 1.3). Therefore, there is a need to analyze and rectify the conflict between research and industrial implementation. During initial product development phases, evaluation of the impact of fed and fasted condition on the product performance should be considered. Dual working technologies could be a better option to rectify the drawbacks associated with various GRDDS. Development of successful GRDDS depends on the selection of appropriate drug and excipients. Excipients must fulfill the basic requirement of drug delivery system without influencing the therapeutic efficacy of drug. The United States Food Drug Administration (USFDA) provides the database of excipients that have been used in appropriate drug product (USFDA, 2011). Detailed preformulation studies should be carried out before product development as it provides rationale for product design. Physicochemical properties of drug decides the drug performance and the development of efficacious dosage forms.

Table 1.3. Summary of Marketed Gastroretentive Floating Drug Delivery Systems

Technology	Company	Product	Active pharmaceutical ingredient
Effervescent floating system	Ranbaxy, India	Zanocin OD	Ofloxacin
		Riomet OD	Metformin hydrochloride
		Cifran OD	Ciprofloxacin
Coated multi-layer floating and swelling system	Sun Pharma, India	Baclofen GRS	Baclofen
Minextab Floating® system	Galenix, France	Metformin hydrochloride	Metformin hydrochloride
		Cafeclor LP	Cefaclor
		Tramadol LP	Tramadol
Floating, CR capsule	Roche, UK	Madopar	Levodopa and benserazide
		Valrelease	Diazepam
Effervescent floating liquid alginate preparation	Reckitt Benckiser Healthcare, UK	Liquid gaviscon	Alginic acid and sodium bicarbonate
Bilayer floating capsule	Pharmacia Ltd., UK	Cytotec	Misoprostol (100/200 µg)
Floating liquid alginate	Pierre Fabre Medicament, France	Topalkan	Aluminum magnesium antacid
Effervescent and swelling-based floating system	Sun Pharma, Japan	Prazopress XL	Prazosin hydrochloride
Foam-based floating system	Sato Pharma, Japan	Inon Ace Tablets	Simethicone

Regulatory guidelines released by various regulatory bodies must be followed to evaluate the product performance precisely. International conference on harmonization provides the specifications (ICHQ6A) for test procedure and acceptance criteria for new drug substance and new drug product and chemical substance (ICH, 1999). In addition, as per the current good manufacturing practices, there is need to identify and optimize potential and critical in-process controls in order to design a quality product (GMP, 2009). Statistical tests can be applied to optimize the critical formulation and process variables as it influences the quality and performance of GRDDS. Different statistical design such as factorial design (Varshosaz et al., 2007; Nagarwal et al., 2009; Narkar et al., 2010), central composite design (Badhan et al., 2009; Pal and Nayak, 2011; Awasthi et al., 2012), Box-Behnken design (Miyazaki et al., 2008) and simple lattice design (Patel et al., 2007; Kamila et al., 2009) are used for optimization of GRDDS. Successful optimization studies should be followed by scale up and process validation studies to assess the reproducibility and adaptability of developed manufacturing process by large scale production machine. Final phase of development of GRDDS is bioequivalence and bioavailability studies. In vitro-in vivo correlation (IVIVC) explains the relationship between in vitro release and in vivo absorption. Therefore, by using the IVIVC data in vivo fate of GRDDS can be predicted. GRDDS shows excellent in vitro results but fail to give desirable in vivo performance. In consequence, dual working system based on the combined mucoadhesive and floating principles may improve its in vivo performance thus it has more potential to increase industrial implementation of the GRDDS.

1.9. Summary

Despite the enormous development in the drug delivery systems, delivery systems usually suffers from the major problems such as poor physical properties leading to instability and/or poor bioavailability, non-specific drug release leading to a poor bioavailability, intra and inter-subject pharmacokinetic variations, poor control on drug release and fluctuating plasma levels. Most of these reasons are interlinked but need to be addressed individually. Gastroretentive drug delivery systems are becoming increasingly important for successful delivery of various drugs in order to improve their therapeutic outcome. Multiple-unit approach for gastroretention provides various advantages over single unit system. Among

the gastroretention techniques, floating delivery systems and mucoadhesive systems have more industrial applicability. Therefore, these delivery systems need to be developed and optimized in order to meet specific requirements of the drug and disease conditions. Moreover, preparation of tailor-made floating delivery system may offer significant advantage in case of drug which specifically need to be delivered in upper GIT.

1.10. Problem Definition and Research Objectives

In 1817, James Parkinson was the first to describe 'paralysis agitans' (Parkinson, 1817). This was later named Parkinson's disease (PD). PD is second most common progressive neurodegenerative disorder of the central nervous system manifested by death of dopamine-containing cells in the substantial nigra, a region of the midbrain (Bowman and Rand, 1980). PD is characterized by motor impairment which includes tremor at rest, akinesia, bradykinesia, rigidity and loss of postural reflexes (Fahn and Przedborski, 2005). Currently PD is treated symptomatically. Levodopa (LD) is a precursor of neurotransmitter dopamine is used in clinical management of PD and syndrome. In the central nervous system, it converts to the naturally occurring neurotransmitter dopamine, which is necessary for proper motor function and cognitive processes. In contrast, D-dopa is not used in treatment of PD as it does not convert to dopamine and causes granulocytopenia (Cotzias et al., 1967). LD is administered concomitantly with aromatic-L-amino-acid decarboxylase (dopa-decarboxylase) inhibitor (e.g. Carbidopa, Benserazide), catechol-O-methyltransferase (COMT) inhibitor (e.g. Tolcapone, Entacapone) and monoamine oxidase (MAO) inhibitor (e.g. Selegilline) in order to avoid its peripheral metabolism (LeWitt, 2008). LD therapy shows clinical response in case of all signs and symptoms of PD. At early stage of PD, LD therapy shows beneficial clinical response due to dopamine buffering capacity. Among the five receptors located in the various areas of basal ganglia only D1 and D2 receptors are located in the striatum (Deogaonkar and Subramanian, 2005). Depletion of dopamine level at these two receptors leads to development of PD. With PD progression, duration of clinical response to LD therapy decreases because of progressive degeneration of nigral dopaminergic neurons and loss of dopamine buffering capacity, which leads to motor fluctuations named dyskinesia and “on-off” effects (Warren Olanow et al., 2013). A gradual dose escalation is recommended in order to avoid decline in the

function. Studies reported that the "on-off" fluctuations in the responses are associated with the LD dose and frequency of administration. Therefore, there is continuous interest in development of strategies for slowing and targeting the release of LD to prolong and improve therapeutic outcome and to reduce the frequency of the administration. Chronic use of LD results in development of motor complications and dose related dyskinesia (Fahn, 1974; Marsden and Parkes, 1976; Nutt et al., 1984). In addition, motor complications are associated partly with the fluctuating plasma level of LD (Fabbnni et al., 1988; Mouradian et al., 1988). Various research scientists in pharmaceutical field are continuously implementing numerous strategies to improve the dopaminergic function in PD for better therapeutic outcome which includes delay of the need for LD, reduction of the cumulative dose of LD, avoidance of the pulsatile stimulation of dopamine receptors and neuroprotection to slow down disease progression. In addition, various dosage forms and delivery systems have already been developed for LD like oral drug delivery system (Hauser et al., 2013; Hsu et al., 2015), pulmonary drug delivery system by nasal inhalation (Jackson et al., 2013; Sharma et al., 2014), subcutaneous injection, intraduodenal (Chang et al., 2016) and intravenous infusion etc. to improve therapeutic efficacy in PD.

Sinemet® is the first marketed immediate release (IR) oral dosage form containing a combination of LD and Carbidopa (CD) (Hsu et al., 2015). Madopar® is the other commercialized product containing LD and the other peripheral decarboxylase inhibitor (Benserazide)(Gasser et al., 2013). Liquid and dispersible formulations have also been reported for delivery of LD (Djaldetti et al., 2002). At early stage of PD, buffering and compensatory mechanisms are intact and hence, conventional oral medication shows beneficial therapeutic response. As PD progresses, dopaminergic terminals continue to degenerate and are no longer able to buffer the exogenous LD adequately which leads to "wearing off phenomenon" (Sage and Mark, 1992; Nyholm, 2007). In addition, fluctuating LD concentrations associated with immediate release system leads to abnormal and intermittent stimulation of dopaminergic receptor which results into oscillating clinical response during chronic treatment of PD with conventional tablets present in market. Pulsatile stimulation of D1 and D2 receptors and subsequent desensitization induces abnormal involuntary movements called as dyskinesia (Deogaonkar and Subramanian, 2005). Although solid dosage forms as well as liquid/dispersible preparations are found to

provide immediate supply of LD, due to short half-life of LD it needs to be administered frequently which leads to fluctuating plasma concentration and associated motor complications. These formulations fail to provide adequate relief due to narrowing of therapeutic index with PD progression (Figure 1.6). Therefore, various sustained release formulations have been investigated in order to maintain stable plasma concentration and to improve therapeutic efficacy which includes Sinemet® CR 50/200, Madopar® CR 50/200.

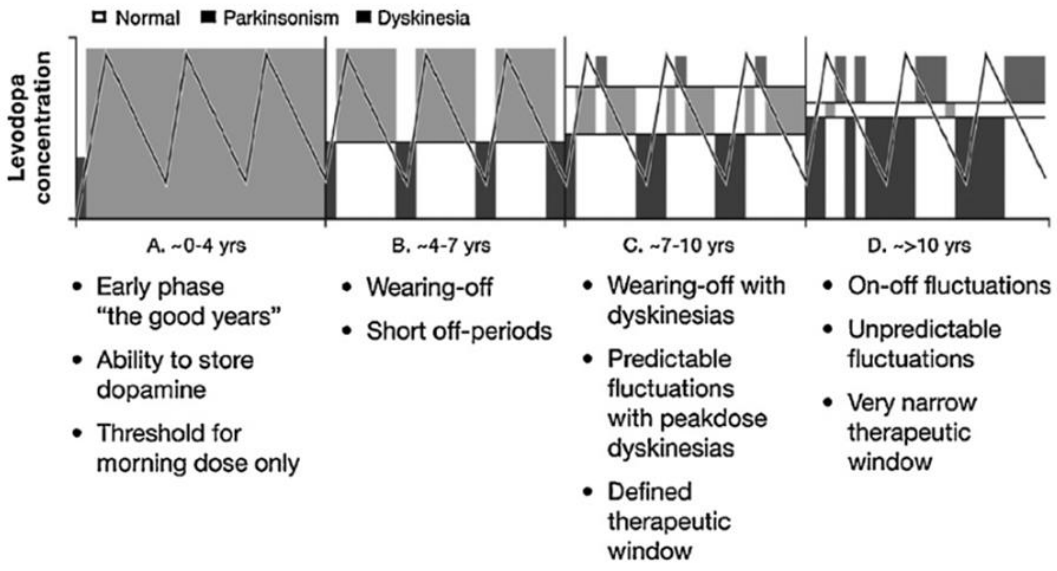


Figure 1.6. Narrowing of therapeutic window for oral Levodopa with disease progression (Nyholm, 2007)

LD exhibits narrow absorption window at upper part of the small intestine (specially duodenum) therefore amount of drug released from sustained release formulations above absorption site would get absorbed and the remaining drug in sustained release formulation becomes unavailable for absorption once controlled release (CR) tablet goes below absorption site of LD. In addition, delayed onset of action in case of sustained release formulation makes them unsuitable for LD delivery. Therefore use of dual release (DR) formulations could be alternative solution for sustained release dosage forms (Madopar® DR). As discussed earlier, gastric emptying determines the rate and extent of LD

absorption, because LD has narrow absorption window at the upper part of the small intestine (Rozenberg et al., 2013). In addition to this, LD and CD both have pH dependent solubility and stability (Goole et al., 2007). Both LD and CD are more stable and soluble at acidic condition which is also responsible for uneven absorption of LD through GIT resulting in to fluctuating plasma level. Fluctuating LD plasma level further leads to "on-off' fluctuations and motor complications in PD patients (Goole et al., 2007). Further, LD delivery also suffers from inter subject variability probably due to variable gastric emptying (Contin and Martinelli, 2010). Fluctuations in LD plasma concentration are associated with IR, CR and dual release formulations as these dosage forms fails to provide constant supply of LD at its absorption site. Therefore, gastroretentive dosage forms have been designed in order to obtain stable clinical effect by reduction of fluctuations.

Controlled release gastroretentive dosage forms (CR-GRDF) based on the unfolding membranes has also been reported to improve LD oral delivery (Klausner et al., 2003c). Although unfolding membrane showed encouraging results in dogs, therapeutic use of such dosage forms in humans is avoided due to the potential risk associated with it, like undesirable extended gastric retention time which may further cause GI problems. Further, dosage forms based on hydrodynamically based systems (HBS) have also been reported (Timmermans and Möes, 1990; Goole et al., 2008c). Goole and co-workers studied floating properties of the marketed product, Prolopa® HBS125 (Madopar® HBS) using Resultant Weight (RW) method (Timmermans and Möes, 1990; Goole et al., 2008c). There was no initial floating lag time for HBS capsule due to very low initial density. Results indicated that capsule failed to maintained longer floating probably due to development of hydrodynamic equilibrium (Goole et al., 2008c). Further, Prolopa® HBS 125 failed to maintain similar release profile of LD and Benserazide after 5 h (Goole et al., 2008c). Compared to the single unit floating systems, multiple unit floating systems are found to be more reliable and reproducible in prolonging gastroretention and in maintaining stable plasma concentration in vivo. In this regards swellable floating minitablets were designed which showed most even distribution of the plasma level of LD (Goole et al., 2008c). However, no statistically significant difference was observed between gastroretention time, area under curve (AUC), maximum plasma concentration (C_{max}), and time to maximum concentration (T_{max}) values obtained from floating minitablets and Prolopa® HBS 125. In

addition, continuous infusion of LD and CD through intraduodenum has been reported for the severely fluctuating PD patients. Researchers observed normal control of moment in patient treated with intermittent oral LD dosing due to constant supply of dopamine by avoiding fluctuating dopamine levels (Nutt et al., 2000). On this basis, suspension of LD and CD (Duodopa®, Solvay Pharmaceuticals) was developed that can be administered in duodenum by using portable pump through gastrostomy. Although, higher improvement in motor function has been reported, intra duodenal infusion suffers from certain disadvantages which makes it less convenient for patient use. Infusion treatment has most common problems like technical difficulties with the pumps, transient infection within the gastrostomy, change or adjustment of the catheter by gastroscopy or X-ray guidance and sliding back of the tip from the duodenum into the stomach during infusion etc. These mechanical limitations are more frequent in patient with severe PD as major tremors are associated with dementia (Nyholm et al., 2008). Recently, sustained release pellet formulations have also been proposed for LD and CD delivery (Rubin, 2000; Hsu et al., 2013; Kulkarni et al., 2015). Reported pellet delivery system comprised of the IR pellets, CR pellets and tartaric acid pellets. In this system, tartaric acid pellet are also administered along with LD and CD pellets to maintain acidic microenvironment thereby stability and solubility of both LD and CD can be improved. Further, pellets releases LD and CD at controlled rate. Thereby, stable plasma concentration of LD can be achieved. Although theses delivery systems have been proposed to overcome the problems associated to LD delivery like unpredictable gastric emptying, pH dependent solubility and stability, "on-off" fluctuations etc, erratic absorption of the LD still appears with the dosage forms. Therefore, already reported GRDDS and other delivery systems for LD need to be improved in order to provide successful treatment in PD patients.

Thus, the principle objective of the present research work was to design and develop modified release floating multi unit particulate delivery system to deliver LD and CD at controlled rate to its site of absorption in order to improve bioavailability and to maintain stable plasma concentration. To achieve this broad objective following specific studies were carried out in the present work.

1. For efficient product development, preformulation studies were performed to establish necessary physicochemical data of LD and CD prior to the formulation development. Studies were performed to address product specific questions related to the drug solubility, stability, drug-excipient compatibility etc.

2. Formulation development was carried out by identifying and optimizing the critical factors in the design and the process using optimization techniques. The prepared floating pellets were extensively investigated for surface morphology and shape, particle size and size distribution, drug content (loading and entrapment efficiency) and in vitro drug release. Further, optimized formulations were studied for the drug product stability and reproducibility.

3. Pharmacokinetic studies were performed to investigate in vivo performance of the prepared floating pellets in rats. Pharmacodynamic studies were carried out in lesioned rats to evaluate the benefit of designed delivery systems with respect to the oral solution and immediate release pellets containing LD and CD. Obtained pharmacokinetic data was modeled using non-compartmental analysis to predict the preclinical efficacy and clinical efficacy of the formulations in diseased animals.

4. Suitable analytical and bioanalytical methods were developed based on the high performance thin layer chromatography and liquid chromatography technique for determination of the drugs in preformulation samples, formulation samples and biological matrix.

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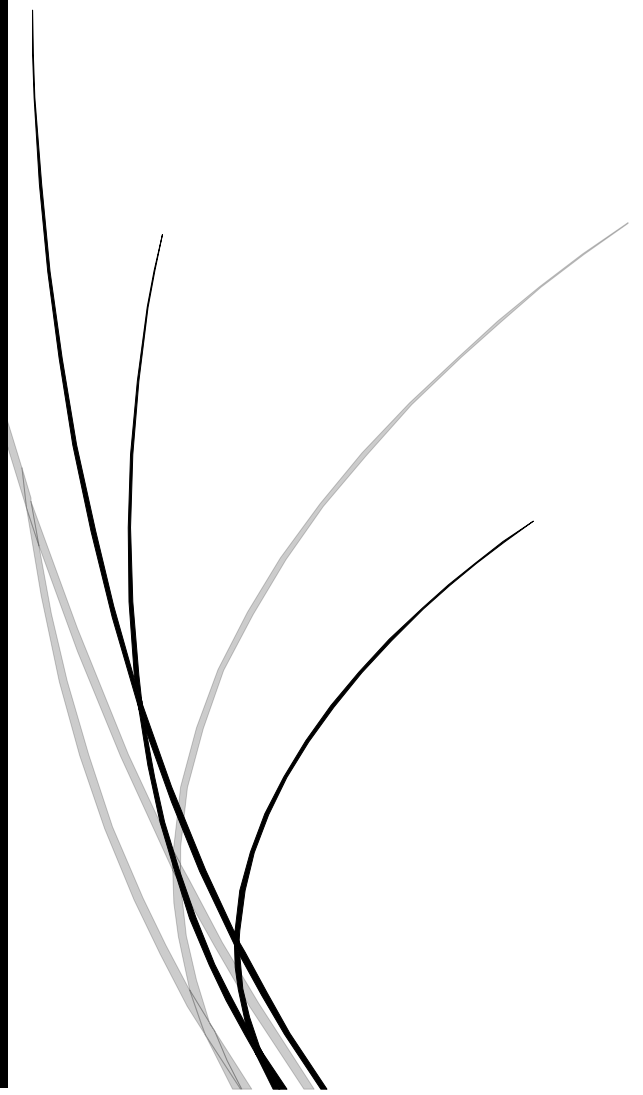
2 DRUG PROFILES: LEVODOPA AND CARBIDOPA



2.1. Introduction

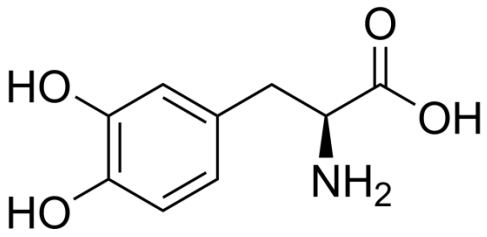
Levodopa (LD) is an endogenous chemical produced and used as part of the normal biology of human being, some animals and plants. It is produced in animals and human via biosynthesis from L-tyrosine. LD is precursor of various neurotransmitters such as dopamine, norepinephrine (noradrenaline) and epinephrine (adrenaline) collectively known as catecholamines. LD is manufactured as a psychoactive drug and used in clinical management of Parkinson's disease (PD) and syndrome. In the central nervous system, LD converts to a naturally occurring neurotransmitter dopamine, which is necessary for proper motor function and cognitive processes. LD is co-administered with Carbidopa (CD) in pharmaceutical formulations containing 10-25% of CD for better therapeutic effect and to reduce side effects associated with LD treatment.

2.2. Drug Information

2.2.1. Levodopa

Parameters	**Description**
Synonym:	L-Dopa, Levodopum, Dopa, dihydroxyphenylalanine
Category:	Antiparkinson's agent
Therapeutic class:	Dopaminergic agent Antidyskinetics
Chemical class:	Catecholamines and derivatives
Approval status:	United States of America, United Kingdom, European Union, Japan, India and other countries
Chemical name:	(2S)-2-amino-3-(3, 4-dihydroxyphenyl) propanoic acid (IUPAC)
Chemical formula:	$C_9H_{11}NO_4$
Generic name:	Levodopa
Proprietary name:	Dopar® (LD alone), [LD and CD combination: Sinemet®, Sinemet® CR (US), Parcopa®, Atamet®]

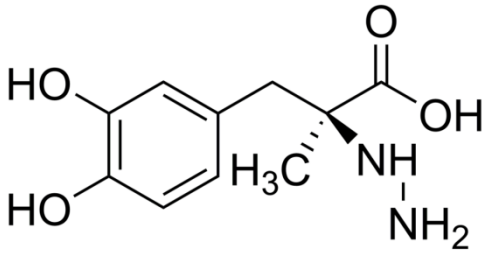
Chemical structure:



CAS registry number:	59-92-7
Melting point:	284 - 285°C
Molecular weight:	Average: 197.19, Monoisotopic: 197.07
Water solubility profile:	Slightly soluble
BCS Class:	Class 1 (Max. dose 250 mg, WHO), Class 2 (Max. dose 500 mg, UK).
Physical state:	Solid (white odorless powder)
pH of 1%, w/v suspension:	4.5 to 7
Hydrophobicity (Log P):	-2.39
Ionization constant:	2.3, 8.7, 9.7, 13.4 (Reynolds, 1982; Moffat et al., 1986)
Optical activity:	$[\alpha]_{25/D}$ = -11.5°± 1°, c = 1 in 1 M hydrochloric acid (L)
Physical stability:	Stable in solid state
Photo stability:	Protect from light and air

2.2.2. Carbidopa

Parameters	**Description**
Synonyms:	α-Methyldopa Hydrazine, Carbidopum, Carbidopum Monohydricum, Karbidopa, Karbidopa monohydrate,
Category:	Antiparkinson’s agent

Therapeutic class:	Enzyme inhibitor, Dopaminergic agent, Antidyskinetic
Chemical class:	Catecholamines and derivatives
Approval status:	United States of America, United Kingdom, European Union, Japan, India and other countries
Chemical name:	(2S)-3-(3,4-dihydroxyphenyl)-2-hydrazinyl-2-methyl propanoic acid (IUPAC)
Chemical formula:	$C_{10}H_{16}N_2O_5$
Generic name:	Carbidopa
Proprietary name:	Lodosyn® (tablet contains only carbidopa)
Chemical structure:	
CAS registry number:	28860-95-9 (anhydrous), 38821-49-7 (monohydrate)
Melting point:	208 - 210°C
Molecular weight:	Average: 244.24, Monoisotopic: 244.11
Water solubility profile:	Slightly soluble
BCS Class:	Class 3
Physical state:	Solid (white odorless powder)
pH of 1%, w/v suspension:	4 to 5
Hydrophobicity (Log P):	-1.9
Optical activity:	$[\alpha]_{26/D} = -13.82°$, c = 0.3 in methanol (L)
Physical stability:	Stable in solid state
Photo stability:	Protect from light and air

2.3. Pharmacodynamics and Therapeutic Applications

PD is a degenerative disorder of the central nervous system (Bowman and Rand, 1980) which is also known as idiopathic (unknown cause) or primary parkinsonism, hypokinetic rigid syndrome (HRS) or paralysis agitans. A pathological hallmark of PD is death of pigmented, dopaminergic neurons of the substantia nigra pars compacta (SNpc) that provide dopaminergic innervations to the striatum (caudate and putamen), a region of the midbrain. At early stage of the PD, the symptoms are mostly related to the movement like shaking, muscular rigidity, bradykinesia (slowness and poverty of movement), impairment of postural balance leading to difficulty with walking, gait etc. At later stage of PD, thinking and behavioral problems may arise. Dementia commonly occurs in the advanced stages of the PD. Further, depression is the most common psychiatric symptom associated with advanced PD. Other symptoms include sensory, sleep and emotional problems. Death frequently results from complications of immobility, including aspiration pneumonia or pulmonary embolism.

The prognosis of PD can be controlled by pharmacological treatment. In most cases, good mobility can be maintained for many years and the life expectancy increases substantially. PD can be treated by maintaining appropriate level of dopamine inside brain. Exogenous dopamine cannot be administered directly to restore normal levels due to its inability to cross blood-brain barrier. Therefore, LD (metabolic precursor of the dopamine) is used as gold standard in the treatment of PD. LD permeates across blood brain barrier (BBB) easily by a membrane aromatic amino acid transporter. Inside brain, LD is metabolized to dopamine by enzyme dopa decarboxylase. This conversion occurs primarily within the presynaptic terminal of dopaminergic neurons in the striatum. This synthesized dopamine is responsible for the therapeutic effectiveness of LD in PD (Figure 2.1). After secretion, dopamine present in synaptic cleft is transported, either back to dopaminergic terminals or into postsynaptic cells, where it may be restored in granules (neurons) or metabolized by monoamine oxidase (MAO) and catechol O-methyl transferase (COMT) (neurons and non-neurons) (Goodman, 1996).

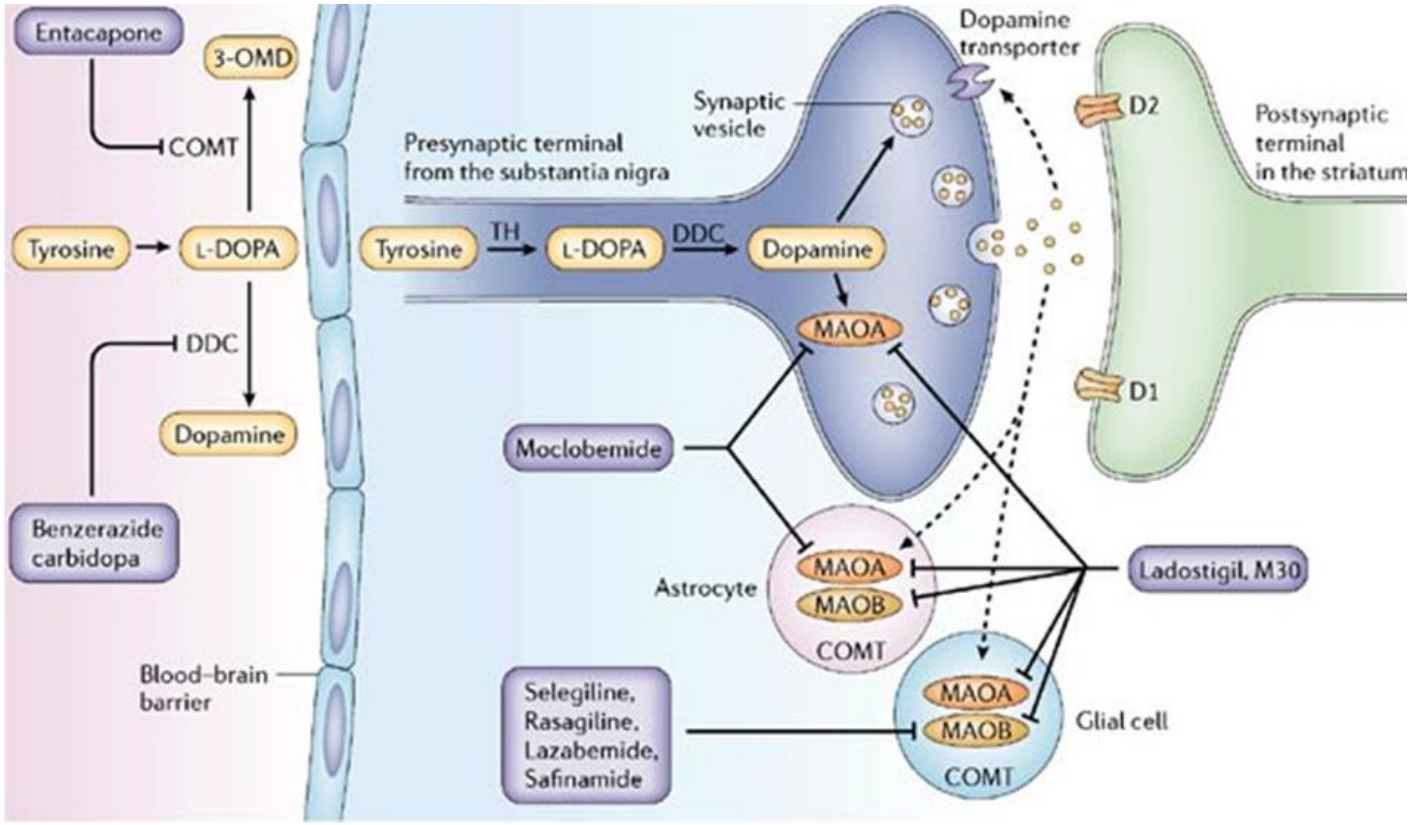


Figure 2.1. Dopamine synthesis and mechanism of action (Youdim et al., 2006).

[**L-DOPA:** Levodopa; **3-OMD:** 3-O-methyldopa; **COMT:** catechol-O-methyl transferase; **DDC:** dopa decarboxylase (aromatic L-amino acid decarboxylase); **TH:** tyrosine hydroxylase; **MAOA:** monoamine oxidase-A; **MAOB:** monoamine oxidase-B].

The LD therapy shows pharmacological response in case of all signs and symptoms of PD. In early stage of PD, LD shows some beneficial effects suggesting the capability of the nigrostriatal system to store and release dopamine. However, long term LD therapy cannot be useful because nigrostriatal system loses this "buffering" capacity with time and therefore patient's motor state may fluctuate dramatically with each dose of LD. Most common problem associated with the long term LD therapy is "wearing off" phenomenon resulting from loss of dopamine producing cells in brain with disease progression. After each dose of LD, mobility improves effectively for short period but at the end of dosing interval rigidity and akinesia returns rapidly. This can be treated by increasing the dose and frequency of LD administration, but high LD plasma concentration further leads to excessive and abnormal involuntary movements a condition called dyskinesia. Dyskinesias or dystonias may be triggered due to fluctuating LD level and these movements can be as uncomfortable and disabling as rigidity and akinesia.

In later stage of PD, patient on LD treatment for long duration shows fluctuating benefit from their medication called as "on-off phenomenon". On off phenomenon in PD refers to a switch between mobility and immobility in LD treated patients (Bhidayasiri and Tarsy, 2012). "On" refers to mobility due to beneficial effect from their medication and "off" refers to loss of effect that usually occur at the end of the dose or "wearing off" worsening of motor functions or much less commonly, as sudden and unpredictable motor fluctuations (Bhidayasiri and Tarsy, 2012). LD treatment associated dyskinesia and fluctuations are greatly reduced when LD levels are maintained constant by intravenous infusion. In addition, on off phenomenon can also be reduced by use of sustained release formulations and administration of total daily dose into divided forms more frequently.

2.4. Pharmacokinetics

In modern clinical practice, LD is usually administered in the form of fixed dose combination formulation with aromatic L-amino acid decarboxylase (AADC) inhibitors, either CD or benserazide (BEN)(Nutt et al., 1985). Formulations contains LD/CD dose in the ratio of 10:1 (w/w) or 4:1 (w/w), while LD/BEN dose is in the ratio of 4:1 (w/w). Therefore, pharmacokinetics of LD is discussed along with CD.

2.4.1. Absorption

Pharmacokinetic studies in humans demonstrate that, LD is absorbed rapidly from upper gastrointestinal tract (Gundert-Remy et al., 1983) by the large neutral amino acid (LNAA) transport carrier system (Nutl and Fellman, 1984; Koller and Rueda, 1998) with peak plasma concentration level (C_{max}) reaching within 0.5 to 2 h (Jubelt and Miller, 1995) Absorption of aromatic and branched chain neutral amino acids (e.g. histidine, phenylalanine, threonine, leucine, isoleucine, tyrosine, valine and tryptophan) is also facilitated by this active transport mechanism which is saturable and sodium independent (Wade et al., 1973; Nutl and Fellman, 1984; Tamai et al., 1998). Because of limited transport capacity of this transport system, dietary proteins competes with LD during absorption from energy dependent proximal small intestine absorption site (Cedarbaum, 1987; Goetz, 1992) and thus delays absorption of LD administered with diet rich in protein (Frankel et al., 1989; Robertson et al., 1991).

During absorption, LD undergoes metabolism by AADC enzyme present in the stomach, intestinal wall and at other peripheral sites. Thus, this enzyme serves as a significant barrier to the absorption and transport of the intact LD (Nutl and Fellman, 1984; Cedarbaum, 1987). Only about 30% of administered dose of LD reaches to the systemic circulation as unmetabolized LD and probably less than 1% penetrate in to the CNS (Nutl and Fellman, 1984). Therefore, absorption can be enhanced by simultaneous administration of peripheral AADC inhibitor like CD (Bowman and Rand, 1980; Cedarbaum, 1987; Goetz, 1992; Goodman, 1996) or COMT inhibitors such as tolcapone (Männistö and Kaakkola, 1990; Kaakkola et al., 1994). Although, AADC inhibitors does not cross blood brain barrier, they inhibits peripheral metabolism and markedly increases the fraction of unmetabolized LD available to cross blood brain barrier, thus appropriate level of dopamine is maintained inside the brain.

AADC inhibitors (e.g. CD) increases C_{max} and decreases time to peak serum concentration (t_{max}) (Table 2.1) (Cedarbaum, 1987). Administration of LD along with CD results in approximately 2 fold increase in the plasma LD elimination half-life ($t_{1/2}$) (Nutt et al., 1985; Gancher et al., 1987) and 70% reduction in the total daily LD dose required to produce therapeutic effect. After oral administration absolute bioavailability of LD was reported in the range of 29 to 37% when administered alone (Sasahara et al.,

1980) and in the range of 80 to 98%, when administered with CD (Table 2.1). Moreover, population PK studies demonstrated higher bioavailability of LD in women compared to men. However, sex-related differences in drug disposition are likely to be of minor relevance in administration of drugs (Martinelli et al., 2003). The side effects associated with LD therapy are due to peripheral conversion of LD in to dopamine which can also be reduced by use of AADC inhibitor. Therefore, LD is typically co-administered with CD in pharmaceutical formulations for better therapeutic effect and to reduce the incidence of nausea and other gastrointestinal (GI) side effects due to peripheral conversion of the drug to dopamine.

Table 2.1. Summary of Pharmacokinetic Properties of LD in Combination with Peripheral Decarboxylase Inhibitor

Drug	Dose (mg)	C_{max}[a] (µg/L)	t_{max}[b] (h)	F[c] (%)	Vd[d] (L/Kg)	CL[e] (L/h)	$t_{1/2}$[f] (h)
LD alone	300	1.8	1.4	33.0	0.9	46.8	1.1
LD/ BEN	100/25	4.0 - 35.9	0.50 - 1.0	80.0	0.8	25.8	1.5 - 2.0
LD/ CD	250/25 250/50	1.9 2.7	0.5 - 2.0	80.0 - 98.0	1.7	25.2	1.5

[a] C_{max}: peak (maximum) plasma concentration
[b] t_{max}: time to C_{max}
[c] F: oral bioavailability
[d] Vd: volume of distribution
[e] CL: clearance
[f] $t_{1/2}$: elimination half life

High gastric acidity, delayed stomach emptying and presence of certain amino acids in gastric content may influence the absorption of LD (Cedarbaum, 1987). The gastric emptying rate is the principle determinant of absorption of LD. Erratic absorption of LD is observed in human volunteers with the altered pattern of gastric emptying (Evans et al., 1981; Kurlan et al., 1988; Robertson et al., 1990; Djaldetti et al., 1996; Müller et al., 2006). Patient above 65 years suffering from PD showed increased area under curve (AUC) for LD due to reduced gastric emptying. Moreover, high incidence of multiple peak plasma concentrations has been observed in this subpopulation which leads to

fluctuating clinical response (Evans et al., 1981; Horowitz et al., 1984). The t_{max} has been reported to be increased in presence of food (especially fat) resulting from delayed gastric emptying (Baruzzi et al., 1987; Robertson et al., 1991; Crevoisier et al., 2003). Therefore, it has been recommended that the LD should be administered 30 min before food (Baruzzi et al., 1987). In addition, coadministration of some drugs like antimuscarinic drugs, tricyclic antidepressants and ferrous sulphate may reduce absorption of LD (Pfeiffer, 1996) while benzamide derivatives and cisapride seem to improve LD absorption and thereby motor functions (Neira et al., 1995). Excessive exercise and other activities that influences mesenteric circulation may delays LD absorption (Cedarbaum, 1987; Carter et al., 1992). Moreover, age of patient also influences the pharmacokinetic properties of LD (Robertson et al., 1989; Contin et al., 1991). In order to minimize LD absorption problems, liquid formulations of LD are also used which are reported for faster LD absorption than solid dosage forms like tablets (Contin et al., 1996) and shows faster onset of action.

2.4.2. Distribution

LD is distributed widely to most body tissues except central nervous system (CNS) because of extensive peripheral metabolism. LD crosses BBB by LNAA transport system which is similar to the intestinal epithelium transporter system (Mouradian and Chase, 1988; Frankel et al., 1989; Tsuji, 2005). This active transport system is saturable and stereospecific. Studies in PD patients revealed that the orally administered proteins reverse the antiparkinsons effect of orally (Pincus and Barry, 1987), intraduodenally (Pincus and Barry, 1987) or intravenously (Nutt et al., 1989) administered LD without decreasing the plasma LD concentration level. Further, studies reported PD symptoms even at high plasma LD concentration in patient with high plasma LNAA level. These studies suggest that dietary protein and neutral amino acids are also transported to brain by this LNAA transport mechanism which competes with LD (Avery, 1980; Frankel et al., 1989; Alexander et al., 1994). Further, rate of LD transport across BBB is dependent on the plasma LD concentration and concentration of competing amino acids (Goetz, 1992). Similar to the flux of amino acids across BBB, flux of LD is bidirectional and the net flux of LD is from brain to plasma when plasma concentration of LD falls (Nutl and

Fellman, 1984). Both CD and BEN fail to cross BBB at usual dosages. LD disappears from plasma by biphasic process. However, unlike the apparent volume of distribution, plasma LD clearance is age-dependent (Contin et al., 1991) and it is significantly decreased in the presence of AADC inhibitors (Fabbrini et al., 1987). Studies revealed that the PD progression does not influence the pharmacokinetic properties of LD suggesting the pharmacodynamic origin of motor complications (Fabbrini et al., 1987; Gancher et al., 1987). Motor fluctuations are associated with dopamine deficit with disease progression and fluctuating LD plasma level resulting from administration of immediate release formulations and short $t_{1/2}$ of LD (Gancher et al., 1987; Chase et al., 1988). Small change in LD disposition is insignificant in early stage of PD but can dramatically contribute to the therapeutic response in the advanced stage of PD (Fabbrini et al., 1987).

2.4.3. Metabolism

L-tyrosine is the metabolic precursor of LD. L-tyrosine is converted to LD by enzyme tyrosine hydroxylase. Enzyme tyrosine hydroxylase is expressed in catecholaminergic neurons in the brain, in noradrenergic neurons of sympathetic ganglia and nerves and in epinephrine and norepinephrine containing cells of the adrenal medulla. Orally administered LD (approximately 95%) is metabolized pre-systemically by extracerebral AADC enzyme in stomach, lumen of intestine, kidney and liver (Rahman et al., 1981; Goetz, 1992; Baas and Rietbrock, 1995; Harder et al., 1995). Newly synthesized extracerebral dopamine is unable to cross BBB and thus it does not elicit its effect in brain (Goetz, 1992). This dopamine synthesized from LD peripherally is believed to be responsible for many LD adverse effects which includes cardiovascular complications, GI disturbance etc. These adverse effects of LD can be reduced by increasing the dose ratio of CD to LD from 5:1 to 10:1 (Kaakkola et al., 1985). Inside nigrostriatal neurons, LD is decarboxylated to dopamine by pyridoxine-dependent enzyme AADC. This synthesized dopamine is normally stored in presynaptic terminals of dopaminergic neurons in the striatum (Harder et al., 1995). Dopamine is converted to norepinephrine by enzyme dopamine β-hydroxylase in the adrenal medulla and noradrenergic neurons in the central nervous system (Goodman, 1996) (Figure 2.2).

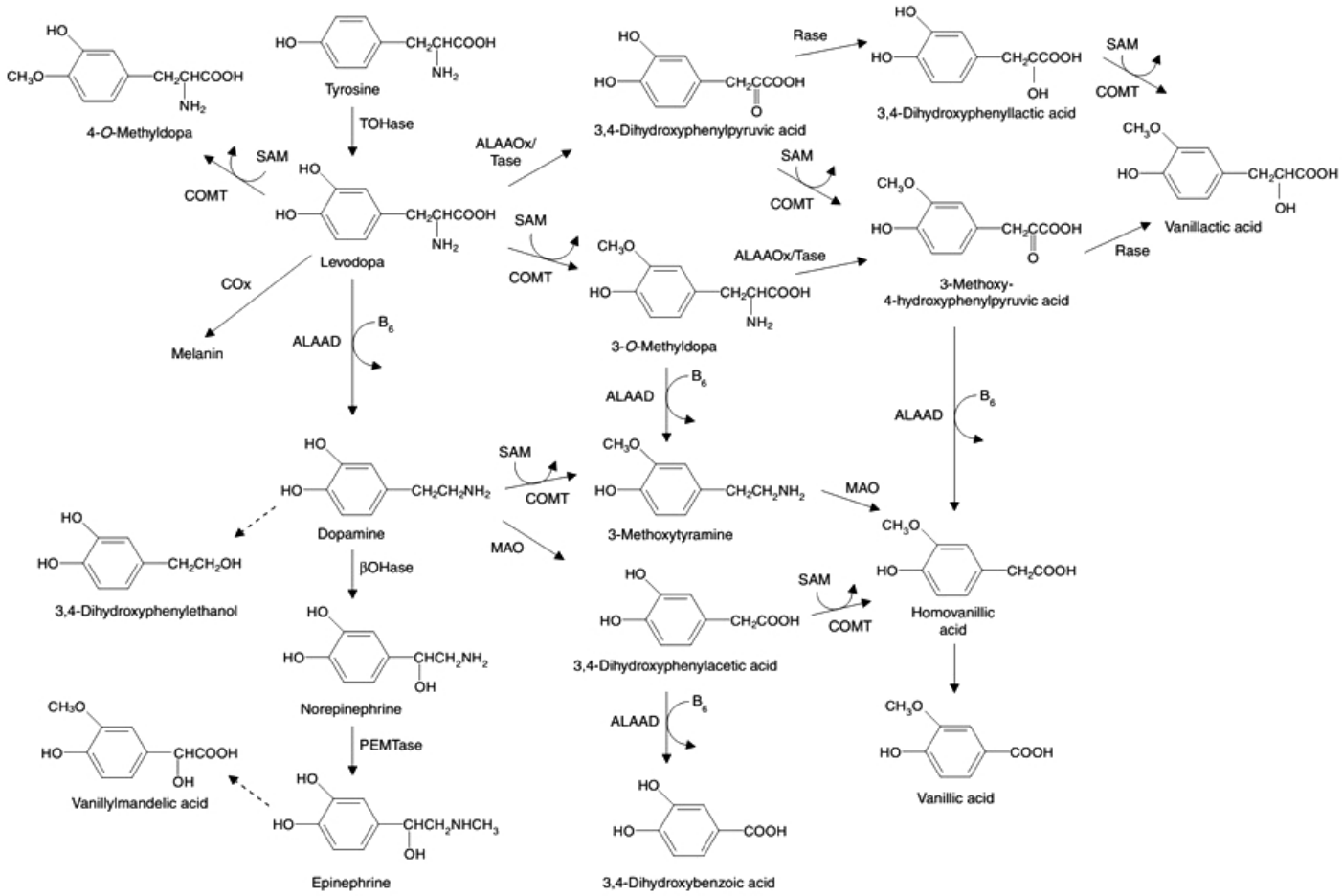


Figure 2.2. Major pathways of LD metabolism (Deleu et al., 1991).

[**ALAAD**: aromatic L-amino acid decarboxylase; **ALAAOx**: aromatic L-amino acid oxidase; **βOHase**: β-hydroxylase; **COMT**: catechol-O-methyltransferase; **COx**: catechol oxidase; **MAO**: monoamine oxidase; **PEMTase**: phenylethanolmethyltransaminase; **Rase**: reductase; **SAM**: S-adenosyl-L-methionine; **Tase**: transaminase; **TOHase**: tyrosine hydroxylase].

In liver, LD is methylated by hepatic COMT enzyme to 3-O-methyl dopa (3-OMD). COMT is an extracellular, nonspecific enzyme responsible for the catalysis of transfer of a methyl group from *S*-adenosyl-L-methionine to the 3-hydroxyl group of many endogenous substances consisting catechol substrate which includes LD, norepinephrine, epinephrine etc (Figure 2.2). In addition, COMT also metabolize catechol steroids, α-methyldopa and apomorphine (Axelrod, 1966). In humans, COMT is distributed widely, with highest activity in the mucosal layer of the gastrointestinal tract (GIT), liver, brain and kidney (Axelrod et al., 1959; Brannan et al., 1997; Vieira-Coelho and Soares-da-Silva, 1999). The catalysis of oxidative deamination of neuroactive and vasoactive amines is carried out by MAO which is a flavin-containing integral protein of the outer mitochondrial membrane. MAO exists in two forms viz. MAO-A and MAO-B. They differ from each other in their substrate preference, inhibitor specificity, tissue and cell distribution and immunological properties. MAO-A oxidizes norepinephrine and indolamines while MAO-B involved in oxidation of phenylethylamines and benzylamines. MAO-A is irreversibly inhibited by clorgyline whereas pargyline and selegiline are the irreversible inhibitor of MAO-B (Figure 2.1). Further, radioligand studies suggested that there is significant difference in distribution and abundance of MAO-A and MAO-B in tissues investigated (Saura et al., 1992). MAO-A is expressed in catecholamine containing neurons and is present in abundant levels in certain brain areas and various peripheral tissues including liver, whereas MAO-B is expressed in serotonin containing neurons and is present in abundant levels in ependyma, hippocampal formation, mammillary nuclei, posterior pituitary, pancreas and liver.

Although LD is coadministered with peripheral decarboxylase inhibitor, only 5 to 10% of the administered drug reaches to brain due to metabolism of LD by peripheral COMT (Kaakkola, 2000). Metabolite of LD (3-OMD) is a large neutral amino acid with a long half-life (15 h). It crosses BBB but does not show antiparkinsons activity. Further, 3-OMD is reported to competes with LD and amino acids for same transport mechanism across BBB (Juncos, 1992; Alexander et al., 1994). Moreover, 3-OMD diminishes allosteric regulation of the biopterin cofactor on activity of tyrosine hydroxylase (rate-limiting enzyme of catecholamine synthesis). This may influence the buffer action of endogenous LD synthesis that regulates appropriate dopamine level in the brain. The

ratio of 3-OMD/homovanillic acid (HVA) in cerebrospinal fluid (CSF) of PD patient was reported to be approximately 1:15 (Olanow et al., 1991). In plasma and CSF, LD, 3, 4-dihydroxyphenylacitic acid (DOPAC) and HVA are less subject to sulphate conjugation compared to dopamine. The loss of the hydrazine functional group (probably as molecular nitrogen) represents the major metabolic pathway for CD. There are several metabolites of CD which includes 3 - (3, 4- dihydroxy phenyl) - 2 -methyl propionic acid, 3- (4 - hydroxy - 3 - meth - oxyphenyl) 2 - methyl propionic acid, 3 - (3 -hydroxy phenyl) -2-methylpropionic acid, 3 - (4 - hydroxy - 3 - methoxy phenyl) 2 - methyl actic acid, 3 - (3 - hydroxy phenyl) - 2 - methyl - actic acid and 3, 4 - dihydroxy phenyl acetone (Vickers et al., 1975; Durso et al., 1993; Chen et al., 2002). LD and CD exhibits short plasma half-life (Goodman, 1996). Further, therapeutic effects of LD in the brain are affected by the rate and extent of cerebral conversion to dopamine, rate of movement of the synthesized dopamine to the striatal receptors and rate of metabolism of newly synthesized dopamine.

2.4.4. Elimination

LD is excreted in urine as dopamine metabolites. Approximately 70 to 80% of radiolabelled dose is eliminated within 24 h. The principle dopamine metabolites in urine are DOPAC and HVA. In urine, DOPAC and HVA account for up to 50% of administered dose whereas negligible amounts are found in faeces. LD is also excreted in urine in unchanged form but the amount is less than 1% of administered dose. Urine color may change to red because of presence of some metabolites. In addition, these metabolites undergo oxidation when urine is exposed to air which causes darkening of urine color (Budavari et al., 1989). Further, in chronic administration of LD, ratio of DOPAC and HVA excreted may increase resulting from a depletion of methyl donors necessary for metabolism by catechol-O-methyl transferase. It is estimated that approximately $3/4^{th}$ of dietary methionine is utilized for metabolism of large doses of LD (Goodman, 1996).

2.4.5. Concentration-Effect Relationship

Understanding of pharmacokinetic and pharmacodynamic (PK-PD) relationship of LD is necessary for effective treatment of PD patient experiencing motor fluctuations. Numerous studies have been carried out to find out the PK-PD relationship of LD. Although, some researcher did not observe clear cut correlation between plasma LD concentration and effect (Rinne et al., 1973), studies revealed that an immediate onset of the motor response to LD was observed when a threshold plasma concentration was high whereas the response fade off when plasma concentration decline below this level (Baas and Rietbrock, 1995; Contin et al., 1998). Further, Nutt and Holford (1996) reported four types of responses to LD viz. the short-duration response (SDR), long-duration response (LDR), negative response and dyskinesias (Nutt and Holford, 1996). Studies revealed that SDR is associated with unpredictable fluctuations in motor performance and positively correlated with the pharmacokinetic profile of LD. In case of LDR, patient treated for long duration with LD showed gradual increase in motor disability. Negative response is observed due to deterioration of motor function which is usually observed at the end of SDR of LD. Further, dyskinesia is most common motor complication observed in patient treated with LD for longer duration. In order to show clinical response, LD concentration should reach the threshold concentration of plasma compartment and central-effect compartment. Studies revealed that, there is a lag time between peak plasma concentration and clinical response. In addition to this, once threshold LD concentration is reached, the intensity of motor response cannot be further increased and duration of clinical response is linearly related to the plasma LD concentration (Olanow et al., 1991).

Weak drug concentration-effect relationship has been reported at early stage of PD but disease progression reveals a close temporal relationship between the duration of motor response and the plasma concentration of the drug (Contin et al., 1994). It was also reported that the age of the patient inversely affect the magnitude of clinical response irrespective of duration of the disease (Durso et al., 1993). In addition, duration of clinical response to the LD is dose and concentration dependent but it is not influenced by age (Durso et al., 1993). It has also been reported that dopamine agonists reduces the threshold and shorten the time-lag between plasma LD concentration and therapeutic response (Harder et al., 1995).

PD disease progression can be identified by pharmacodynamic changes. The baseline motor function in absence of any drug decreases without decrease in maximal therapeutic response (E_{max}) (Harder et al., 1995). As LD therapy progresses, LD concentration required to obtain 50% of E_{max} (EC_{50}) increases and is 2.5 times higher in fluctuating responders in comparison with stable responders. This reduction in LD potency results from receptor desensitization. Further the lag time for onset and duration of motor response also decreases with PD progression. Reduction in the half-life of LD with disease progression is related to the nigrostriatal dopamine turnover like loss of dopamine storage capacity, disturbed synthesis and release mechanisms. Furthermore, motor complications reported with long LD treatment are associated with fluctuating plasma LD concentration resulting from reduction in $t_{1/2}$ of LD at advanced stage of PD and administration of immediate release formulation.

Studies have reported that PD patient treated with LD/CD (Sinemet® tablet) every 6 h, showed time dependent improvement in motor function. In addition, continuous intravenous infusion, constant rate duodenal administration of liquid LD (Quinn et al., 1984; Kurlan et al., 1986; Kurlan et al., 1988; Sage et al., 1988; Deleu et al., 1991; Kurth et al., 1993) and administration of controlled release LD/CD (Sinemet® CR) (McHale et al., 1990) containing formulation maintains more stable plasma concentration of LD by reducing fluctuations. These findings suggested that stable plasma LD concentration may results in better motor response and improvement in quality of patient life (Pahwa et al., 1997). In addition increasing the peripheral level of LD may increases amount of LD reaching to the brain for active transport across BBB (Rinne and Mölsä, 1979; Cedarbaum et al., 1990; Contin et al., 1993; Dietz et al., 2001). Although the phenomenon of motor function is not fully understood, researchers suggested that some pharmacokinetic factors influencing the delivery of LD to brain and PD factors like alteration of dopamine receptor function as possible mechanism responsible for fluctuating response to LD (Mouradian and Chase, 1988; Kempster et al., 1989; Deleu et al., 1991).

2.4.6. Dose Escalation

Studies have been carried out to investigate the effect of increasing dose of LD in PD patient treated with LD. The outcomes suggested that dose can be increased on weekly

basis until substantial improvement in motor functions occurs. If several doses are equally effective then patient can be treated with lowest of those equipotent dose. If side effects are observed after dose increment then dose should be reduced and patient should be treated with previous dose. In addition, immediate release formulation should be administered 1 h before meal in order to avoid absorption problem. Practitioners suggest that the starting dose should be LD (100 mg)/CD (25 mg) three times a day. A common dose escalation scheme suggests addition of one half of LD (100 mg)/CD (25 mg) tablet to all three doses weekly (50 mg LD per dose). Escalation then can be continued [LD/CD: 100/25 (three times daily) to 300 - 250 mg of LD (maximum LD dose) (three times daily)] until there is marked improvement in motor function (or side effects) or up to 2.5 or 3 tablets three times daily. However, literature suggested that the amount of LD beyond 300 mg/dose does not add further benefit (Ronald et al., 2012). Most patients have been adequately treated with 400 to 1600 mg of LD/day, which is administered as divided doses at intervals ranging from 4 to 8 h. Higher doses (2400 mg or more of LD/day) and shorter intervals (less than 4 h) have also been used, but are not usually recommended due to fluctuating clinical response. It is recommended that maximum daily dosage of CD should not exceed 200 mg. Although increase in LD dose results in improved motor response, dose escalation has to be considered on the basis of PD status, age of patient and therapeutic response to LD.

2.4.7. Population Pharmacokinetics

Population pharmacokinetic studies revealed that pharmacokinetic parameters for LD in patients with mild to severe PD are independent of patient characteristics. However, population PK - PD studies of oral LD in PD patient demonstrate that changes in pharmacodynamic parameters are also responsible for the motor complications associated with LD therapy (Triggs et al., 1996). Pharmacodynamic parameters of LD vary with disease severity (Hoehn and Yahr status) and duration (Triggs et al., 1996). In PD patients, standard LD dose can maintain drug concentration above therapeutic threshold values but for shorter duration which may be responsible for reduced duration of motor response. In addition to this, fluctuating response to LD at advanced stage of PD indicate that higher degree of drug receptor occupancy is required to obtain clinical response.

Triggs and coworkers predicted motor activity (tapping times) of LD by determination of effect of Hoehn and Yahr status and duration of disease on the equilibrium rate-constant, plasma concentration corresponding to EC_{50} and sigmoidicity factor (i.e. steepness) of the plasma concentration-effect curve (γ) (Triggs et al., 1996). Triggs and coworkers observed significant shortening of the equilibration half-life from 173 min in a PD patient whose Hoehn and Yahr status is I, to a value of 43 min where the patient has a Hoehn and Yahr status of IV. Similarly value of EC_{50} increased from 0.35 mg/L (Hoehn and Yahr status of I) to 1.40 mg/L (Hoehn and Yahr status of IV), and γ increased from 0.8 (Hoehn and Yahr status of I and duration of disease of 1 year) to 2.7 (Hoehn and Yahr status of IV and duration of disease of 24 years). From study findings Triggs et al. (1996) concluded that motor response changes significantly with progressive shortening of the equilibration $t_{1/2}$. In addition, increased ED_{50} value demonstrated reduction in sensitivity to drug with disease progression and shortening of duration of therapeutic effect. Moreover, steepening of concentration response curve demonstrate prolonged duration of the peak response but with shorter overall response function. Combined effect of these pharmacodynamic changes observed with PD progression is responsible for motor complications (on-off fluctuations) associated with LD treated patient for long duration. Therefore, it is recommended that the dose LD/CD must be titrated on the basis of response produced and status of PD instead of plasma concentration of LD.

2.4.8. Drug-Drug and Drug-Food Interaction

PK and PD studies revealed that, LD/CD combination shows interaction with various drugs. Administration of LD/CD with amoxapine should be avoided due to risk of hypertensive episodes and postural hypotension (Cedarbaum, 1987). In addition, amoxapine also reduces LD response and aggravate PD symptoms. LD/CD also demonstrates interaction with amiodarone. Anticonvulsant agents like phenytoin, valproate, vigabatrin etc. and antineoplastic agents (e.g. cytarabine, methotrexate, dacarbazine, fluorouracil, paclitaxel) may aggravate the parkinsons symptoms and induce extrapyramidal adverse effect when use concomitantly with LD/CD (Pfeiffer, 1996). Furthermore, coadministration of procarbazine (weak MAO inhibitor) with LD may results in hypertensive crisis (Haefeli, 2007). Antipsychotic agents (e.g. haloperidol,

chlorpromazine, trifluoperazine) show dopamine receptor antagonistic effect and thereby reduce the pharmacological response to LD (Hardie and Lees, 1988; Rabey et al., 1995). In addition, calcium channel antagonist like flunarizine, cinnarizine, verapamil, diltiazem, amlodipine may reduce the response to LD by inhibition of striatal dopamine release when used concomitantly with LD (Winterkorn and Teman, 1991; Bezprozvanny, 2009).

The dose of LD needs to be reduced by 30% when COMT inhibiters are administered with LD/CD in order to produce equal therapeutic response as that of LD/CD alone (Nutt et al., 1994; Dingemanse et al., 1995). This is because of increase in the bioavailability of LD due to COMT inhibition (Dingemanse et al., 1995). Literature suggests use of 200 mg of entacapone with LD/CD increases AUC of LD by 35% (Myllylä et al., 1993; Kaakkola et al., 1994). Penicillamine increases plasma LD concentration by 50% when administered concomitantly, which may further increase the risk of dyskinesia (Mizuta and Kuno, 1993). Isoniazide may cause symptomatic deterioration of PD or reduction of dyskinesia (Gershanik et al., 1988; Wenning et al., 1995). In addition, it also shows inhibitory effect on AADC inhibitors. Concomitant use of LD/CD with either reserpine or methyldopa may reduce antiparkinsons activity of LD. The reduction in response in case of reserpine may be due to depletion of presynaptic dopaminergic stores whereas methyldopa competes with dopamine at dopamine receptor.

Drugs like cisapridec (Djaldetti et al., 1995; Neira et al., 1995; Bedford and Rowbotham, 1996), metoclopramide (Tarsy et al., 1975; Berkowitz and McCallum, 1980; Avorn et al., 1995) and domperidone (Shindler et al., 1984; Bradbrook et al., 1986; Langdon et al., 1986; Nishikawa et al., 2012) may increase the incidence of extrapyramidal symptoms either by affecting gastric emptying and gastrointestinal absorption of LD. In addition, LD also demonstrate interaction with other drugs influencing the gastric acidity and emptying like antacids (Hurwitz, 1977; Bradbrook et al., 1986), ferrous sulphate (Campbell and Hasinoff, 1989; Campbell et al., 1990; Greene et al., 1990) etc. The effect of food on the pharmacokinetics of LD was studied previously in healthy volunteers (Crevoisier et al., 2003). Studies demonstrate that protein containing food may reduce LD absorption. The beneficial effects of a low protein diet in the treatment of patients with PD probably results from reduced competition for LD transport at gastrointestinal site and across the blood-brain barrier (Robertson et al., 1991; Karstaedt and Pincus, 1992).

Therefore, understanding of LD interactions with other drugs and food is necessary in order to improve therapeutic outcome during treatment of PD patient.

2.4.9. Side Effects

Side effects of LD therapy are frequent and often troublesome (Ray, 1972; Scheidtmann et al., 2001). Most are dose related and limit the dose that can be administered but are usually reversible. Some are prominent in beginning of the therapy while others appear late. The adverse effect like nausea, vomiting, postural hypotension, cardiac arrhythmias, exacerbation of angina and alteration of taste sensation are associated with initiation of LD therapy with low dose. In addition, prolonged LD therapy causes abnormal movements, behavioral effects and fluctuations in motor performance (Ahlskog and Muenter, 2001). Appropriate dose adjustment and the use of AADC inhibitors may reduce the incidence of side effect associated with LD therapy.

2.5. Pharmaceutical Formulations

LD is available in many different formulations to suit individual patient needs which are summarized in Table 2.2.

Table 2.2. Pharmaceutical Formulations of LD and CD

Brand name	Content	Formulation type	Strength (mg)
Dopar®	LD	Tablet	LD: 100, 250, 500
Lodosyn®	CD	Tablet	CD: 25
Rytary®	LD + CD	Extended release capsule	LD/CD: 95/23.75, 145/36.25, 195/48.75, 245/61.25
Sinemet®	LD + CD	Tablet	LD/CD: 100/25, 100/10, 250/25
Sinemet® CR	LD + CD	Controlled release tablet	LD/CD: 100/25, 200/50
Parcopa®	LD + CD	Orally disintegrating tablet	LD/CD: 100/25, 100/10, 250/25
Duopa®	LD + CD	Enteral suspension	-

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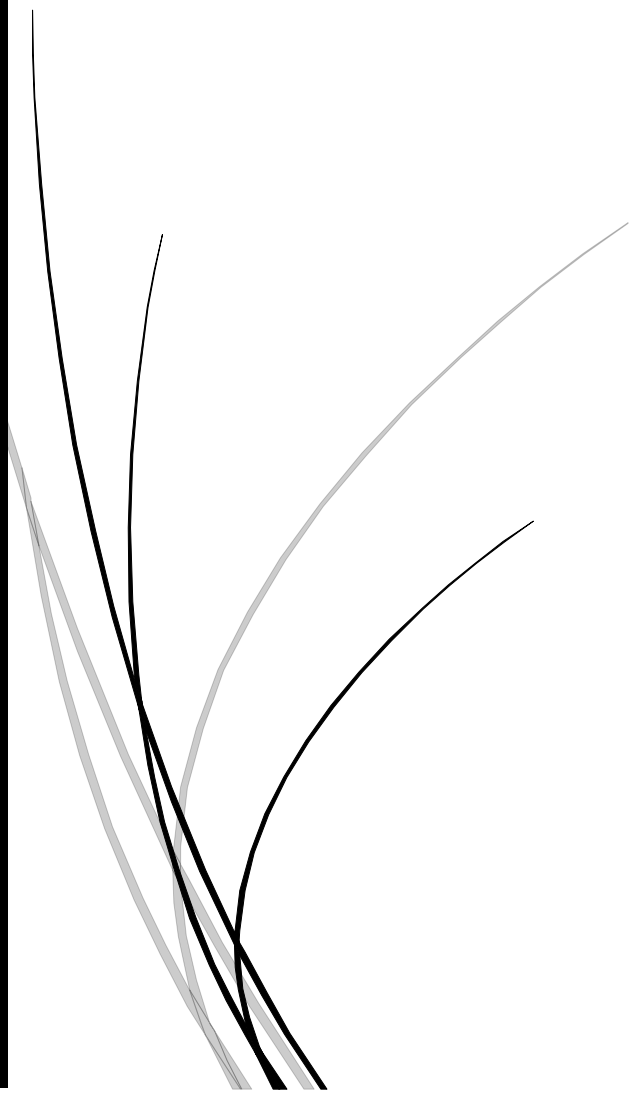
3 ANALYTICAL METHODS



3.1. Introduction

The main aim of any pharmaceutical formulation development is to investigate design and process that would consistently deliver a pharmaceutical formulation of predetermined quality. Appropriate analytical methods are needed for the evaluation or to assess the quality of invented product. Analytical methods play important role in product design and development process and it is essential to ensure that the employed analytical method(s) should provide accurate and reliable data (Peters et al., 2007; Rozet et al., 2011). Therefore, it is necessary to develop simple, sensitive and accurate analytical method(s) for accessing critical quality and performance attributes (Ermer, 2001; Ermer et al., 2005; Ermer and Ploss, 2005).

The science of analyzing chemical characteristics such as identity and purity is well established and still advancing with the use of hyphenated analytical technique coupled with mathematical principles like multivariate design, design of experimentation etc. (Wieling et al., 1996; Ermer, 1998; Ermer and Vogel, 2000; Ragonese et al., 2002). Most of the regulatory agencies across the globe emphasize the use of stability indicating methods for the monitoring of the stability profile of the drug substance and drug product (Rozet et al., 2007). Requirements also demands the strict monitoring of the impurities and degradation products. Such analytical methods are also useful in preformulation and product stability studies (Bakshi and Singh, 2002). Similarly, presence of the complex biological environment demands the specific considerations for estimation of the pure drug in biological samples for accurate determination of the in vivo pharmacokinetic parameters (Braggio et al., 1996; Kelley and DeSilva, 2007; Araujo, 2009).

With FDA's process analytical technology initiative, the current view of 'quality by design' is further strengthened by stating that the quality should be built in the product and should not be inspected within. Drug control and regulatory agencies of various countries have recognized the importance of the analytical sciences in the product design and development process and have released extensive guidelines on the validation requirement in recent years (FDA, 2001; ICH, 2005; USP, 2007). Although analytical validation requirements depend on the type of the analyte and analytical instrument, it broadly includes the specificity and selectivity, linearity and range, accuracy and precision, sensitivity, reproducibility, stability etc. (Chandran and Singh, 2007).

In the literature, a number of analytical methods have been reported for the estimation of LD and CD alone in variety of study samples like bulk, formulation and biological samples. Most of these methods used spectrophotometric (Nagaraja et al., 1998; Coello et al., 2000; Nagaraja et al., 2001), high performance liquid chromatography (HPLC) (Cannazza et al., 2005), gas chromatographic (Doshi and Edwards, 1981), chemiluminescence (Deftereos et al., 1993; Yang et al., 1998), amperometric and voltammetric principles (Garrido et al., 1997; Teixeira et al., 2004; Bergamini et al., 2005). Few methods for estimation of LD and CD have also been reported using potentiometry (Badawy et al., 1996), radio-immunoassay (Waugh et al., 1989) and flow injection analysis (Fatibello-Filho and da Cruz Vieira, 1997; Marcolino-Junior et al., 2001; Pistonesi et al., 2004).

A stability indicating method using HPLC has also been reported for LD and CD (Kafil and Dhingra, 1994). In this method, stability of LD and CD was reported only in mobile phase of the HPLC method developed for the simultaneous estimation of LD and CD. Pappert and coworkers reported the stability of LD in water and in presence of ascorbic acid at different storage conditions (Pappert et al., 1996). In addition, stability of LD in water at different pH has also been reported (Kankkunen et al., 2002). Although there are few reports on the stability of LD in water at different climatic condition, there is a need of detailed study of solution state and solid state stability of LD and CD at different storage conditions before product development. Further, high performance thin layer chromatography (HPTLC) methods for estimation of CD alone were not found in the literature. Therefore, to study the stability and solubility behavior of LD and CD alone, a sensitive, selective analytical method is needed. Thus, it was planned to develop individual HPTLC methods for quantitative estimation of LD and CD individually for routine research analysis.

In pharmaceutical formulations, LD and CD are present in combination therefore appropriate analytical method for simultaneous estimation of LD and CD in pharmaceutical formulation is of great importance. Various analytical techniques for simultaneous estimation of LD and CD have been reported in literature. A kinetic H-point standard addition method has been reported for the simultaneous determination of LD and CD without any interference (Safavi and Tohidi, 2007). Methods using high

performance liquid chromatography (Kafil and Dhingra, 1994; Tolokan et al., 1997; Sagar and Smyth, 2000), nuclear magnetic resonance spectroscopy (Talebpour et al., 2004), capillary electrophoresis and enzymatic stopped-flow-injection (Grünhut et al., 2007) have also been reported for simultaneous estimation of LD and CD. Some of these methods can also be employed for estimation of LD and CD with other catecholamines (noradrenaline, dopamine, L-tyrosine etc) (Törnkvist et al., 2004). Most of these methods have disadvantages like high cost, low selectivity, use of organic solvents, complex sample preparation procedures, addition of derivatizing agent, long analysis time etc. Further, liquid chromatography mass spectrometry technique for simultaneous estimation of LD and CD has also been reported in literature (Törnkvist et al., 2004). Extensive literature survey did not reveal simple and sensitive method for simultaneous estimation of LD and CD using HPLC and fluorescence detector. Thus, it was planned to develop a HPLC method for simultaneous estimation of LD and CD using fluorescence detector that will help to overcome disadvantages of reported method. The developed and validated method was used for simultaneous estimation of LD and CD in bulk, pharmaceutical formulations, in vitro drug release study samples and stability samples.

Among the methods reported for estimation of LD and CD in biosamples, some methods although found to be very sensitive, precise and accurate, mostly use costly and sophisticated analytical instruments such as LCMS (Pan et al., 2010; Zhu and Hua, 2010; César et al., 2011; Van de Merbel et al., 2011; Cho et al., 2012; Junnotula and Licea-Perez, 2013; Martins et al., 2013; De Oliveira Vilhena et al., 2014) making these methods unsuitable for routine analysis in laboratories with relatively modest infrastructure. Moreover, the reported methods have some disadvantages like derivatization before analysis, gradient elution (Muzzi et al., 2008), complex sample extraction procedure and column switching for separation (Sagar and Smyth, 2000). In addition to this, LD and CD as well as naturally occurring neurotransmitter DA are not stable in biological matrix for long time. To the best of our knowledge there is no detailed report on stability data of these analytes in rat plasma and brain. Thus, method using HPLC coupled with electrochemical detector (HPLC-ECD) was developed for estimation of LD, CD and 3-oxymethyl dopa (3-OMD) in rat plasma using simple sample preparation technique. The

concentration levels of the 3-OMD (metabolite of LD) was also monitored as an index of the turnover of LD.

The objective of the present investigation was to develop simple, sensitive, accurate, reproducible, reliable and economical analytical methods for individual estimation of LD and CD in order to evaluate physicochemical properties and compatibility with various excipients. In addition, a sensitive, accurate, selective and robust method was required for the simultaneous determination of the LD and CD in pharmaceutical formulation for routine drug analysis. Moreover, for evaluation of the formulations in animal model, a selective and sensitive bioanalytical method for determination of LD and CD in rat biomatrices was also essential. In the present work, in-house developed methods used most of the common mathematical and statistical treatments for analytical method validation in modern technique of experimental design and orthogonality (Pellett et al., 2006). In addition, in-house developed methods were validated using appropriate statistical tests as per the standard regulatory guidelines set for analytical and bioanalytical methods (FDA, 2001; ICH, 2005; USP, 2007; Bolton and Bon, 2009). Furthermore, methods were successfully assessed for the determination of LD and CD in respective matrices.

3.2. Materials

Levodopa (purity > 98.0%) and Carbidopa (purity > 98.0%) were purchased from Shaanxi Hygethy Biotechnology Co., Ltd. Xi'an, China and Smruthi Organics Pvt. Ltd., Solapur, India, respectively. Deionized ultra-pure water (18.2 MΩ.cm at 25°C) was produced using Milli-Q water purification system (Millipore®, MA, USA). 3-O-methyldopa (3-OMD, purity ≥ 98.0%), dopamine hydrochloride (DA, purity ≥ 98.0%), 3,4-dihydroxy phenyl acetic acid (DOPAC, purity ≥ 98.0%), glacial acetic acid (purity ≥ 99.85%), n-butanol (purity > 99.8%) sodium metabisulfite ($Na_2S_2O_5$, purity ≥ 99.00%), trichloroacetic acid (TCA, purity ≥ 99.0%) and 1-heptanesulphonic acid monohydrate sodium salt (HAS, purity ≥ 98.0%), hydrogen peroxide (H_2O_2, purity ≥ 30.00%), sodium hydroxide (NaOH, purity ≥ 98.00%) and talc were purchased from Sigma-Aldrich® Corporation, Mumbai, India. Hydrochloric acid (HCl, purity = 37.00%), perchloric acid ($HClO_4$, purity= 70.00%), potassium dihydrogen orthophosphate (purity > 99.5%),

orthophosphoric acid (purity ≥ 85%), sodium ethylene diaminetetraacetic acid (Na_2EDTA), sodium metabisulfite ($Na_2S_2O_5$) (purity ≥ 99.0%) and potassium chloride were purchased from Merck, Mumbai, India. The HPLC grade methanol (purity > 99.7) was purchased from Merck, Mumbai, India. All chemicals were of analytical and HPLC grade and used as received without any modification.

Excipients used for the preparation of tablet placebo blend such as glidant (Aerosil® NF), lubricant (magnesium stearate NF), disintegrant (Polyplasdone XL-10 NF), binder (Hypromellose USP) etc. were obtained from Medreich Pharmaceuticals, Bangalore, India. Microcrystalline cellulose (Avicel® PH101) was purchased from FMC BioPolymer, USA. Povidone (PVPK30) was supplied as a gift sample by BASF Ludwigshafen, Germany. One commercially available tablet preparation SYNDOPA® 275 (Sun Pharmaceutical, India) labeled to contain LD (250 mg) and CD (25 mg) in combination was purchased from the local Indian market.

For bioanalytical methods, drug free plasma pool and brain homogenate was obtained from healthy male Wistar rats and it was stored at -80°C in sealed cryovials.

3.3. Analytical Method I: High Performance Thin Layer Chromatographic Method for Individual Estimation of LD and CD

3.3.1. Instrumentation

The high performance thin layer chromatography system used was CAMAG HPTLC system (CAMAG, Muttenz, Switzerland). CAMAG microliter syringe (100 µL) was used for spotting the sample in the form of 4 mm bands on precoated silica gel aluminium plate G60 F254 (200 µm thickness; Merck, Darmstadt, Germany) using a CAMAG Linomat 5 applicator (CAMAG, Muttenz, Switzerland). Chromatograms were developed in 10 cm × 10 cm twin trough glass chamber (CAMAG, Muttenz, Switzerland) using linear ascending development technique. The densitometric analysis was performed using CAMAG TLC Scanner 3 (CAMAG, Muttenz, Switzerland) in absorbance mode at 280 nm for all measurements. Obtained data was collected and integrated using winCATS software (version 1.4.1.8154, CAMAG).

3.3.2. Chromatographic Conditions

Separate HPTLC methods were developed for the individual estimation of LD and CD. In case of both methods, except the compositions of mobile phases, all other chromatographic conditions were kept similar. A precoated silica gel aluminium plate G60 F254 (5 mm × 7.5 mm, 0.2 mm thickness, Merck, Darmstadt, Germany) was washed with methanol and dried. Dried plate was then developed in the optimized mobile phase and activated at 120°C for 10 min prior to actual analysis. After activation of the plates, samples were applied at constant application rate of 50 nL/s using CAMAG Linomat 5 automatic sample applicator at predefined position over the band length of 4 mm and keeping 6.3 mm distance between two bands. Sample loading was followed by the development of the plates in chamber saturated with mobile phase. Each plate was developed in approximately, 10 mL of mobile phase (5 mL in trough containing the plate and 5 mL in other trough). Development chamber was saturated for 1 h using optimized mobile phase prior to the plate development. The optimized compositions of mobile phases consisted of n-butanol/glacial acetic acid/water in the ratio of 3.5:1:1 (v/v/v) and 1.4:1:1 (v/v/v) for estimation of LD and CD, respectively. Developed TLC plates were dried in hot air oven at 150 ± 2°C for 20 min. After development, the plates were dried at 150°C in an oven and then densitometric analysis was carried out. The procedure of the thermochemical activation of analyte was optimized in order to get reproducibility in the signal. The slit dimension was set at 3 mm × 0.45 mm, and scanning was done at scanning speed of 20 mm/s and data resolution of 100 µm/step. Analysis was performed at ambient temperature.

3.3.3. Preparation of Stock and Standard Solutions

Individual primary stock solutions of LD and CD were prepared in 0.05 N HCl with 0.1%, w/v sodium metabisulfite solution each having concentration of 1 mg/mL. Individual secondary stock solutions of LD and CD each having concentration of 100 µg/mL were prepared by appropriate dilution of the primary stock solutions using mixture of methanol and 0.05 N HCl (9:1, v/v). From LD secondary stock solution, nine separate series of six calibration standards were spotted to obtain concentration range of 100 - 500 ng/spot for LD. Similarly, from secondary stock solution of CD, nine separate

series of six calibration standards were spotted to obtain concentration range of 100 - 600 ng/spot for CD.

Formulations standards of LD and CD were prepared individually by spiking known amount of LD and CD from the respective secondary stock solutions into the placebo blend of the pellets. Individual formulation standards were prepared at five concentration levels so as to contain 100, 200, 300, 400, 500 ng/spot of LD and 100, 200, 400, 500, 600 ng/spot of CD. Placebo blanks were prepared without addition of any drug. Prepared standards were processed independently, as described in their respective sample preparation section and analyzed. All solutions were protected from light and stored in refrigerator at 5 ± 3°C.

3.3.4. Sample Preparation

Pellets loaded individually with LD and CD were manufactured in-house by extrusion spheronization technique. Developed methods were used for individual analysis of LD and CD from the respective pellet formulation. To estimate the content of LD in-house manufactured pellets (containing 480 mg of LD per 1 g of pellets); pellets (1 g) were triturated using mortar and pestle. A powder equivalent to 10 mg of LD was weighed and transferred to a 10 mL calibrated flask containing 7 mL of mixture of 0.05 N HCl and 0.1%, w/v sodium metabisulfite and sonicated for 15 min. The volume was made up with same solvent. The resulting solution was centrifuged at 5000 rpm for 5 min and supernatant was filtered through 0.22 µm Millipore® syringe filters (Millex® Syringe Filter, Millipore). Finally, 1 mL of the filtrate was transferred to a 10 mL volumetric flask and volume was made up with mixture of methanol and 0.05 N HCl (9:1, v/v). Similarly, for CD, pellet (containing 120 mg of CD per 1 g of pellets) powder equivalent to 10 mg of CD was weighed and transferred to a 10 mL calibrated flask and volume made up with 0.1%, w/v sodium metabisulfite solution in 0.05 N HCl. Resulting solution was further processed similar to the LD solution as discussed above.

3.3.5. Analytical Method Validation

The developed HPTLC methods [for estimation of LD (LD method) and CD (CD method)] were validated separately for selectivity, linearity, range, precision, accuracy

and sensitivity. The methods were also applied in preformulation studies of LD and CD and for the drug content analysis in in-house prepared pellet formulations.

a. Linearity and Range

For LD Method

Calibration standards were spotted at six concentration levels in the range of 100 to 500 ng/spot in replicates of nine to establish the linearity of the proposed method. Average peak areas were calculated at each concentration level and least square linear regression analysis was performed to obtain calibration equation. The calibration equation was used to calculate the corresponding predicted concentrations. One way analysis of variance (ANOVA) test was performed for the peak areas obtained at each concentration during the replicate measurement at six concentration levels (Bolton and Bon, 2009). The range of proposed analytical method was estimated by residual analysis.

For CD Method

Linearity of proposed method was estimated by spotting nine series of calibration standards of CD at six concentration levels in the range of 100 to 600 ng/spot. Least square linear regression analysis, ANOVA and analysis of residuals were performed similar to the method developed for LD estimation.

b. Accuracy

For both methods, recovery study was carried out by placebo spiking and standard addition technique.

For LD Method

In placebo spiking method, a known amount of standard secondary stock solution of LD was spiked in placebo blank at five different concentration levels 100, 200, 300, 400, 500 ng/spot for LD. For standard addition technique, a known amount of pure LD was added at 50, 100 and 150% concentration level of the label claim in previously analyzed pellet samples.

For CD Method

For placebo spiking method, five different concentrations (100, 200, 400, 500, 600 ng/spot) were prepared by spiking appropriate amount of standard secondary stock

solution of CD in placebo blank whereas for standard addition method, previously analyzed pellet samples were spiked with pure CD at 50, 100 and 150% concentration level of the label claim.

For both LD and CD methods, each concentration level was prepared and processed using respective method individually in six replicate on three different days. Obtained results were expressed as mean absolute recovery, relative standard deviation (RSD) and bias.

c. Precision

Repeatability (intraday) and intermediate (interday) precision studies were carried out to assess the preciseness of the proposed methods. Precision study was performed by preparation of the quality control standards (QC) at three different concentration levels, viz. lower quality control (LQC), middle quality control (MQC) and higher quality control (HQC) for both methods.

For LD Method

Three series of three QC standards (LQC= 150 ng/spot; MQC= 250 ng/spot and HQC= 500 ng/spot) were prepared freshly and analyzed for intraday variation (repeatability). On three consecutive days, QC standards were prepared similarly and analyzed in triplicate for intermediate precision (interday).

For CD Method

Three QC standards namely LQC (150 ng/spot), MQC (300 ng/spot) and HQC (550 ng/spot) were prepared and analyzed for intraday (repeatability) and interday (intermediate precision) variation.

For both methods, predicted concentrations were obtained from regression equation at three QC levels. Repeatability and intermediate precision was represented by RSD of the predicted concentrations at three QC levels.

d. Selectivity and Specificity

For both methods, selectivity was assessed by comparison of placebo and spiked placebo sample analysis. Placebo and formulation standards (pellets) were prepared in triplicate and processed on three consecutive days, as described in the respective sample

processing section. Prepared standards were analyzed using chromatographic conditions mentioned earlier and checked for peak area and interference of excipients at drug peaks.

For LD Method

A spot in formulation sample was confirmed by comparing the retention factor (R_f) values and spectra of the spot with that of standard. Further, peak purity of LD was assessed by comparing the spectra at three different levels, i.e., peak start (S), peak apex (M), and peak end (E) positions of the spot. In addition, standards were prepared from pure drug stock (200 ng/spot) and in-house prepared pellet formulation sample stock. The prepared standards were analyzed as mentioned earlier (n = 6). The obtained mean of peak areas were compared by using unpaired t-test at 5 % level of significance.

For CD Method

Spectra at three QC levels were compared in order to assess the peak purity of CD similar to the LD method. Also, standard (200 ng/spot) was compared with the in-housed prepared formulation sample (n = 6). Unpaired t-test was used at 5% level of significance for comparison of the obtained mean peak areas as mentioned above.

e. Sensitivity

For LD Method

The sensitivity of the proposed methods was expressed in the form of limit of quantitation (LOQ) and limit of detection (LOD). A lower part of linear range of the calibration curve was used for estimation of the LOD and LOQ. Samples containing 10, 11, 12, 13, 14 and 15 µg/mL of LD were prepared and spotted (10 µL each) in triplicate on the TLC plate. The amount of LD versus corresponding average peak area was plotted and straight line equation for this was calculated. The standard deviation of these peak areas was calculated. The average of the standard deviation (A.S.D.) was also calculated. The LOD and LOQ were calculated by (3.3 × A.S.D.)/B and (10 × A.S.D.)/B, respectively. Where "B" corresponds to the slope obtained in the linearity study of method.

For CD Method

The LOQ and LOD for CD method were also calculated similar to LD method. Samples containing 10, 11, 12, 13, 14 and 15 µg/mL of CD were prepared. Fixed volume (10 µL)

from each sample was spotted in triplicate on the TLC plate and analyzed. The LOQ and LOD for CD method then calculated using the equation computed as described in LD method.

f. Robustness

The chromatographic parameters were changed deliberately to study the robustness of the developed methods.

For LD Method

Robustness of the method was studied by introducing small deliberate change in the mobile phase composition. Different mobile phase compositions like n-butanol/glacial acetic acid/water (3.5:1:0.9, v/v/v), n-butanol/glacial acetic acid/water (3.4:1.1:1, v/v/v), n-butanol/glacial acetic acid/water (3.4:1.1:0.9, v/v/v), n-butanol/glacial acetic acid/water (3.6:1:1, v/v/v), and n-butanol/glacial acetic acid/water (3.5:1.1:1, v/v/v) were studied. The effect of change in the amount of mobile phase (± 5%) on the response function was investigated. The plates were prewashed by mobile phase and activated at 120 ± 5°C for 3, 6, and 10 min, respectively, prior to chromatography. Further time from spotting to chromatography and chromatography to scanning was varied from 0, 15, 30, and 45 min to evaluate robustness of the developed method. Robustness study of the method was carried out at three different concentration levels 150, 250, and 500 ng/spot.

For CD Method

Robustness study of the CD method was performed similar to the LD method. The effect of different mobile phase compositions like n-butanol/glacial acetic acid/water (1.4:1:0.9, v/v/v), n-butanol/glacial acetic acid/water (1.3:1:1, v/v/v), n-butanol/glacial acetic acid/water (1.3:1.1:1, v/v/v), n-butanol/glacial acetic acid/water (1.5:1:1, v/v/v), and n-butanol/glacial acetic acid/water (1.4:1.1:1, v/v/v) on the response function was studied. Robustness study was carried out at the three QC levels (150, 300 and 550 ng/spot).

g. Application of Method

Developed methods were applied for estimation of the drug content in in-house prepared formulation and forced degradation studies. Further, these methods were also used in preformulation studies and detailed procedures are mentioned in Chapter 4.

(i) Estimation of LD in In-house Manufactured Pellets

The powder of pellets equivalent to 10 mg of the LD was weighed accurately and processed as per the sample preparation procedure described in the sample preparation section so as to contain 200 ng/µL of LD. Finally, 1 µL of sample was spotted and analyzed by developed method. Study was done in triplicate.

(ii) Estimation of CD in In-house Manufactured Pellets

The powder of CD loaded pellet equivalent to 10 mg of CD was processed as per the sample preparation procedure discussed earlier so as to contain 300 ng/µL of CD. Sample volume of 1 µL was spotted and analyzed in triplicate.

(iii) Forced Degradation Studies

Forced degradation studies were performed by exposing drugs to hydrolytic (acidic, basic and neutral) and oxidative stress conditions. For each stress treatment, LD and CD were exposed separately. Individual stock solution of LD (1 mg/mL) and CD (0.25 mg/mL) was prepared in water and protected from the light. For hydrolytic stress treatment, HCl (0.1 N), phosphate buffer pH 6.8 and NaOH (0.1 N) were added individually to the individual stock solutions of the LD and CD in the 1:1 (v/v) ratio then solutions were kept at 40°C for 2 h. Resulting solutions were analyzed using developed chromatographic methods. For oxidative stress treatment, H_2O_2 (10%, v/v) was added individually to the stock solution of LD and CD in the ratio of 1:1 (v/v). Resulting mixtures were kept at 40°C for 2 h. During stress treatment; all the samples were protected from the light in order to avoid the possible degradative effect of the light. After completion of each treatment samples were analyzed by the respective methods proposed for estimation of LD and CD.

3.3.6. Results and Discussion

a. Analytical Method Development

During preliminary stage of method development peak properties and response function were optimized by changing the type of organic phase and the organic to aqueous phase ratio. Methanol and water in the ratio of 5:5 (v/v) was tried for the both drugs

individually. The LD spot did not move and dragging was observed while comparatively less dragging was observed for CD spot. When n-butanol and water was tried in the ratio of 4:6 (v/v) individually for LD and CD, the distance travelled by both spots was less and dragging was observed in case of CD spot. Then, n-butanol and water in the ratio 6:4 (v/v) was tried. The R_f of LD was observed near to the solvent front whereas dragging was observed in case of the CD spot. To obtain the compact spot, chloroform, n-butanol and water were also tried in different ratios with ethyl acetate for both cases individually but developed spot were found to be diffused, less compact and showed dragging in both cases. The LD and CD were then individually loaded on TLC plates and then plates were developed separately in n-butanol, formic acid and water in the ratio of 3:2:1 (v/v/v), compact spot was observed for LD but CD spot showed diffusion and dragging. In the above mobile phase, when formic acid was replaced by glacial acetic acid, R_f of CD was observed near to solvent front and spot lacked compactness whereas R_f of LD was found to be at 0.35. Then volume of n-butanol was increased and the volume of glacial acetic acid and water was reduced to obtain compact spot. Finally, mobile phase consisting of n-butanol, acetic acid and water in the ratio of 3.5:1:1 (v/v/v) showed compact and dense spot for LD. This mobile phase was modified little to obtain compact and dense spot for the CD. Ultimately, mobile phase consisting of n-butanol, acetic acid and water in the ratio of 1.4:1:1 (v/v/v) was optimized for CD. For LD and CD, peaks were found to be symmetrical in nature and no tailing was observed when plates were scanned at 280 nm. Sharp peaks were obtained for both LD (Figure 3.1a and 3.1c) and CD (Figure 3.1b and 3.1d), when plate was activated at 120°C for 10 min and development chamber was saturated for 1 h at room temperature prior to analysis.

b. Linearity and Range

For optimized chromatographic conditions, excellent linearity was observed over the range of 100 to 500 ng/spot for LD and 100 to 600 ng/spot for CD which was further confirmed by statistical analysis. The slope and intercept values were calculated by using linear regression analysis. For LD, the slope and intercept values were found to be 14.87 and 482.40, respectively with regression coefficient of 0.9997 while for CD slope and

intercept values were found to be 11.63 and -100.90, respectively with regression coefficient of 0.9998 (Table 3.1).

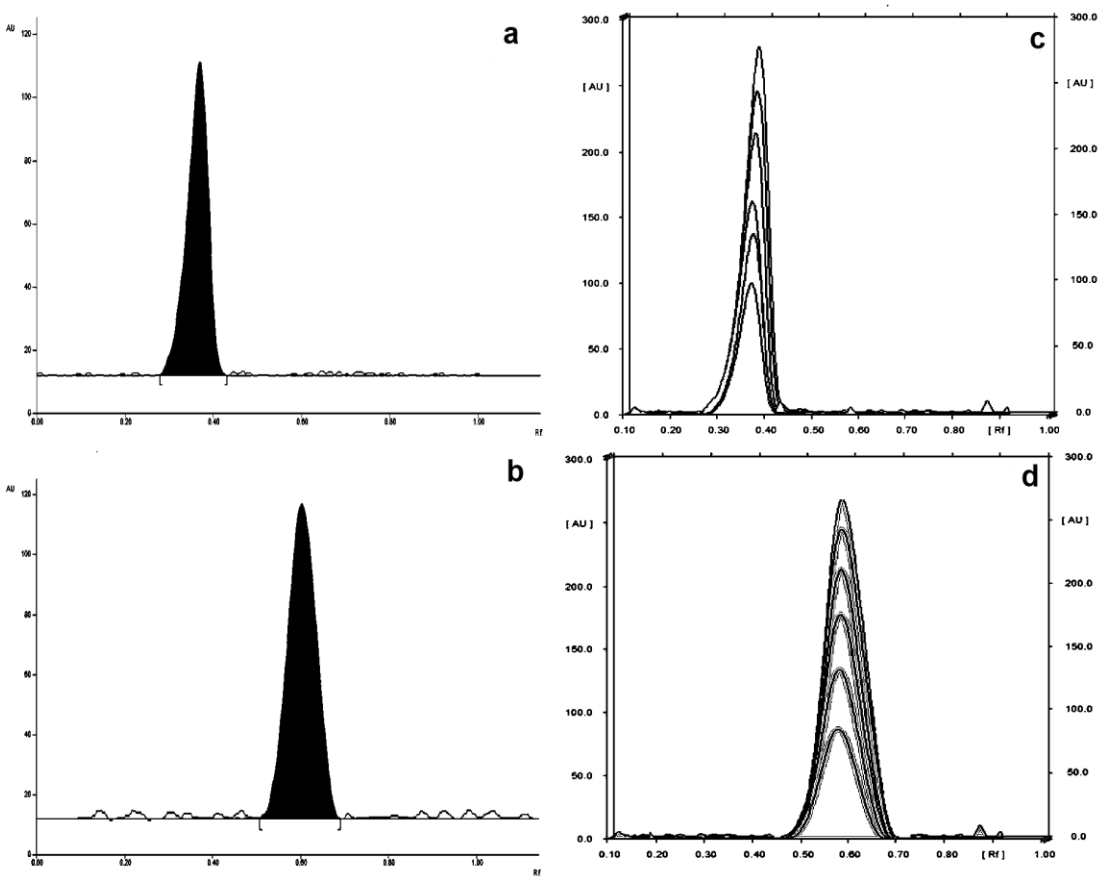


Figure 3.1. A typical HPTLC chromatogram demonstrating (a) estimation of LD (200 ng/spot; R_f, 0.44 ± 0.01; mobile phase n-butanol/glacial acetic acid/water = 3.5:1:1, v/v/v); (b) estimation of CD (200 ng/spot; R_f, 0.65 ± 0.01; mobile phase n-butanol/glacial acetic acid/water = 1.4:1:1, v/v/v); (c) overlaid chromatograms of LD and (d) overlaid chromatograms of CD.

Standard deviation of peak area at all selected concentration levels of LD was significantly low. Similar results were observed for CD method. The RSD values of peak areas corresponding to the each concentration level were observed below 2% for both the methods. The minimum values of bias calculated using linear model using univariant regression confirmed the goodness of fit in both cases. In addition to this, low standard

error of estimate and mean sum of squared residuals values, further confirm the precision of the developed methods for LD and CD estimation. Residuals calculated for both methods were found to be normally distributed around the mean with uniform variance, confirming the homoscedastic nature of the obtained data. Finally, one way ANOVA was carried out for both methods, low calculated F-value (8, 45) (2.16×10^{-4}) in comparison to critical F value ($LD_{FCrit} = 2.15$) at 5% level of significance confirmed precision of LD method (Table 3.1). Similarly, calculated F value (3.91×10^{-4}) was found to be lower than critical F value ($CD_{FCrit} = 2.15$) for CD method (Table 3.1).

c. Accuracy

For LD method, consistent and high absolute recovery values were found at all concentration levels for both placebo spiking and standard addition technique. For both techniques, mean absolute recovery of 98.61 - 100.07% for LD suggested that optimized method was accurate for estimation of LD (Table 3.2). For CD method, mean absolute recovery of 98.43 - 100.75% for CD confirmed the accuracy of method developed for CD estimation (Table 3.2). In case of the both methods, calculated absolute recovery values were normally distributed around the mean at selected concentration levels suggesting that obtained data is homoscedastic in nature. The lower values of bias (-1.57 to 0.75%) for both methods confirmed that there was no significant interference of the excipient and proposed methods were accurate (Table 3.2). In addition to this, for standard addition technique, consistent and high absolute recoveries obtained were in good agreement with placebo spiking technique. Therefore, it can be concluded that the proposed methods were suitable for the individual estimation of the LD and CD.

d. Precision

In precision study, deviation in measured responses of the freshly prepared standards at three QC levels were found to be insignificant which demonstrated that developed method for LD estimation was repeatable with RSD value below 1.42%. The low RSD values obtained for CD (RSD ≤ 1.46%) indicated the repeatability of method proposed for the CD estimation. For intermediate precision, RSD of LD and CD for inter-day variation were found to be ≤ 1.56% and ≤ 1.30%, respectively (Table 3.3).

Table 3.1. Statistical Data Summary for Developed HPTLC Methods

Parameter	Values	
	LD method	**CD method**
Calibration range (ng/spot)	100 - 500	100 - 600
Linearity (Regression coefficient)	0.9997	0.9998
Regression equation	Peak area = 14.87 Conc. (ng/spot) + 482.40	Peak area = 11.63 Conc. (ng/spot) - 100.90
Confidence interval of slope[a]	14.81 to 14.94	11.44 to 11.83
Confidence interval of intercept[a]	461.6 to 503.3	-178.20 to -23.54
F-value[b] (8,45)	2.16×10^{-4}	3.91×10^{-4}
Standard error of estimate	34.77	29.92
Specificity and selectivity t_{Cal} (t_{Crit})[c]	1.18 (2.57)	1.78 (2.57)
Limit of detection (ng/spot)	35.00	50.00
Limit of quantitation (ng/spot)	50.00	75.00
Absolute recovery (%)	98.61 to 100.07	98.43 to 100.75
Precision (RSD, %)		
Repeatability of application	1.23	1.12
Repeatability of measurement	1.46	1.39
Interbatch (n = 27)	1.56	1.30
Intrabatch (n = 9)	1.42	1.46
Robustness	Robust	Robust

[a]Calculated at 5% level of significance.
[b]Calculated using Fisher test with one way ANOVA at 5% level of significance. (LD_{FCrit} = 2.15 and CD_{FCrit} = 2.15).
[c]Calculated t-value (t_{Cal}) and theoretical t-value (t_{Crit}) at 5 degree of freedom are based on the paired t-test at 5% level of significance.

Table 3.2. Recovery Studies by Placebo Spiking and Standard Addition Technique

Product	Technique	Method (Drug)	Amount of drug added (ng) (% of excess drug added to the analyte)[d]	Mean amount recovered (ng) (Mean ± SD) (n = 6)	RSD (%)	Mean absolute recovery (%)	Bias (%)
In-house manufactured pellets	Placebo spiking[a]	LD	100	98.61 ± 1.51	1.54	98.61	-1.39
			200	199.89 ± 0.44	0.22	99.94	-0.06
			300	299.60 ± 1.60	0.54	99.87	-0.13
			400	399.17 ± 1.88	0.47	99.79	-0.21
			500	499.02 ± 1.45	0.29	99.80	-0.20
		CD	100	98.43 ± 1.07	1.09	98.43	-1.57
			200	198.01 ± 0.90	0.45	99.01	-0.99
			400	399.59 ± 3.46	0.86	99.90	-0.10
			500	503.73 ± 5.00	0.99	100.75	0.75
			600	598.67 ± 1.00	0.17	99.78	-0.22
	Standard addition	LD[b]	200 (0)	199.51 ± 0.74	0.37	99.75	-0.25
			300 (50)	298.19 ± 2.06	0.69	99.40	-0.60
			400 (100)	399.29 ± 1.62	0.40	99.82	-0.18
			500 (150)	500.36 ± 1.84	0.37	100.07	0.07
		CD[c]	200 (0)	197.01 ± 0.50	0.25	98.50	-1.50
			300 (50)	298.63 ± 2.20	0.74	99.54	-0.46
			400 (100)	399.16 ± 3.73	0.93	99.79	-0.21
			500 (150)	497.48 ± 2.82	0.57	99.50	-0.50

[a]Placebo capsule containing blank pellets equivalent to the unit dose weight.
[b]In-house manufactured pellets containing 480 mg of LD per grams of pellets.
[c]In-house manufactured pellets containing 120 mg of CD per grams of pellets.
[d]Values in parentheses indicates percentage of excess drug added to the analyte.

Table 3.3. Results of Repeatability and Intermediate Precision Study

QC levels (ng/spot)	Repeatability (intrabatch) (n = 9)									Intermediate precision (interbatch) (n = 27)		
	Batch 1			Batch 2			Batch 3					
	Range	Mean ± SD	RSD (%)	Range	Mean ± SD	RSD (%)	Range	Mean ± SD	RSD (%)	Range	Mean ± SD	RSD (%)
LD method												
LQC (150)	148.03-152.13	149.75 ± 2.13	1.42	147.08-150.85	148.99 ± 1.88	1.26	148.36-150.04	149.28 ± 0.85	0.57	146.75-153.47	150.01 ± 2.35	1.56
MQC (250)	247.00-252.64	249.17 ± 3.04	1.22	249.55-250.76	250.20 ± 0.61	0.24	247.00-249.01	248.07 ± 1.42	0.57	247.00-252.64	249.75 ± 1.75	0.70
HQC (500)	497.99-501.69	500.14 ± 1.92	0.38	497.72-499.47	498.53 ± 0.88	0.78	496.37-499.94	498.08 ± 1.79	0.36	493.69-501.69	498.09 ± 2.44	0.49
CD method												
LQC (150)	147.65-151.77	149.31 ± 2.18	1.46	147.47-150.91	148.91 ± 1.79	1.20	148.33-151.34	150.20 ± 1.63	1.08	146.62-152.89	149.77 ± 1.95	1.30
MQC (300)	298.31-300.46	299.14 ± 1.15	0.39	297.80-300.98	299.37 ± 1.59	0.53	297.88-299.60	298.77 ± 0.86	0.29	298.48-302.09	299.00 ± 2.42	0.81
HQC (550)	547.47-550.05	549.17 ± 1.46	0.27	548.08-550.14	548.94 ± 1.07	0.20	546.62-551.17	549.08 ± 2.30	0.42	546.87-551.51	549.45 ± 1.81	0.33

The lower RSD values demonstrated the intermediate precision and repeatability for the methods proposed for individual estimation of LD and CD (Table 3.3).

e. Specificity and Selectivity

During the analysis of placebo samples, peaks obtained for LD and CD were compared with the blank placebo samples to study the specificity of the proposed methods. The response and the peak properties did not changed significantly at LOQ level of LD (Figure 3.2a).

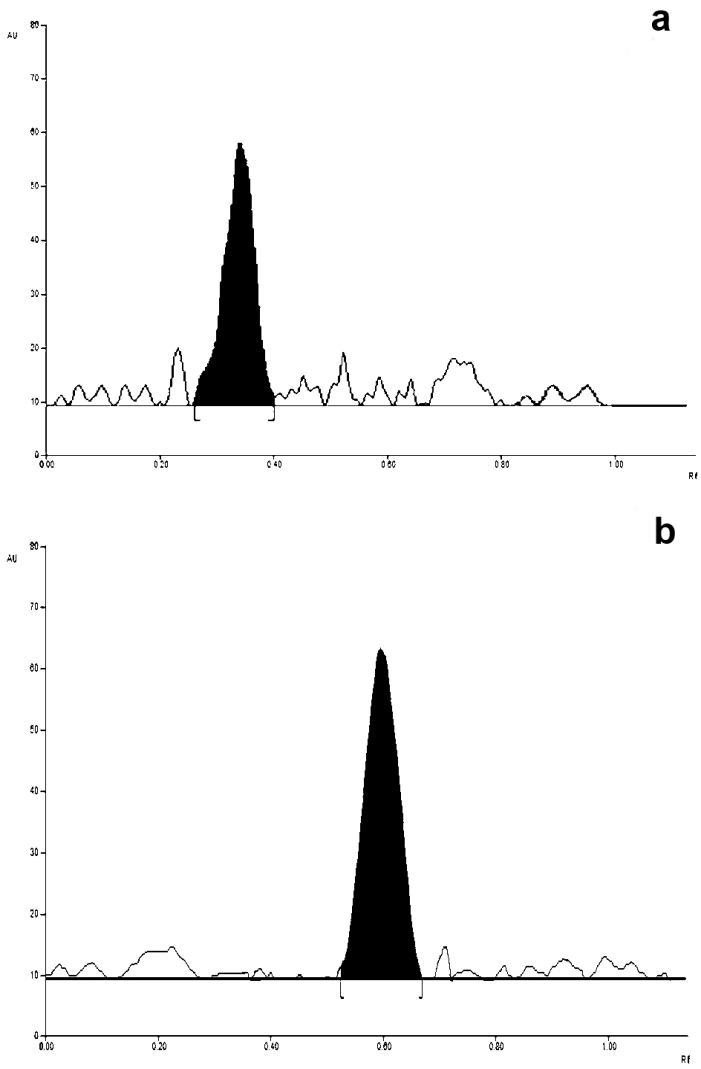


Figure 3.2. Representative HPTLC chromatograms of (a) LD at quantitation limit (LOQ = 50 ng/spot) and (b) CD at quantitation limit (LOQ = 75 ng/spot).

Similarly, acceptable deviation in the response and the peak properties of CD at LOQ level further confirmed the specificity of the method proposed for estimation of CD (Figure 3.2b). Absence of the interfering peak within the vicinity of the drug peaks in case of the both methods demonstrated the specificity of the proposed methods (Figure 3.3a and 3.3b).

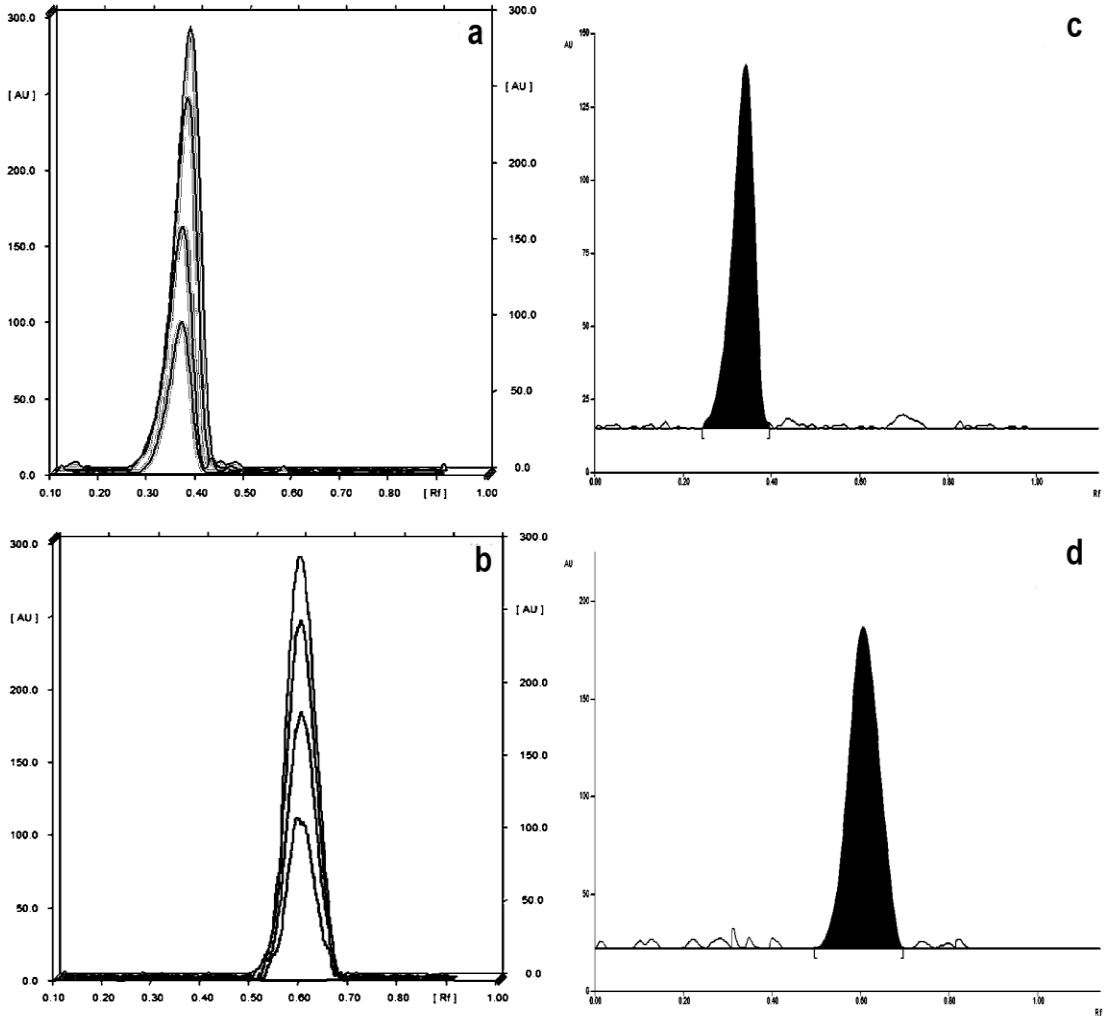


Figure 3.3. The representative HPTLC chromatograms demonstrating (a) selectivity for placebo and formulation standards of LD; (b) selectivity for placebo and formulation standards of CD; (c) test sample for in-house manufactured pellets of LD and (d) test sample for in-house manufactured pellets of CD.

In addition, for both methods calculated t values (LDt_{Cal} = 1.18; CDt_{Cal} = 1.78) were found to be lower than the critical t values (LDt_{Crit} = 2.57 and CDt_{Crit} = 2.57) indicating statistically insignificant difference between mean peak areas of standard prepared from pure drug sample and in-house prepared pellet formulation sample (Figure 3.3C and Figure 3.3d) (Table 3.1).

f. Sensitivity

Sensitivity of the proposed methods was assessed using calculation of LOQ and LOD. For estimation of the LOQ and LOD, the calibration curve was plotted between the amount of the analyte and the corresponding response (peak area). Regression equations were obtained by linear regression analysis for both methods. The regression equations for LD and CD obtained were Y= 15.07X + 436.60 with regression coefficient value of 0.9993 and Y= 11.41X - 73.11 with regression coefficient value of 0.9991, respectively. Where, Y is peak area and X is the slope. The LOD and LOQ for the proposed methods were calculated by equation discussed in "*analytical method validation"* section. The LOQ and LOD values calculated by equation were 24.18 ng/spot and 7.98 ng/spot, for LD respectively, whereas LOQ and LOD values for CD were 45.21 ng/spot and 14.92 ng/spot, respectively. However, practically calculated LOQ and LOD values were found to be different than the values calculated by equation. Practically determined values of LOD and LOQ were 35.00 ng/spot and 50.00 ng/spot for LD, respectively, whereas for CD value of LOD was 50.00 ng/spot and LOQ was 75.00 ng/spot (Table 3.1). For both the methods, upon repeated analysis at LOQ, observed peak properties (retention time, peak area) were not affected. In addition, mean absolute recoveries were consistently high with acceptable bias and RSD values at LOQ for both methods. Practically found LOQ for both the proposed methods is adequate for in vitro analysis and stability studies. Therefore, it can be concluded that proposed methods were found to be sensitive.

g. Robustness Study

Standard deviation was calculated for the response (peak area) at each parameter selected for robustness study for both methods. The obtained RSD values were found to be lower

than the 2% for both methods. The low RSD values in Table 3.4 confirmed the robustness of the proposed methods.

h. Drug Content Estimation of LD and CD in In-house Manufactured Pellets

The chromatograms of LD and CD extracted from in-house prepared pellet formulations are shown in Figure 3.3c and 3.3d. For both the methods, observed mean recoveries were found to be in good agreement with the claim of respective label. In addition to this, RSD was found to be in acceptable limit (< 2%) which further confirmed the precision of both the methods (Table 3.2). In addition to this, formulation excipients did not show any interference within the vicinity of the LD and CD peaks for both proposed methods. The bias observed for the LD and CD methods were found to be lower than 0.07% and 0.75% respectively, which further confirmed the accuracy of the proposed methods. Thus, proposed methods were found to be suitable for individual estimation of LD and CD from their respective formulations.

i. Forced Degradation Study

The individual chromatographs of LD and CD samples degraded in presence of acid, base, neutral conditions and H_2O_2 showed well resolved peaks of LD and CD from the degradation product peaks. The well resolved peaks of LD from its degradation products peaks are shown in Figure 3.4a for acid, Figure 3.4b for base, Figure 3.4c for neutral and Figure 3.4d for oxidation, while the chromatograph with resolved peaks of CD from its degradation product peaks are shown in Figure 3.4e for acid, Figure 3.4f for base, Figure 3.4g for neutral and Figure 3.4h for oxidation. The amount of drug recovered and R_f values of degradation products values were calculated and summarized in Table 3.5. Thus these methods were found to be suitable for selective estimation of analytes even in presence of degradation products.

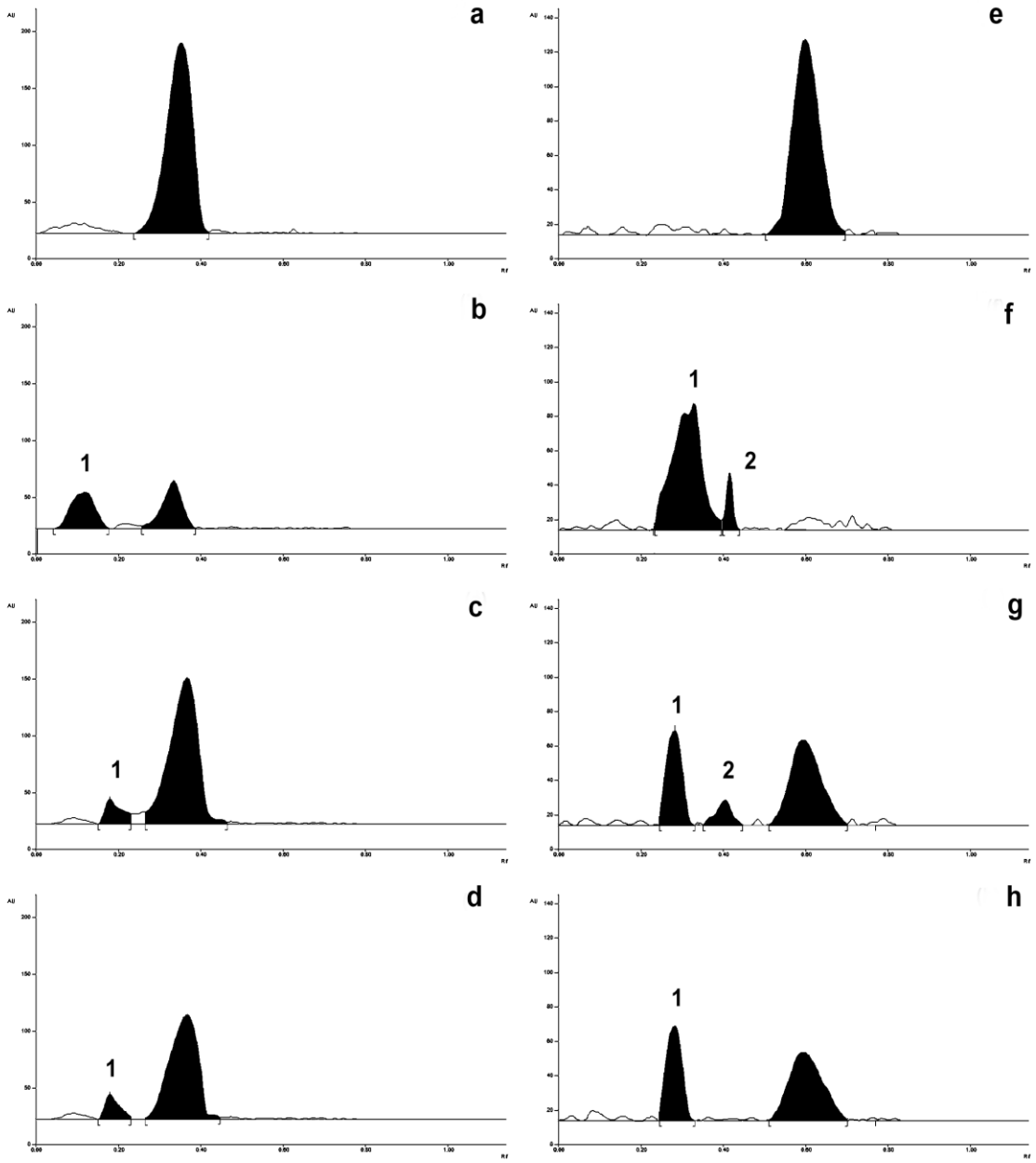


Figure 3.4. HPTLC chromatograms of (a) acid degradation products of LD; (b) base degradation products of LD; (c) degradation products of LD at neutral pH; (d) oxidative degradation product of LD; (e) acid degradation products of CD; (f) base degradation products of CD; (g) degradation products of CD at neutral pH and (h) oxidative degradation product of CD.

Table 3.4. Robustness Study Results

Parameter	Methods					
	LD [RSD (%), n = 3]			CD [RSD (%), n = 3]		
	LQC (150 ng/spot)	MQC (250 ng/spot)	HQC (500 ng/spot)	LQC (150 ng/spot)	MQC (300 ng/spot)	HQC (550 ng/spot)
Mobile phase composition	1.64	0.57	0.40	1.99	1.04	0.78
Amount of mobile phase	1.29	0.62	0.45	1.87	0.97	0.44
Plate pretreatment	1.62	1.03	0.44	1.32	0.91	0.66
Time from spotting to chromatography	1.54	0.48	0.55	1.73	0.69	0.43
Time from chromatography to scanning	1.66	0.97	0.58	1.57	0.78	0.32

Table 3.5. Results of Forced Degradation Study of LD and CD

Drug	Sample exposure condition	Number of degradation product (R_f value)	LD recovery (%) (Mean ± SD) (n = 3)	CD recovery (%) (Mean ± SD) (n = 3)
LD	Acid (0.1 N HCl, 2 h, 40°C)	-	99.23 ± 1.23	-
	Base (0.1 N NaOH, 2 h, 40°C)	1 (0.11)	Not detected	-
	Neutral (PBS pH 6.8, 2 h, 40°C)	1 (0.20)	48.23 ± 2.31	-
	Oxidation (H_2O_2, 10% v/v, 2 h, 40°C)	1 (0.21)	34.23 ± 2.45	-
CD	Acid (0.1 N HCl, 2 h, 40°C)	-	-	99.85 ± 1.48
	Base (0.1 N NaOH, 2 h, 40°C)	1(0.33), 2 (0.42)	-	Not detected
	Neutral (PBS pH 6.8, 2 h, 40°C)	1(0.31), 2 (0.41)	-	52.31 ± 2.12
	Oxidation (H_2O_2, 10% v/v, 2 h, 40°C)	1 (0.32)	-	57.23 ± 1.25

3.4. Analytical Method II: Liquid Chromatographic Method

Simultaneous Estimation of LD and CD by Reverse Phase High Performance Liquid Chromatographic Method Using Fluorescence Detector

3.4.1. Instrumentation

The liquid chromatography system employed was Jasco HPLC (Jasco Corporation, Tokyo, Japan) with solvent delivery system of two intelligent pumps (Jasco-PU-1580, Jasco, Japan), intelligent auto injector (Jasco AS-1559, Jasco, Japan), intelligent fluorescence detector (Jasco FP-1520, Jasco, Japan) and Jasco Model Interface box (LC-NET II/ADC, Jasco, Japan). Data collection and integration was accomplished using Borwin software version 1.50.

3.4.2. Chromatographic Conditions

The chromatographic separation was achieved on an endcapped C_{18} reverse phase column (Lichrospher®, 250 mm long and 4.6 mm internal diameter, particle size 5 µm, E. Merck, Darmstadt, Germany). The optimized mobile phase consisted of an aqueous phase (10 mM potassium dihydrogen phosphate buffer, pH adjusted to 4.0 using 0.1 M orthophosphoric acid) and methanol in the ratio of 90:10 v/v. The buffer was filtered through 0.22 µm Millipore® filtration membrane. The HPLC system was equilibrated for minimum 1 h by passing mobile phase through system at a flow rate of 1 mL/min, prior to actual analysis. LD and CD were monitored at an excitation wavelength of 280 nm and emission wavelength of 310 nm with mobile phase flow rate of 1 mL/min. The injection volume was 50 µL. Analysis was carried out at an ambient temperature (25 ± 2°C).

3.4.3. Preparation of Stock and Standard Solutions

Individual stock solutions (100 µg/mL) of LD and CD were prepared by dissolving suitable amount of each pure substance in a mixture consisting of 0.1% orthophosphoric acid (v/v), 0.1% sodium metabisulfite (w/v) and water. These stock solutions were used to prepare secondary stock solution containing 1 µg/mL of LD and CD. From secondary stock solution, three separate series of seven calibration standards containing 5, 10, 25, 50, 100, 250 and 500 ng/mL of LD and CD were prepared freshly by serial dilution in mobile phase on three different days of validation. Formulation standards were prepared

by addition of known amounts of LD and CD from secondary stock solution in placebo blend of tablets. The formulation standards were prepared at five levels to contain 5, 25, 50, 250 and 500 ng/mL of LD and CD. Similarly, placebo standards were prepared without adding any drug. Prepared standards were processed independently, as described in their respective sample preparation section and analyzed.

3.4.4. Sample Preparation

A quantity of the product equivalent to 10 mg of LD and 1 mg of CD was weighed and transferred to a 100 mL calibrated flask and volume was made up with a mixture consisting of 0.1% orthophosphoric acid (v/v), 0.1% sodium metabisulfite (w/v) and water. After vortex mixing for 5 min, samples were filtered through Whatman filter paper (0.22 µm). Finally, 0.5 mL of the filtrate was transferred to a 100 mL calibrated flask and diluted to volume with mobile phase.

3.4.5. Analytical Method Optimization

A high height to peak area ratio and good peak symmetry were objective in the development of a selective and sensitive method during the development phase. For mobile phase optimization, different buffer types of varying pH such as phosphate buffer (pH 3 - 6, 10 mM), citrate buffer (pH 3 - 5, 10 mM), ammonium acetate buffer (pH 3 - 5, 10 mM) and acetic acid buffer (pH 3 - 5, 10 mM) were studied in combination with methanol (10, 20 and 40 %, v/v). The effects of various organic modifiers on peak properties (peak height, peak area, peak symmetry, retention time etc.) and response function were observed. The mobile phase that was finally selected consisted of an aqueous phase (10 mM potassium dihydrogen phosphate buffer, pH adjusted to 4.0 using 0.1 M orthophosphoric acid) and methanol in the ratio of 90:10 v/v. The criteria employed for selection of mobile phase were: peak properties [retention time (R_t) and asymmetric factor (T_f)], retention factor (k), sensitivity (height and area), number of theoretical plates (N), height equivalent to theoretical plates (HETP), ease of preparation and applicability of the method for estimation of LD and CD in pharmaceutical formulations. During the final stage of method development robustness study was carried out using design of experimentation technique (DOE).

3.4.6. Analytical Method Validation

The developed liquid chromatographic method was validated for selectivity, linearity, range, precision, accuracy, sensitivity, robustness and system suitability. The method was also applied for drug content analysis of commercial and in-house prepared tablet formulations.

a. Linearity and Range

To establish linearity of proposed method, nine separate series of calibration standards were prepared at seven concentration levels ranging from 5 to 500 ng/mL for LD as well as for CD and analyzed using developed method. Least square linear regression analysis was performed for average peak area obtained at each concentration level. Calibration equation obtained from regression analysis was used to calculate the corresponding predicted concentrations. ANOVA test (one-way) was performed based on the peak area observed for each concentration during the replicate measurement at seven concentration levels (Bolton and Bon, 2009). The analytical range of the method was established by analysis of residuals and a test of the intercept was carried out using the *t*-statistic.

b. Accuracy

To estimate accuracy of proposed method, a recovery study was carried out using two different techniques, viz. the placebo spiking method and the standard addition method for tablets. In the placebo spiking method, a known amount of standard solution was added to placebo blank at five concentrations 5, 25, 50, 250 and 500 ng/mL for LD as well as for CD. In the standard addition technique, a known amount of pure drug was added to the sample solution at 40, 80 and 160% concentration level of the labeled claim of previously analyzed tablet preparation for both LD and CD. Each concentration was processed in six replicates on three different days and the results were expressed as mean absolute recovery, RSD and bias.

c. Precision

The precision of the proposed method was determined as repeatability (intraday) and intermediate (interday) precision. The study was conducted using QC standards prepared

at three concentrations LQC (5 ng/mL), MQC (50 ng/mL) and HQC (500 ng/mL). Three series of three QC standards were prepared freshly and analyzed for intraday variation (repeatability). Similarly, standards were prepared and analyzed on three consecutive days for intermediate precision (interday). For intraday and interday assay, RSD of the predicted concentrations obtained from the regression equation at three QC levels were used to assess repeatability and intermediate precision, respectively.

d. Selectivity and Specificity

Placebo and spiked-placebo analysis techniques were applied to assess selectivity of proposed method. On three consecutive days, placebo and formulation standards (tablets) were prepared in triplicate. Standards were processed as described in the respective sample processing section. Prepared samples were then analyzed using chromatographic conditions mentioned earlier. After analysis of samples, the obtained chromatograms were checked for peak area and interference of excipients at retention time of drug peaks. In a separate study, standard (combination of LD = 100 ng/mL and CD = 10 ng/mL) was prepared independently from pure drug stock and commercial sample stock in selected media and analyzed as mentioned earlier (n = 6). A paired t-test at 5% level of significance was applied to compare the means of peak areas.

e. Sensitivity

The LOD and LOQ were calculated on the basis of signal-to-noise ratio of 3:1 and 10:1 respectively (Shah et al., 2000; USP, 2007). The validity of calculated LOD and LOQ was further confirmed by injecting these concentrations onto the column at chromatographic condition mentioned earlier.

f. Robustness

Robustness of proposed method was estimated by testing reliability of analysis with respect to small but deliberate variation in optimized method parameters. Critical chromatographic factors and their effects on method performance were identified using DOE (Snyder et al., 1997; Lewis et al., 1998; Vander Heyden et al., 2001). A DOE technique allows the execution of a minimum number of experiments for study of the

selected factors. It is an effective tool for maximizing the amount of information gained from a study while minimizing the amount of data to be collected. Further, DOE is an efficient procedure for planning experiments so that the data obtained can be analyzed to yield valid and objective conclusions.

In the proposed study during method development, the mobile phase was identified as major cause of variability in the analysis. Three factors related to mobile phase preparation, viz. percent organic component (%MET), buffer strength and pH of buffer phase, were identified as critical sources of variability in the operating procedure. A three-factor face centered design consisting of 17 experiments was carried out. The %MET (A = 7.5 - 12.5%, v/v), pH of aqueous phase (B = 3.75 - 4.25) and buffer strength of aqueous component (C = 5 - 15 mM) were considered critical factors. Drug standards were injected in triplicate after baseline stabilization for each experiment. Chromatographic parameters such as retention time and peak area along with system suitability parameters (*k*, T_f, *N* and HETP) were recorded as experimental responses. Validation was carried out by one-way ANOVA and lack of fit analysis. Data was processed using Design-Expert Software (version 8.0.7.1, Stat-Ease Inc., Minneapolis, MN, USA).

g. System Suitability and Drug Stability Study

In system suitability study, chromatographic performance parameters (*k*, T_f, *N* and HETP) were recorded as an essential part of analytical procedure. System precision was determined by performing injection repeatability of the calibration standards with ten replicates. The stability of the drugs in mobile phase was estimated by injecting calibration standards as well as formulation standards at 0, 24 and 48 h in triplicates.

h. Forced Degradation Studies

Forced degradation studies of LD and CD were carried out to explore stability indicating nature and specificity of the developed HPLC method. Forced degradation of LD and CD was carried out by exposing drugs to hydrolytic (acid and base), oxidative, photolytic and thermal stress conditions. The stock solution of LD (1 mg/mL) and CD (0.25 mg/mL) was prepared in water and protected from light.

For hydrolytic stress treatment, stock solution of LD and CD were diluted in 0.1 N HCl (1:1, v/v) and 0.1 N NaOH (1:1, v/v), separately. Resulting solutions were kept at 60°C for 2 h. For oxidative stress treatment, H_2O_2 (10%, v/v) was added to stock solution of LD and CD in the ratio of 1:1 (v/v) and kept at 60°C for 2 h. Further, for photolytic degradation study, stock solution of LD and CD was exposed to UV light at a wavelength of 254 nm, at a distance of 20 cm for a period of 24 h. Thermal degradation study, was performed by keeping physical mixture of LD and CD (4:1, w/w) at 150° C for 8 h. After completion of stress treatment, samples were allowed to cool to room temperature (if needed) and appropriately neutralized when required (samples of acid and base degradation studies). During stress treatment, all the samples except photolytic treatment sample were protected from light in order to avoid the possible degradative effect of light. All the degradation samples were suitably diluted using mobile phase before injecting into the HPLC system. The degraded samples were compared with control sample (freshly prepared samples lacking degradation treatment).

i. Drug Content Estimation of LD and CD in Pharmaceutical Preparations

As a part of the validation procedure, the applicability of the method was tested for drug content analysis of real world samples such as marketed tablets as part of the validation procedure. For estimation of LD and CD in commercial tablets, 20 tablets were weighed and powdered. A quantity of powder equivalent to 10 mg of LD and 1 mg of CD was weighed accurately and processed as described in sample processing section so as to contains 250, 100, 50 ng/mL of LD and 25, 10, 5 ng/mL of CD. The resulting solutions (50 µL) were injected in triplicate and analyzed using the proposed method.

3.4.7. Results and Discussion

a. Analytical Method Optimization

In preliminary studies, peak properties and response function were optimized by changing the type of organic phase, the organic to aqueous phase ratio, buffer type, buffer strength and the pH of aqueous phase. The use of organic modifiers led to varying degrees of improvement in peak symmetry of CD (T_f = 1.18 to 1.45) over simple buffer (T_f > 1.7) whereas in case of LD, consistent peak symmetry was observed. In case of CD,

peak properties were largely affected by the use of different organic modifiers and by change in the pH of the buffer phase. The retention time and peak symmetry of LD were almost unaffected by changes in pH of buffer phase and the use of different organic modifiers. For better peak symmetry and resolution, a double endcapped C_{18} column was used. Moreover, excitation and emission wavelength for both LD and CD were optimized for better sensitivity and selectivity from degradation products. Thus, an optimized mobile phase consisting of an aqueous phase (10 mM potassium dihydrogen phosphate buffer, pH adjusted to 4.0 using 0.1 M orthophosphoric acid) and methanol in the ratio of 90:10 v/v was found to provide good retention (LD_{Rt} = 3.6 ± 0.01 min, CD_{Rt} = 5.6 ± 0.04 min) with better peak properties, selectivity and reproducibility. The representative chromatogram of simultaneous estimation of LD and CD is shown in Figure 3.5a.

b. Linearity, Accuracy and Precision

Statistical analysis showed excellent linearity over the range 5 - 500 ng/mL for both LD and CD with the selected mobile phase. According to linear regression analysis, in the case of LD, the slope and intercept were found to be 6128.80 and 107.60 with a regression coefficient of 0.9999, whereas in the case of CD, the slope was 4173.57 and the intercept was 159.70 with a regression coefficient of 0.9998 (Table 3.6). In both cases the intercepts were not significantly different from zero at the 95% confidence interval (LDt_{df} = 0.12, CDt_{df} = 0.55) as confirmed by the application of a test of intercept. At all LD and CD concentrations, the standard deviation of the peak area was significantly low and RSD values were below 1.58% and 1.59% for LD and CD, respectively. The selected linear model with univariant regression showed minimum bias indicating goodness of fit in both cases. Moreover, low values for the standard error of estimate (LD = 0.29 ng/mL, CD = 0.11 ng/mL) and mean sum of the squared residuals for LD, as well as for CD, indicates that the proposed method may be useful for the simultaneous estimation of LD and CD with high precision (Table 3.6). Analysis of residuals indicated that the residuals were normally distributed around the mean with uniform variance across all concentrations, suggesting the homoscedastic nature of the data, in both cases. Finally, in both cases, one-way ANOVA was performed for the peak area obtained at individual concentration levels and lower calculated F values (8, 54) ($LD_{Fcal} = 7.105 \times 10^{-5}$, CD_{Fcal} =

1.209×10^{-4} in comparison with critical F values ($LD_{FCrit} = 2.02$, $CD_{FCrit} = 2.02$) at the 5% level of significance further confirming the precision of the method.

Consistent and high absolute recovery at all concentrations in the placebo spiking and standard addition methods with a mean absolute recovery of 99.63 - 100.80% for LD and 98.97 - 100.94% for CD confirmed the accuracy of the optimized method (Table 3.7). Moreover, in the placebo spiking method, the obtained recoveries were normally distributed around the mean with low RSD across five concentrations suggesting the homoscedastic nature of the data for both drugs. Thus, it can be concluded that there was no significant interference of excipients and the method was found to be accurate with a low bias (LD ≤ 0.80%, CD ≤ 0.94%). In the standard addition method, consistent and high absolute recoveries obtained were in good agreement with the placebo spiking technique. The recovery study indicated that the method was suitable for the simultaneous determination of LD and CD from tablet preparations.

In the repeatability study, variation in the measured response of six freshly prepared standards at three QC levels was found to be insignificant, which showed that the developed method was repeatable with RSD values below 1.65% for LD and 1.85% for CD. Further, RSD values for interday variation were significantly low ($RSD_{LD} \leq 1.35\%$, $RSD_{CD} \leq 1.37\%$) for intermediate precision. Acceptable RSD values indicated the intermediate precision of the method and its repeatability (Table 3.8).

c. Specificity, Selectivity and Sensitivity

At LOQ level, drug spiked placebo samples showed no significant change in response and peak properties (Figure 3.5b). In the case of placebo samples, no interference was observed within the vicinity of the drug peak, which demonstrate the selectivity of the method for LD and CD in the presence of formulation excipients (Figure 3.6a).

The means of peak areas were compared by paired t-test at 95% confidence interval. Calculated t-values below the critical t-value, revealed that, statistically there was no significant difference between the mean peak areas of standards prepared from pure drug sample and marketed formulation sample (Table 3.7), indicating specificity and selectivity of proposed method (Figure 3.6b).

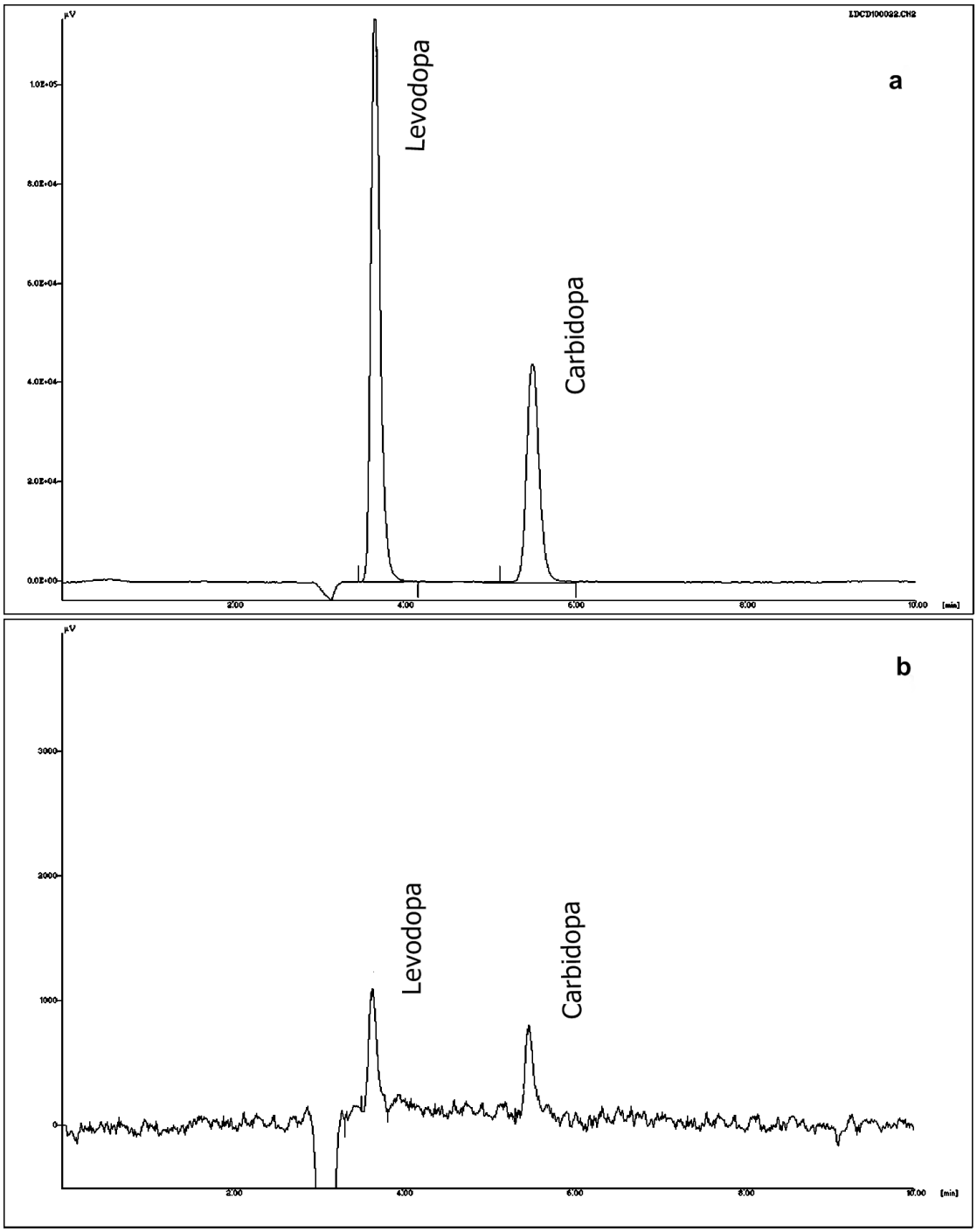


Figure 3.5. The representative chromatograms demonstrating (a) peaks of LD (100 ng/mL) and CD (100 ng/mL) and (b) standard at quantitation limit (LD = 0.70 ng/mL, CD = 1.20 ng/mL)

Table 3.6. Statistical Data Summary for Liquid Chromatographic Method

Parameter	Values	
	LD	**CD**
Calibration range (ng/mL)	5 - 500	5 - 500
Linearity (Regression coefficient)	0.9999	0.9998
Regression equation	Peak area(µVs) = 6128.80 Conc. (ng/mL) + 107.60	Peak area(µVs) = 4173.57 Conc. (ng/mL) + 159.70
Confidence interval of slope[a]	6118.29 to 6139.71	4170.53 to 4177.47
Confidence interval of intercept[a]	-2203.45 to 2418.64	-587.99 to 907.39
Standard deviation of intercept (Sa)	8.99×10^2	2.91×10^2
t-value of intercept[a,b] (tab = 2.57)	0.12 (P-value 0.91)	0.55 (P-value 0.61)
F-value[c]	7.105×10^{-5}	1.209×10^{-4}
Standard error of estimate	1.861×10^3 (0.29 ng/mL)	6.02×10^2 (0.11 ng/mL)
Specificity and selectivity - t_{Cal} (t_{Crit})[d]	1.53 (2.57)	0.85 (2.57)
Limit of detection (ng/mL)	0.30	0.60
Limit of quantitation (ng/mL)	0.70	1.20
Absolute recovery	99.63 - 100.80%	98.97 - 100.94%
Precision (RSD, %)	Repeatability = 1.65% (Intraday) Intermediate precision = 1.35% (Interday)	Repeatability = 1.85% (Intraday) Intermediate precision = 1.37% (Interday)
System suitability	System precision = 0.65% (n = 10) Tailing factor = 1.13 Number of plates = 6852.42 HETP= 36.40 µm	System precision = 0.93% (n = 10) Tailing factor = 1.08 Number of plates = 8012.35 HETP = 31.20 µm
Selectivity (Resolution)[e]	-	8.99
Robustness	%MET ± 2.50 pH ± 0.25	%MET ± 2.50 pH ± 0.25

[a]Calculated at 0.05 level of significance.
[b]Calculated using the test of the intercept ($t_{df} = |a-\alpha|/Sa$).
[c]Calculated using Fisher test with one way ANOVA (5% level of significance).
[d]t_{Cal} is calculated value and t_{Crit} is theoretical value (at 5 degree of freedom) based on paired t-test at 5% level of significance.
[e]Acceptable resolution is > 2.

Table 3.7. Recovery Studies by Placebo Spiking and Standard Addition Methods

Product	Method		Amount of drug added (ng/mL)	Mean amount recovered (ng/mL) (Mean ± SD) (n= 6)	Mean absolute recovery (%)	RSD (%)	Bias (%)
Tablet	Placebo spiking [a]	LD	5	4.99 ± 0.06	99.80	1.18	-0.20
			25	24.98 ± 0.16	99.91	0.66	-0.09
			50	50.22 ± 0.05	100.44	0.10	0.44
			250	251.28 ± 0.63	100.51	0.25	0.51
			500	504.01 ± 1.55	100.80	0.31	0.80
		CD	5	4.99 ± 0.05	99.71	0.94	-0.29
			25	24.74 ± 0.46	98.97	1.86	-1.03
			50	50.47 ± 0.21	100.94	0.41	0.94
			250	251.82 ± 0.61	100.73	0.24	0.73
			500	504.56 ± 2.80	100.91	0.55	0.91
	Standard addition [b]	LD	100	351.44 ± 0.66	100.41	0.19	0.41
			200	298.90 ± 2.41	99.63	0.81	-0.37
			400	449.00 ± 1.15	99.78	0.26	-0.22
		CD	100	124.85 ± 0.13	99.88	0.10	-0.12
			200	211.25 ± 1.81	100.59	0.86	0.59
			400	407.57 ± 0.66	100.63	0.16	0.63

[a]Placebo tablet matrix equivalent to unit dose weight.
[b]Commercial tablet preparation (SYNDOPA® 275) containing LD (250 mg) and CD (25 mg).

Table 3.8. Results of Repeatability and Intermediate Precision Study

QC levels (ng/mL)	Repeatability (n = 9)									Intermediate precision (n = 27)		
	Batch 1			Batch 2			Batch 3					
	Range	(Mean ± SD)	RSD (%)	Range	(Mean ± SD)	RSD (%)	Range	(Mean ± SD)	RSD (%)	Range	(Mean ± SD)	RSD (%)
LD												
LQC (5)	4.95 - 5.11	5.03 ± 0.08	1.52	4.98 - 5.08	5.01 ± 0.06	1.11	4.99 - 5.11	5.05 ± 0.06	1.25	4.92 - 5.08	5.02 ± 0.06	1.17
MQC (50)	49.76 - 51.41	50.53 ± 0.83	1.65	50.29 - 50.89	50.58 ± 0.30	0.59	49.73 - 50.22	49.94 ± 0.25	0.50	49.01 - 50.58	49.90 ± 0.67	1.35
HQC (500)	498.89 -502.47	500.14 ± 2.02	0.40	5.2.33 - 503.81	502.98 ± 0.75	0.15	499.52 - 501.22	500.47 ± 0.86	0.17	498.67 - 503.45	500.98 ± 1.76	0.35
CD												
LQC (5)	4.99 - 5.15	5.05 ± 0.09	1.85	4.99 - 5.13	5.01 ± 0.07	1.41	4.92 - 5.04	4.98 ± 0.06	1.22	4.95 - 5.16	5.02 ± 0.07	1.37
MQC (50)	49.82 - 50.41	50.54 ± 0.79	1.56	49.36 - 50.30	49.95 ± 0.52	1.03	50.37 - 50.51	50.76 ± 0.54	1.06	49.79 - 51.37	50.55 ± 0.58	1.15
HQC (500)	498.30 -502.73	500.05 ± 2.36	0.47	498.31 - 501.13	499.99 ± 1.49	0.30	499.50 - 500.63	499.95 ± 0.60	0.12	498.89 - 506.02	501.26 ± 2.58	0.52

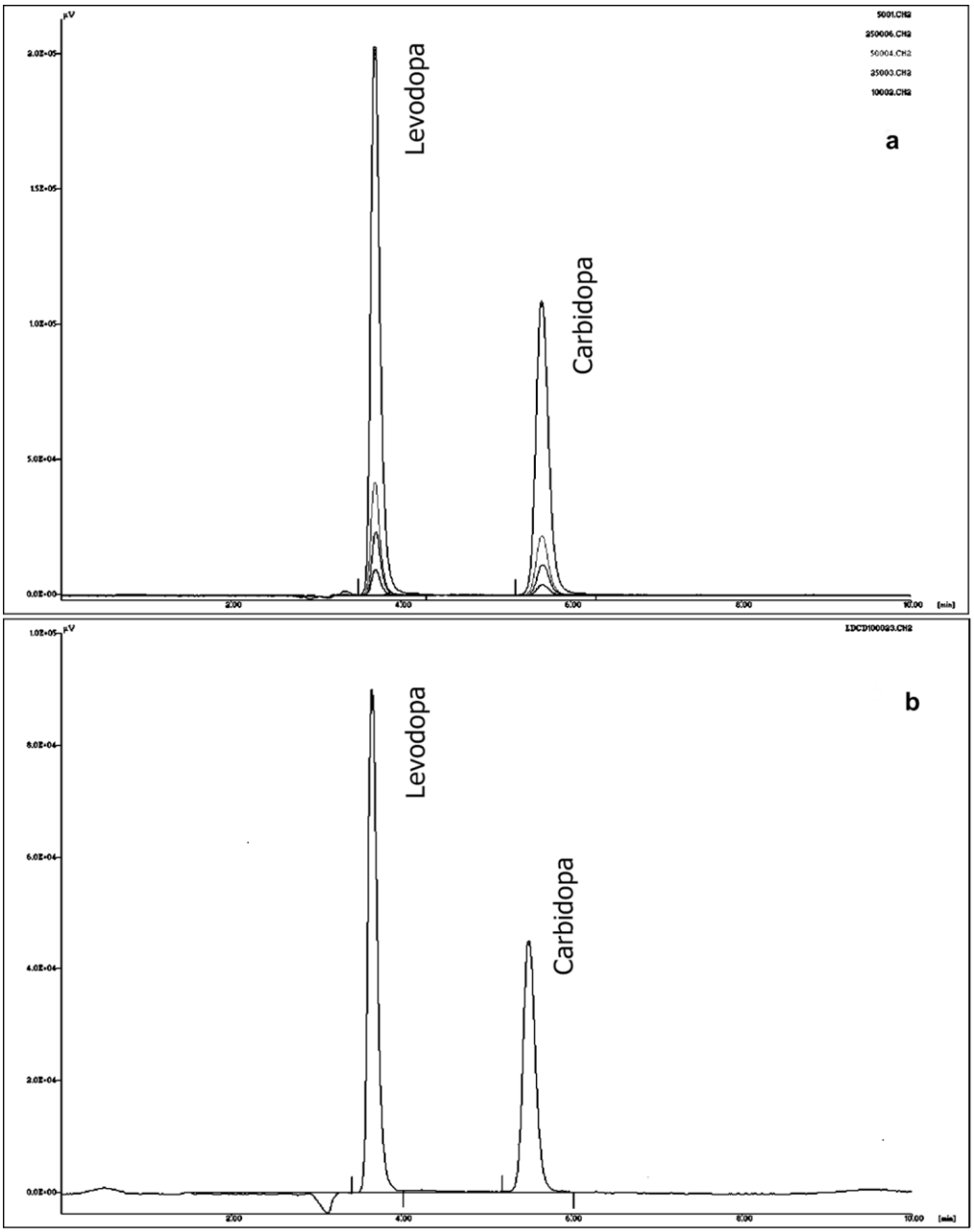


Figure 3.6. Chromatograms demonstrating (a) selectivity for placebo and formulation standards and (b) test sample-commercial tablet (SYNDOPA® 275)

The sensitivity of the method is expressed as the LOD. A more useful way to describe the sensitivity is to calculate the LOD and LOQ. The LOD is the amount of an analyte giving a peak height three times the standard deviation of the base line noise without any matrix interference, whereas the LOQ is the concentration of an analyte in the matrix that could be determined with a precision (a signal-to noise ratio of 10) using the developed

analytical method. In our case, the LOD values for LD and CD was found to be 0.30 and 0.60 ng/mL, respectively. The LOQ was 0.70 ng/mL for LD and 1.20 ng/mL for CD (Table 3.6). The LOD and LOQ found for LD was less than the LOQ and LOD values reported previously (Shah et al., 2000) which demonstrate the sensitivity of the method. Upon repeated injections at the quantitation limit, the peak properties (R_t, peak area and T_f) were not affected and mean absolute recovery was consistently high with acceptable bias and RSD values. This LOQ is adequate for in vitro analysis and stability studies and comparable with values obtained previously using similar techniques (Gelber and Neumeyer, 1983; Kafil and Dhingra, 1994). A HPLC-UV method has been reported for the simultaneous estimation of LD and CD which is sensitive up to the microgram level (Issa et al., 2011) whereas the proposed method shows sensitivity up to the nanogram level. The method was found to be highly sensitive for estimation of LD and CD in in vitro samples.

d. Robustness

Response surface plots indicate that the obtained response remains unaffected by small changes in critical method parameters such as percent organic component, buffer strength and pH. Statistical analysis confirmed that there was good agreement between experimental and predicted values. A good correlation was found between obtained responses and studied factors for the developed quadratic model. The model coefficients were estimated by least square regression analysis between predicted response and selected critical method parameters (Table 3.9). Estimated model coefficients were successfully used to find the qualitative and quantitative relationship between the critical method parameters and chromatographic response function using the following equation.

$$\hat{Y} = \beta 0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2$$

Where, $\hat{Y}$: predicted response; A, B and C are coded variables; β is the model coefficients.

Figure 3.7a-f and Figure 3.8a-f shows the 3-D surface plots of predicted responses (Z axis) for LD and CD as a function of two significant factors (X and Y axis), while the

least significant factor (third factor) was considered constant at optimum level. Figure 3.7a-c shows the peak area of LD against %MET versus pH, %MET versus buffer strength and pH versus buffer strength. An insignificant lack of fit test confirms the selected quadratic model fit the data. Thus, the responses predicted by fitting the quadratic model to the data were valid. The mean peak area of LD did not change significantly over the studied range of all three critical method parameters, demonstrating the robustness of the response for the studied factors (F = 0.93). Significant variation in the retention time of LD was observed with the change in %MET (Figure 3.7d) and pH of the buffer phase (Figure 3.7e; F = 10.34), but peak area of LD did not change significantly at studied range of all three factors (F = 0.93). Among the studied factors, variation in %MET showed a significant effect on retention time of CD as shown in Figure 3.8d-f (F = 6.52). However, change in the peak area of CD was not significant at studied range of all three factors (F = 0.72). Further, the effect of the studied factors on system efficiency and peak symmetry (T_f) was not significant. Peak area, the principle chromatographic response function was almost unaffected by any of the studied factors in both cases (LD and CD) suggesting the robustness of the proposed method (Table 3.9). A few factors showed an effect on retention time and peak symmetry (T_f) but the impact was insignificant.

e. System Suitability and Drug Stability

Primary system suitability parameters such as retention factor ($k \geq 2.5$), resolution (Rs $\geq$ 2.0), and number of theoretical plates ($N \geq 3{,}900$) were above acceptable limits and the height equivalent to theoretical plates ($HETP_{LD}$ ~ 36.40 µm, $HETP_{CD}$ ~ 31.20 µm) was well below the limit in both cases, viz. LD and CD indicating that the optimized method was suitable in terms of system performance (Table 3.6). Moreover, method demonstrated good peak symmetry for LD (T_f ~ 1.13) and CD (T_f ~ 1.08) with consistently low variability in peak areas and retention times after repeated injections. The method was found to be specific, precise and suitable for the simultaneous estimation of LD and CD, as confirmed by system suitability study. Chromatograms of samples stored at room temperature for 48 h showed similar response when compared with chromatograms of freshly prepared samples.

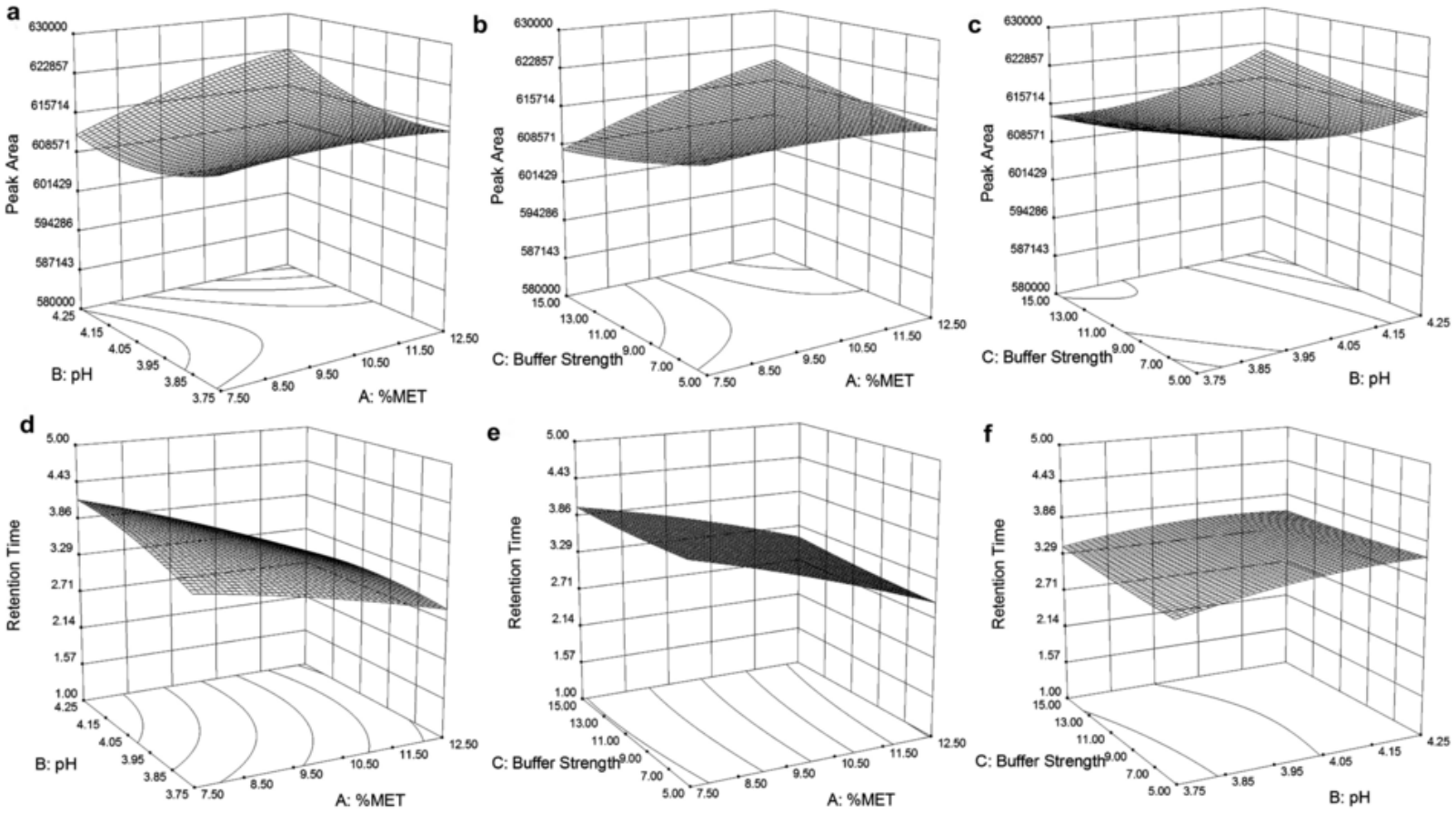


Figure 3.7. 3D response surface plots of predicted responses for LD Peak area (µVs) (a) as a function of % methanol (v/v) and pH; (b) as a function of % methanol (v/v) and buffer strength (mM); (c) as a function of buffer strength (mM) and pH; retention time (min) (d) as a function of % methanol (v/v) and pH; (e) as a function of % methanol (v/v) and buffer strength (mM) and (f) as a function of buffer strength (mM) and pH

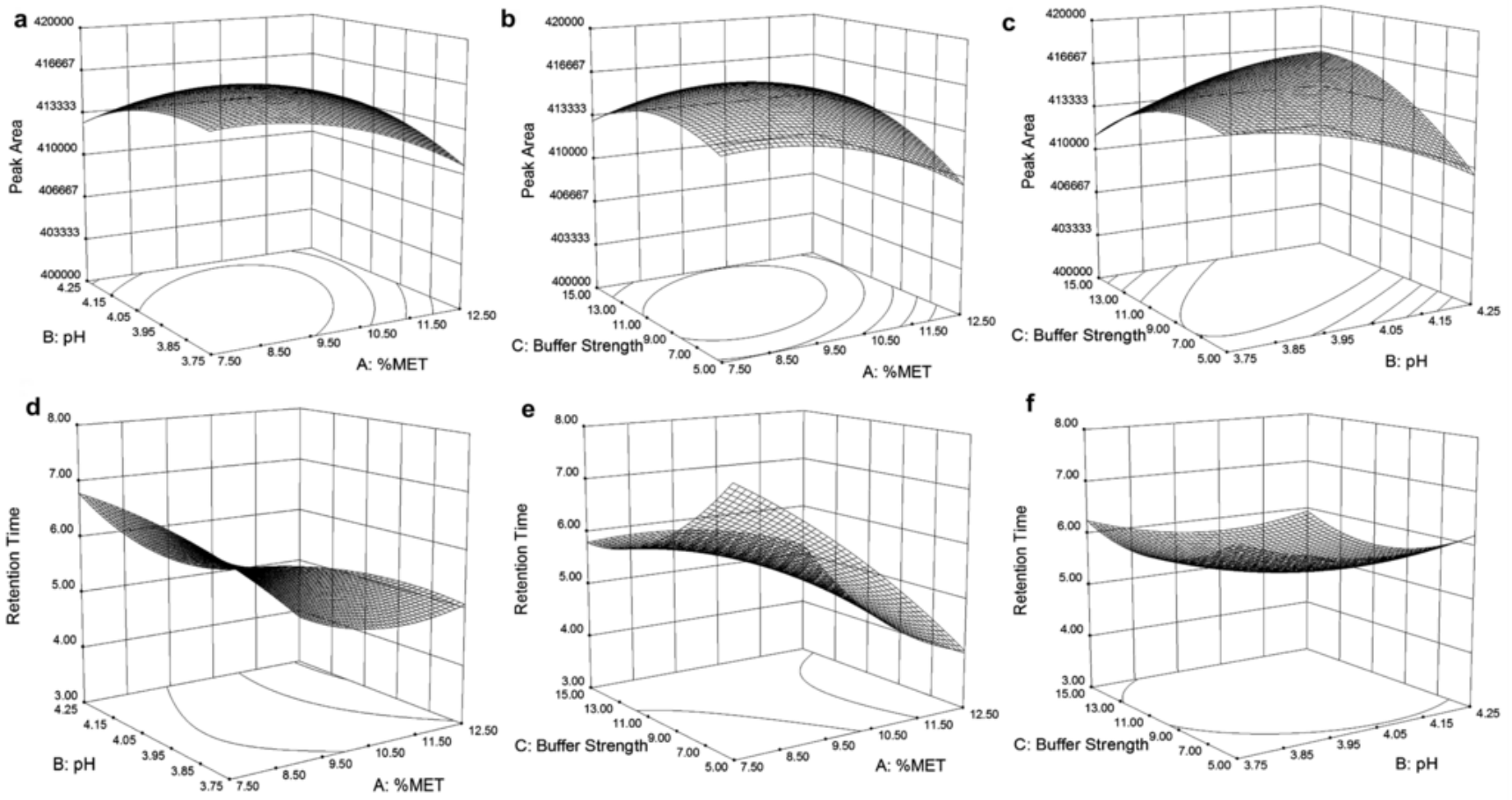


Figure 3.8. 3D response surface plots of predicted responses for CD Peak area (µVs) (a) as a function of % methanol (v/v) and pH; (b) as a function of % methanol (v/v) and buffer strength (mM); (c) as a function of buffer strength (mM) and pH; retention time (min) (d) as a function of % methanol (v/v) and pH; (e) as a function of % methanol (v/v) and buffer strength (mM) and (f) as a function of buffer strength (mM) and pH

Table 3.9. Experimental Design Data Summary for Robustness Study

Parameters		Values	
		LD	**CD**
Least-square second order polynomial equation (Regression value)		**Peak area:** 614692.55 + 2957.95A + 1391.95B - 165.25C + 2479.05AB + 3090.15AC + 2501.72BC - 1074.67A^2 + 2579. 79B^2 + 631.28C^2 (0.5446)	**Peak area:** 415801.27 - 894.00A + 28.03B + 829.21C + 1313.00AB + 1196.99AC + 2484.43BC - 1570.17A^2 – 1170.60B^2 - 1690.60C^2 (0.4805)
Least-square second order polynomial equation (Regression value)		**Retention time:** 3.58 - 0.49A + 0.14B + 0.03C - 0.12AB + 0.05AC - 0.05BC – 0.05A^2 -0.10B^2 + 0.02C^2 (0.9300)	**Retention time:** 5.46 - 1.03A - 0.17B – 0.15C - 0.43AB + 0.73AC + 0.03BC – 0.27A^2 + 0.33B^2 + 0.48C^2 (0.8934)
F-value[a]	Peak area	0.93	0.72
	Retention time	10.34	6.52
Prob > F[b]	Peak area	0.55	0.68
	Retention time	0.003	0.01

[a]Calculated using Fisher test with ANOVA (5% level of significance)
[b]Prob>F values less than 0.05 indicates factors in the model have significant effect on response

This similarity in response and the absence of addition peaks indicated that both drugs were stable in the mobile phase at ambient temperature for at least 48 h.

f. Forced Degradation Studies

The representative chromatogram of LD and CD (freshly prepared samples lacking degradation treatment) is shown in Figure 3.9a. The observed HPLC results of forced degradation study demonstrated that the LD and CD both are susceptible to the basic, oxidative and photolytic stress conditions.

The representative chromatograms of samples of acid, base, oxidative, photolytic and thermal degradation are shown in Figure 3.9b, 3.9c, 3.9d, 3.9e and 3.9f, respectively. During analysis of forced degradation study samples, degradation product peaks were not observed. Further, the amount of drug recovered was calculated and summarized in Table 3.10. The well resolved peaks of LD and CD demonstrated selectivity and stability indicating capability of developed and validated HPLC method.

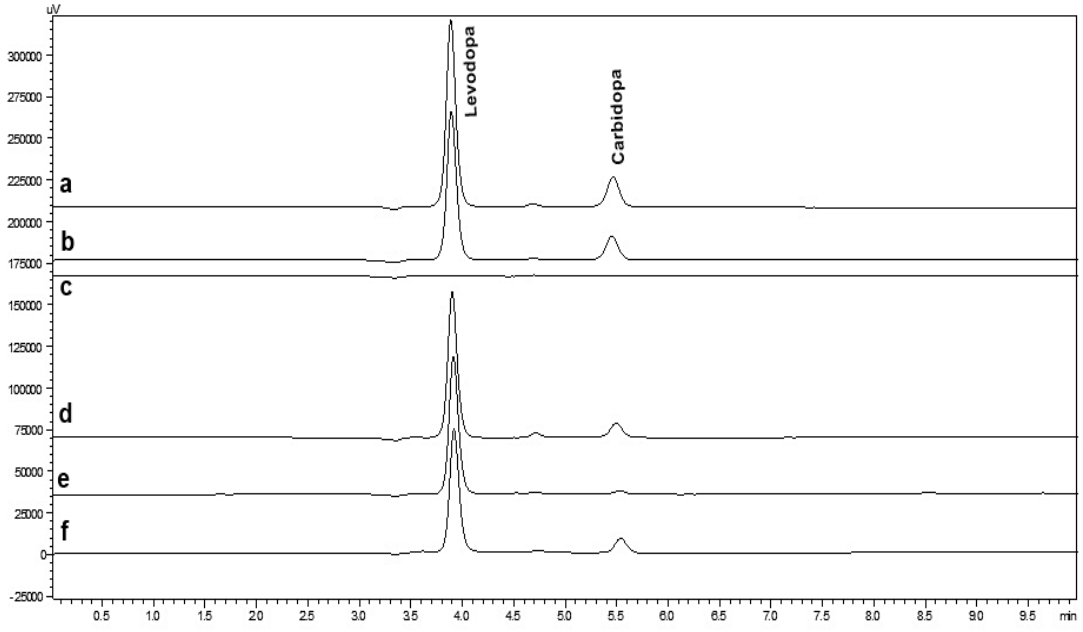


Figure 3.9. Representative chromatograms of samples of (a) pure LD and CD; (b) acid degradation; (c) base degradation; (d) oxidative degradation; (e) photolytic degradation and (f) thermal degradation

Table 3.10. Results of Forced Degradation Study of LD and CD

Sample exposure condition	LD recovery (%) (Mean ± SD) (n = 3)	CD recovery (%) (Mean ± SD) (n = 3)
Acid (0.1 N HCl, 2 h, 60°C)	98.11 ± 1.26	98.91 ± 1.08
Base (0.1 N NaOH, 2 h, 60°C)	Not detected	Not detected
Oxidation (H_2O_2, 10% v/v, 2 h, 60°C)	31.59 ± 3.02	36.26 ± 2.32
Light (254 nm, 24 h)	53.54 ± 2.02	11.34 ± 1.51
Thermal (150°C, 8 h)	93.67 ± 1.59	90.37 ± 1.33

g. Application of HPLC Method

The method was successfully applied to the simultaneous determination of LD and CD in pharmaceutical formulations. A typical chromatogram for simultaneous determination of LD and CD extracted from commercial tablet preparation is shown in Figure 3.6b. The mean recoveries for each formulation were found to be in good agreement with the labeled claim of the individual product. The method was found to be accurate with a mean absolute recovery of 99.63 - 100.80% for LD and 98.97 - 100.94% for CD in tablet. The developed method was precise with a RSD not exceeding 1.18% for LD and 1.86% for CD. Moreover, formulation excipients did not show interference in the simultaneous determination of LD and CD as bias was below 0.80% and 0.94%, respectively for LD and CD (Table 3.7). Thus, the method was found to be suitable for the simultaneous estimation of LD and CD from a tablet formulation. Few methods have been reported for simultaneous estimation of LD and CD using HPLC. Most reported methods are proposed for the estimation of LD along with other neurotransmitters or metabolites of LD in biological matrix. The HPLC method reported uses a combination of detectors (electrochemical and fluorescence detector) for simultaneous estimation of LD and CD in human plasma (Betto et al., 1988). Further, a method is reported for the estimation of LD and CD along with the metabolite of LD in serum by HPLC coupled with UV and fluorescence detector. In addition, HPLC with fluorescence detection using a derivatizing agent to make the method more sensitive than UV detection which makes sample preparation tedious is also reported (Muzzi et al., 2008). Although such methods are efficient for the estimation of LD and CD in a biological matrix, the applicability of such techniques for in vitro drug release sample analysis need to be proved. There is no report

on a simple and sensitive method for the simultaneous estimation of LD and CD using HPLC with a fluorescence detector.

3.5. Analytical method III: Liquid Chromatographic Bioanalytical Method

3.5.1. Instrumentation

The liquid chromatography system employed was liquid chromatograph LC-2010C_{HT} (Shimadzu, Tokyo, Japan) equipped with quaternary pumps, column oven, intelligent autosampler and online degasser. Analytes were monitored using electrochemical detector (791 VA Detector, Metrohm, Switzerland) coupled with liquid chromatograph. Electrochemical detector consisted of carbon glass electrode (working electrode), a silver-silver chloride (Ag/AgCl) electrode (reference electrode) and a gold electrode (auxiliary electrode). Data collection and integration was carried out using LC solution software.

3.5.2. Preparation of Stock and Standard Solutions

Individual stock solutions of LD, CD, 3-OMD, DA and DOPAC were prepared in 0.1 M $HClO_4$ solution containing 0.1%, w/w $Na_2S_2O_5$, each having concentration of 1 mg/mL. Catechol was used as internal standard (IS). Catechol met all typical requirements of compound to be used as IS like stability, good recovery and well resolved peak from peaks of the analyte of interest The stock solution of IS (1 mg/mL) was prepared in methanol. The stock solutions were stable for 1 month at -80°C. Working standard solutions were prepared freshly everyday by diluting stock solution with antioxidant solution (10 mM HCl, 1 g/L $Na_2S_2O_5$, 0.1 g/L Na_2EDTA).

3.5.3. Estimation of LD, CD and 3-OMD in Rat Plasma

a. Chromatographic Conditions

The analytes were separated on the C_{18} reverse phase column (Enable C_{18} G, 250 mm long and 4.6 mm internal diameter, particle size 5µm, Spinco Biotech, India) equipped with the pre column (Enable C_{18}, 30 mm long and 4.6 mm internal diameter, particle size 5µ, Spinco Biotech, India). The optimized mobile phase consisted of a mixture of an aqueous phase and methanol in the ratio of 90:10 v/v. The aqueous phase contained (1.36

g/L potassium dihydrogen phosphate buffer, 20 mg/L of Na_2EDTA, 70 mg/L of HSA and 98 mg/L of potassium chloride, pH adjusted to 3.5 using 0.1 M orthophosphoric acid. The buffer solution was filtered through a 0.22 µm Millipore® (Milford, MA, USA) filtration membrane. Mobile phase flow rate was set at 1.2 mL/min during estimation. The potential applied at electrochemical cell was + 800 mV. The injection volume was 20 µL. Estimation was carried out at 25.0 ± 0.2°C.

b. Preparation of Calibration Standards and Quality Control Samples

Seven calibration standards in plasma were prepared individually by spiking appropriate amount of the stock solution in blank plasma. The volume of stock solution spiked did not exceed the 5% to the total volume of blank plasma. Three QC standards for each analyte were prepared at low (LQC), medium (MQC) and high (HQC) concentration levels of calibration curve. All QC standards were prepared freshly in three replicates on each day of validation.

c. Sample Preparation

A simple and efficient protein precipitation technique was employed for extraction of LD, CD and 3-OMD from real plasma or blank plasma samples. TCA was used as a protein precipitating agent. To aliquot of 200 µL of plasma, 5 µL of IS working solution (and analyte standard solutions for blank plasma samples) was added and vortex mixed for 5 min. To the resulting sample, 195 µL of TCA (10%**,** w/v, water) was added and vortex mixed for 5 min to ensure complete precipitation. Samples were then centrifuged at 17000 rpm for 20 min at 4°C (Eppendorf centrifuge - 5702R) and the resulting supernatant was injected in to HPLC system for analysis.

d. Analytical Method Validation

The developed liquid chromatographic method was validated for linearity and range, recovery, accuracy, precision, selectivity, sensitivity and drug stability in rat plasma.

e. Linearity, Range and Recovery Study

Plasma standards were prepared by spiking 5 µL of working solutions at seven different concentration levels and 5 µL of IS at constant concentration in to 200 µL of blank plasma. To establish linearity of the proposed methods, three separate series of plasma standards were prepared and analyzed. Least square linear regression analysis was performed for analyte/IS peak area ratio obtained at corresponding concentrations of the analyte (Bolton and Bon, 2009).

To determine the extraction yield of the proposed method, LQC, MQC and HQC standards were prepared in blank plasma as discussed above. Spiked plasma samples were processed as per the procedure discussed in sample preparation section. Final supernatants were injected in to the HPLC column for analysis. Analytical standards of the same concentration were prepared by dilution of stock solution in antioxidant solution and analyzed in HPLC. The peaks obtained for both plasma and analytical standards were compared for each QC concentration. Percent absolute drug recovery (extraction yield) was calculated by using the formula [(peak area of plasma standard/peak area of analytical standard of same concentration) × 100].

f. Accuracy and Precision

For estimating accuracy of proposed method, three different QC levels were prepared independently and analyzed. Each concentration level was processed in three replicates and the results were expressed as mean absolute recovery, RSD and bias. The bias was calculated by using equation: bias (%) = [(predicted concentration - nominal concentration)/nominal concentration × 100].

Three separate series of three QC standards were prepared freshly and analyzed for intra-day variation (repeatability). Standards were prepared and analyzed on three consecutive days for intermediate precision (interday). The RSD of the predicted concentrations obtained from the regression equation at three QC levels were used to assess repeatability and intermediate precision, respectively.

g. Specificity, Selectivity and Sensitivity

Selectivity of the method can be defined as absence of interference at the retention time of the analytes by the proteins or other impurities present in the biological matrix. Blank plasma samples from six individual rats were processed and analyzed by proposed method to demonstrate specificity and selectivity. The observed chromatograms of blank plasma samples were compared against analytical and calibration standards for investigating the interference in the determination.

A lowest concentration which can be determined by proposed method with acceptable accuracy and precision (RSD < 20%) was considered as lower limit of quantitation (LLOQ) (FDA, 2001; ICH, 2005). The LLOQ (n = 5) in plasma was prepared for each analyte and analyzed by proposed method.

3.5.4. Estimation of LD, 3-OMD, DA and DOPAC in Rat Brain

a. Chromatographic Conditions

The analytes were separated on the C_{18} reverse phase column (Enable C_{18} G, 250 mm long and 4.6 mm internal diameter, particle size 5 µm, Spinco Biotech, India) equipped with the pre column (Enable C_{18}, 30 mm long and 4.6 mm internal diameter, particle size 5µ, Spinco Biotech, India) at flow rate of 1.4 mL/min. The optimized mobile phase consisted of a mixture of an aqueous phase and methanol in the ratio 92:8 v/v. The aqueous phase composed of 1.36 g/L potassium dihydrogen phosphate buffer 20 mg/L of EDTA, 70 mg/L of HSA and 98 mg/L of potassium chloride, pH adjusted to 3.7 using 0.1 M orthophosphoric acid. The buffer solution was filtered through a 0.22 µm Millipore® (Milford, MA, USA) filtration membrane. The 20 µL of sample was injected in HPLC system. Estimation was carried out at 25.0 ± 0.2°C at + 800 mV potential.

b. Preparation of Calibration Standards and Quality Control Samples

Three separate series of seven calibration standards with concentration ranges from 10 to 1000 ng/mL (10, 20, 50, 100, 200, 400 and 1000 ng/mL) for LD, from 20 to 2000 ng/mL (20, 40, 100, 200, 400, 800, 2000 ng/mL) for 3-OMD, from 5 to 500 ng/mL (5, 10, 25, 50, 100, 200, 500 ng/mL) for both DA and DOPAC were prepared by serial dilution of stock solution in antioxidant solution. Appropriate amount of the working solution was

added to blank brain homogenate to produce quality control standards. The volume of stock solution spiked was not more than 5%, v/v of total volume of brain homogenate. Three QC for each analyte were prepared at low (LQC), medium (MQC) and high (HQC) concentration levels of calibration curve. All QC standards were prepared freshly in three replicate on each day of validation.

c. Sample Preparation

Brain samples were homogenized in ice-cold solution (1 g/mL) containing mixture of 100 ng/mL of catechol, 0.1 M of $HClO_4$, 0.4 mM of $Na_2S_2O_4$ and 1 mM of Na_2EDTA in water using tissue homogenizer. Resulting brain homogenate was centrifuged at 17,000 rpm for 20 min at 4°C (Eppendorf centrifuge - 5702R). The supernatant obtained was injected in HPLC for analysis.

d. Method Validation

To establish linearity of the proposed methods, standards were prepared in triplicate and analyzed. Least square linear regression analysis was performed similar to the plasma samples. The brain QC standards were prepared in brain homogenate and analyzed by HPLC. The peak areas obtained for brain sample for each QC concentration were compared with peak areas obtained by injecting analytical standard solutions at the same theoretical concentrations. Percent absolute drug recovery (extraction yield) was calculated (except for DA) by using formula described in plasma sample analysis section. In case of DA, recovery (R) was calculated by formula: $R = [(A - B)/C] \times 100$, where A is the concentration of DA in the spiked homogenate sample, B is the concentration of DA in the homogenate sample without spiking and C is the added concentration of DA.

For estimation of accuracy of proposed method, brain homogenate was spiked with IS and analyte at three different QC levels similarly like plasma samples. Each concentration level was processed in three replicates and the results were expressed as mean percentage recovery, RSD and bias. The bias was calculated by using equation discussed in plasma samples analysis section. For DA, bias was calculated by using the equation: $\text{bias } (\%) = [(A - B) - C/C] \times 100$; where A is the concentration of DA in the

spiked homogenate sample, B is the concentration of DA in the homogenate sample without spiking and C is the added concentration of DA.

Precision of proposed method was estimated by analysis of three series of freshly prepared three QC standards for intraday variation (repeatability). Standards were prepared and analyzed on three consecutive days for intermediate precision (interday). Precision of proposed method in case of each analyte was expressed as RSD of the predicted concentrations obtained from the respective regression equation at three QC levels.

Chromatogram obtained from the blank brain homogenate samples were compared against the LLOQ of analytical as well as brain calibration standard for investigating the selectivity of the method. Lowest concentration showing RSD less than 20% was considered as lowest limit of quantitation (LLOQ). Brain LLOQ standard was prepared (n = 5) and analyzed by proposed method. The concentration of analytes was calculated from respective linear regression equation and sensitivity is demonstrated as mean concentration, bias and RSD.

3.5.5. Stability

The stability studies of the analytes in rat plasma and rat brain under different storage and operational conditions of proposed methods were carried out which includes bench top stability, three complete freeze thaw cycles, post preparative stability in the autosampler. The stability of analytes was evaluated at two QC levels (LQC and HQC) in triplicate. Stability samples were compared with the freshly prepared QC samples. Analytes were assumed to be stable when concentration in the stored stability sample found in the range of 85% to 115% of the nominal concentration. For bench top stability, QC standards were prepared and stored at controlled room temperature (25 ± 0.2°C) and each set of QC standards were processed and analyzed 4 h after spiking. For freeze thaw stability studies, QC standards were stored in sealed tubes at - 80°C. Samples were processed and analyzed after completion of freeze thaw cycles. For post preparative stability in the autosampler, QC samples were processed and stored in the HPLC autosampler at 4°C for 24 h. The observed results of the stability studies are expressed as accuracy (bias).

3.5.6. Results and Discussion

The preliminary voltammetric assays were carried out to determine the optimum detection potential for analytes. Optimum detection potential was found to be + 800 mV for all analytes. Therefore, analysis was carried out at + 800 mV by amperometric detector for both methods.

A. Estimation of LD, CD and 3-OMD in Rat Plasma

Primarily, a high peak height-to-area ratio, resolution and good peak symmetry were considered in the development of a selective and sensitive method during the development phase. For selection of mobile phase, various buffer types of different pH like phosphate buffer (pH 3 - 6, 10 mM), citrate buffer (pH 3 - 5, 10 mM), ammonium acetate buffer (pH 3 - 5, 10 mM) and acetic acid buffer (pH 3 - 5, 10 mM) in combination of organic solvents such as methanol (10, 20 and 40%, v/v) and acetonitrile (5, 10, 15, and 20%, v/v) were studied. Further, heptanesulfonic acid at different concentrations (20, 40, 80 mg/L) was studied in order to improve resolution from the interfering peaks from the biological matrix. The effect of various organic modifiers and the concentration of ion pairing agent on the peak properties (peak height, peak area, peak symmetry, retention time, resolution etc.) has been studied. Selection of mobile phase was done on the basis of peak properties [retention time (R_t) and asymmetric factor (T_f)], retention factor (k), sensitivity (height and area), number of theoretical plates (N), height equivalent to theoretical plates (HETP), ease of preparation and the applicability of the method for the estimation of analyte in plasma and brain.

The observed resolution and tailing factor for each analyte are summarized in Table 3.11. Well resolved peaks of LD, CD, 3-OMD and IS were observed at retention time (R_t) of 7.11 ± 0.02 min, 12.5 ± 0.01 min, 13.49 ± 0.12 min and 19.2 ± 0.23 min respectively (Figure 3.10). Retention time of analytes was found to be sensitive to the pH of buffer used for mobile phase preparation. Further, during method development various stability problems were encountered. LD, CD and 3-OMD are not stable in biological samples need stabilizers and handling on wet ice (Gorman et al., 2010) during sample preparation. Therefore, in order to improve stability of analytes, sodium metabisulfite and Na_2EDTA were used as stabilizers in current methods. The inconsistent recoveries require the use of

internal standard. Further the potential and the detection sensitivity were optimized on the basis of signal to noise ratio. Further, the potential and detection sensitivity giving maximum signal to noise ratio at LLOQ for all analytes was selected as optimum for complete study.

For sample preparation, various sample preparation techniques were tried for the extraction of analyte. Analytes stability in evaporation stage and solubility in organic solvents limits the use of liquid-liquid extraction technique for sample preparation. In addition, poor and inconsistent recoveries were observed for solid phase extraction technique. Therefore, plasma protein precipitation technique was selected for sample preparation. Various protein precipitating agents like hydrochloric acid, orthophosphoric acid, trichloroacetic acid, acetonitrile and methanol were studied for the plasma protein precipitation and obtained extraction yields were compared. Among the studied protein precipitating agents, TCA was found to be more efficient protein precipitating agent in terms of extraction yield. The methods using TCA as a protein precipitating agent have already been reported in literature for extraction of LD wherein 1 mL of human plasma was used for extraction (Rizzo et al., 1996). Another method is reported using TCA for extraction of LD, CD and 3-OMD from 200 µL of human plasma (Bugamelli et al., 2011). In present study, TCA was used for extraction of LD, CD and 3-OMD from 200 µL of rat plasma. The representative chromatogram of blank plasma, blank plasma spiked with analytes and IS and real pharmacokinetic sample is shown in Figure 3.10a, Figure 3.10b and Figure 3.10c, respectively.

a. Linearity, Range and Recovery Study

The linear regression analysis was carried out between the peak area of analyte and I.S. ratio versus corresponding analyte concentration individually for each analyte. The observed linearity ranges, linear regression equations and regression coefficient are summarized in Table 3.11.

The standard deviation of peak area ratio was significantly low across analytical range of each analyte and RSD was found to be less than 5.55% for LD, 6.56% for CD and 5.99% for 3-OMD. Further, selected linear regression model showed acceptable bias, demonstrating goodness of fit.

The results of extraction yield (absolute recoveries) are summarized in Table 3.11. Mean absolute recovery values were found in the range of 81.25 to 88.25% for LD, 79.73 to 85.23% for CD and 79.96 to 88.53% for 3-OMD. The absolute recovery of IS was found to be 81.25%. Further, the low RSD values for all QC and LLOQ confirmed the precision of proposed method. Therefore obtained results suggest that proposed method was sensitive and precise for estimation of LD, CD and 3-OMD in rat plasma.

b. Accuracy and Precision

The obtained result for bias and RSD are summarized in Table 3.11. The bias for intrabatch ranged between -6.54 to -3.71% and for interbatch ranged between -4.74 to -3.11% at all QC levels for all analytes, confirmed the accuracy of the proposed method. At all QC levels, RSD not exceeding than 6.56% for both intrabatch and interbatch suggested the precision of the proposed method. The results for accuracy and precision study were in acceptable limits indicating that the proposed method was accurate and precise.

c. Specificity, Selectivity and Sensitivity

The chromatograms obtained for the blank plasma sample indicated that there was no interfering peak within the vicinity of the analyte peaks. A lack of response in blank biological matrix originating from endogenous components in the elution window of the analytes confirmed the selectivity of the proposed method. The real plasma samples obtained from rats proved that there was no interference from the plasma proteins, metabolites or degradation products to the drug peaks. Comparison of chromatograms of blank, spiked and test samples demonstrate well resolved peaks of all analytes which further confirmed selectivity of proposed method. Therefore, the proposed method was found to be selective and specific for determination of analytes from spiked plasma and real plasma samples.

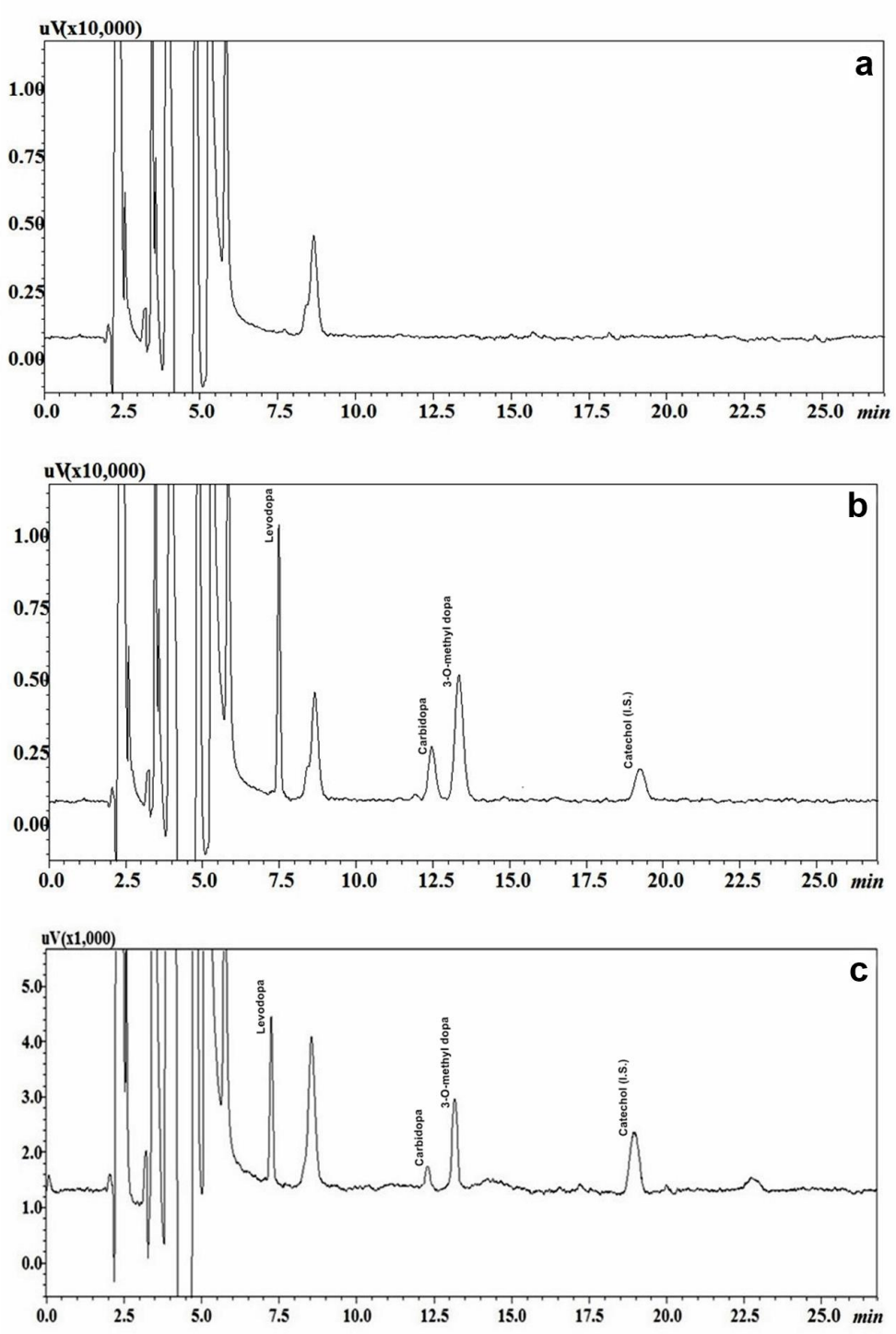


Figure 3.10. Representative chromatograms of (a) blank plasma; (b) spiked plasma [100 ng/mL of LD, 100 ng/mL of CD, 200 ng/mL of 3-OMD and 100 ng/mL of catechol (IS)] and (c) test sample (plasma sample from rats administered with sustained release pellets)

Table 3.11. Validation Parameters of Method Developed for Estimation of Analytes in Rat Plasma

Parameter	Analytes		
	LD	**CD**	**3-OMD**
Calibration range (ng/mL)	10 - 2000	10 - 2000	20 - 4000
Regression coefficient	0.9999	0.9998	0.9997
Regression equation (y = mx + c)[a]	y = 0.022x + 0.041	y = 0.005x + 0.045	y = 0.009x - 0.016
LLOQ (ng/mL) (nominal conc.)	10	10	20
Predicted conc.	8.57	8.69	18.28
RSD (%)	9.44	11.97	4.41
Bias (%)[b]	-14.26	-13.10	-8.59
Repeatability			
RSD (%)	3.67	3.96	3.33
Bias (%)[b]	-4.74	-3.84	-3.11
Intermediate precision			
RSD (%)	5.55	6.56	5.99
Bias (%)[b]	-4.83	-3.71	-6.54
Tailing factor[c]	1.21	1.12	1.02
Resolution (Rs)[d]	21.70	1.93	10.40
Mean absolute recovery (%)[e,f]	81.25 - 88.25	79.23 - 85.23	79.96 - 88.53

[a]y = peak area ratio of analyte and internal standard, m = slope, x = concentration (ng/mL) and c = intercept
[b]Bias (%) = [(predicted concentration - nominal concentration)/nominal concentration × 100]
[c]Tailing factor for internal standard (catechol) is 1.02
[d]Acceptable resolution Rs > 1.5
[e]Mean absolute recovery (%) = [(Peak area ratio of plasma standard/analytical standard of same concentration) × 100]
[f]Mean absolute recovery of IS (catechol) = 81.25%

The method selectivity was also determined by injecting sample solution of DA (R_t = 15.6 min) and DOPAC (R_t = 22.7 min) along with LD, CD and 3-OMD. No interference was observed within the vicinity of analyte of interest from both DA and DOPAC indicating the selectivity of the method. In addition endogenous rat plasma levels of DA and DOPAC are lower than LLOQ of the proposed method. Therefore, determination of DA and DOPAC has not been carried out in rat plasma.

In order to determine the LLOQ, five independent rat plasma samples containing 10 ng/mL of LD, 10 ng/mL of CD and 20 ng/mL of 3-OMD were prepared and analyzed by proposed method. Peaks were processed and concentration predicted by using linear regression equation. The mean predicted concentration for LD, CD and 3-OMD were found to be 8.57 ng/mL, 8.69 ng/mL and 18.28 ng/mL, respectively. The RSD for LD, CD and 3-OMD were 9.44, 11.97 and 4.41%, respectively. Further, bias values for LD, CD and 3-OMD found to be -14.26, -13.10 and -8.59%, respectively. The observed bias values for selected concentration of all analytes were below 20%. Therefore, 10 ng/mL was considered as LLOQ for LD and CD while 20 ng/mL was considered as LLOQ for 3-OMD. The selected potential and sensitivity in electrochemical detector were found to be optimum for the determination of all analytes. The lower values of bias and RSD and acceptable absolute recovery demonstrate that the proposed method was accurate and precise at LLOQ. The proposed method was found to be sensitive with high signal-to-noise ratio and can be used for the pharmacokinetic investigation in rats plasma which demands high sensitivity.

B. Estimation of LD, 3-OMD, DA and DOPAC in Rat Brain

The method for brain estimation was developed by small modification in the developed plasma method. The representative chromatogram of spiked rat brain homogenate is shown in Figure 3.11.

a. Analytical Method Validation

The calibration curves for all analyte were setup on blank brain homogenate spiked with standard stock solution. The linear regression equation, linearity range, regression

coefficient were calculated by means of least square linear regression analysis. The linear regression parameters are represented in Table 3.12.

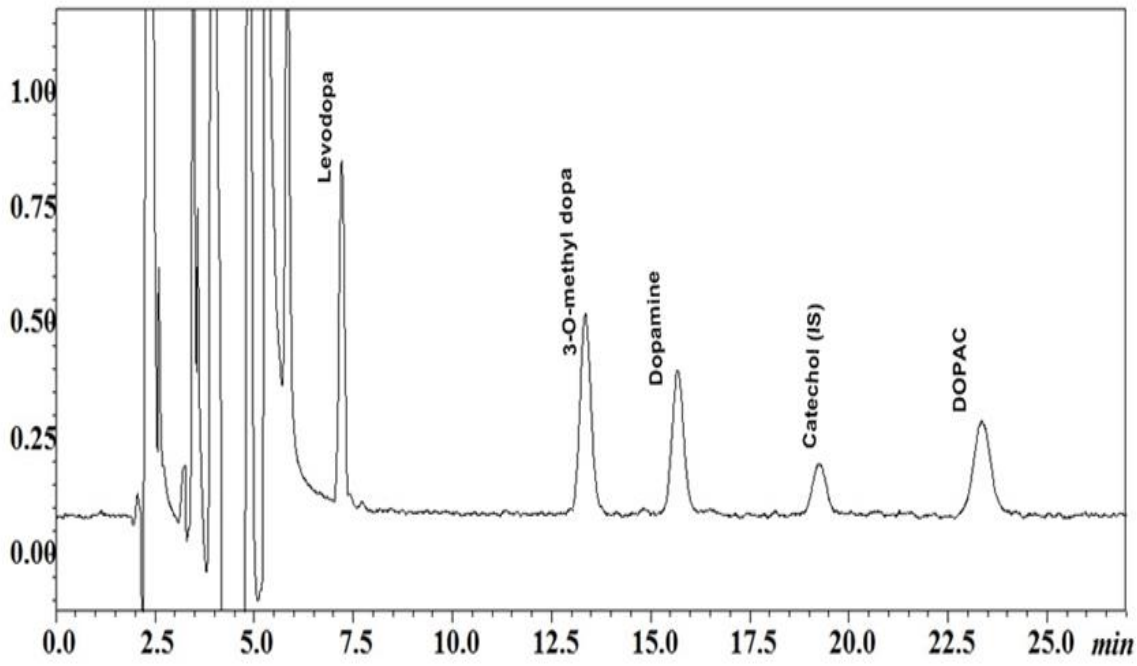


Figure 3.11. Representative chromatograms of spiked rat brain homogenate [100 ng/mL of LD, 200 ng/mL of 3-OMD, 50 ng/mL of DA, 50 ng/mL of DOPAC and 100 ng/mL of catechol (IS)].

The observed data is homoscedastic in nature; since calculated residuals were normally distributed with uniform variance around the mean of observed response at all concentrations for all analytes. The lower values of bias, standard error of estimate and mean sum of squared residuals demonstrates goodness of fit for selected linear regression model. The absolute recovery study results for all analytes is represented in Table 3.13.
The obtained values for bias and RSD for all analytes are summarized in Table 3.13. The bias values were found to be in range of -3.88 to -1.44% and -3.68 to -1.13% for intrabatch and interbatch respectively for all analytes. The acceptable bias values for all analytes demonstrate the accuracy of the proposed method. The recovery values at all QC level for all analytes were found to be more than 96.12%, which further confirms the accuracy of proposed method.

Table 3.12. Validation Parameters of the Method Developed for Estimation of Analytes in Rat Brain

Parameter	Analytes			
	LD	**3-OMD**	**DA**	**DOPAC**
Calibration range (ng/mL)	10 - 1000	20 - 2000	5 - 500	5 - 500
Regression coefficient	0.9997	0.9996	0.9996	0.9997
Regression equation (y=mx+c)[a]	y = 0.02x - 0.05	y = 0.01x + 0.02	y = 0.07x - 0.01	y = 0.06x + 0.02
LLOQ (ng/mL) (nominal conc.)	10.00	20.00	5.00	5.00
Predicted conc.	8.62	18.46	4.37	4.21
RSD (%)	10.23	11.23	7.25	9.59
Bias (%)[b]	-13.80	-7.70	-12.60	15.80
Repeatability				
RSD (%)	4.16	3.36	3.58	3.29
Bias (%)[b]	-3.88	-3.03	-3.06	-2.76
Intermediate precision				
RSD (%)	5.55	5.38	5.86	6.56
Bias (%)[b]	-3.10	-3.65	-3.68	-3..49
Tailing factor[c]	1.02	1.12	1.22	1.18
Resolution (Rs)[d]	21.70	7.93	5.41	6.23
Mean absolute recovery (%)[e, f]	80.96 - 86.23	83.23 - 89.53	81.12 - 87.56	82.81 - 85.23

[a] y = peak area ratio of analyte and internal standard, m = slope, x = concentration (ng/mL) and c = intercept
[b] Bias (%) = [(predicted concentration - nominal concentration)/nominal concentration × 100]
[c] Tailing factor for internal standard (catechol) is 1.02
[d] Acceptable resolution Rs > 1.5
[e] Mean absolute recovery (%) = [(Peak area ratio of plasma standard/analytical standard of same concentration) × 100]
[f] Mean absolute recovery of IS (catechol) = 83.76%

The RSD values at all QC levels for all analyte were not more than 4.16% and 6.56% for intrabatch and interbatch precision study respectively. In addition to this, the RSD at LLOQ was below 11.23% for all analytes. The mean absolute recovery, bias and RSD values obtained for LLOQ level of all analytes are represented in Table 3.12. The acceptable values of bias, RSD and mean absolute recovery for LLOQ indicated that proposed method was sensitive, precise and accurate. The obtained results suggest that the proposed method was accurate and precise and can be used for the estimation of LD, 3-OMD, DA, and DOPAC in rat brain.

The chromatograms obtained from the analytical samples when compared with the chromatograms of blank brain homogenate demonstrated absence of interference within the vicinity of the analyte peaks. The absence of interference from endogenous components in the elution window of the analytes further confirmed the selectivity of the proposed method. The analysis of real brain sample obtained from rats indicated well resolved analyte peaks and there was no interference from the metabolites or degradation products. Therefore, method was found to be selective and specific for determination of analytes from spiked brain homogenate and real brain samples.

The obtained results further demonstrated that the selected potential and detection sensitivity in electrochemical detector were sufficient for sensitive determination of the analytes in rat brain. Therefore, the proposed method can be used for the pharmacokinetic investigation in the rat brain which needs high sensitivity.

3.5.7. Stability

Stability studies demonstrated acceptable stability of analytes under tested condition at LQC and HQC in rat plasma and brain. Stability study results are represented in Table 3.14. All analytes proved to be stable in plasma and brain homogenate for 4 h at room temperature. The bias for plasma and brain homogenate for all analytes was in the range of -10.82 to -0.34% and -9.41 to -0.64% respectively. Further, all analyte were found to be stable in both plasma and brain homogenate after freeze thaw cycles on three consecutive days. Moreover, analytes were also found to be stable for 24 h on wet ice in case of plasma. Acceptable bias (± 15%) at LQC and HQC for all stability samples demonstrating selected processing conditions and storage conditions were appropriate.

Table 3.13. Intrarun and Interrun Precision and Accuracy of Analytes from Quality Control Samples Prepared in Rat Plasma and Brain Homogenate

Meth	Analyte	QC levels (ng/mL)	Repeatability (Intrabatch)			Intermediate precision (Interbatch)		
			Recovery (%)	RSD (%)	Bias (%)[a]	Recovery (%)	RSD (%)	Bias (%)[a]
Plasma	LD	LQC (25)	95.26	3.67	-4.74	95.17	5.55	-4.83
		MQC (900)	96.41	2.95	-3.59	95.73	5.21	-4.27
		HQC (1800)	99.69	1.83	-0.31	98.06	3.59	-1.94
	CD	LQC (25)	98.89	3.96	-1.11	98.09	6.56	-1.91
		MQC (900)	96.16	3.09	-3.84	96.29	5.25	-3.71
		HQC (1800)	99.51	1.82	-0.49	98.00	4.98	-2.00
	3-OMD	LQC (50)	96.89	3.33	-3.11	93.46	5.99	-6.54
		MQC (1800)	97.55	2.00	-2.45	96.29	5.06	-3.71
		HQC (3600)	99.17	0.94	-0.83	98.51	3.89	-1.49
Brain homogenate	LD	LQC (20)	98.02	4.16	-1.98	98.39	5.55	-1.61
		MQC (400)	98.17	2.43	-1.83	97.18	4.39	-2.82
		HQC (900)	96.12	1.84	-3.88	96.90	3.04	-3.10
	3-OMD	LQC (40)	96.97	3.36	-3.03	96.55	5.38	-3.45
		MQC (800)	97.46	2.71	-2.54	96.35	3.71	-3.65
		HQC (1800)	97.23	1.33	-2.77	98.87	4.03	-1.13
	DA	LQC (10)	97.11	3.58	-2.89	97.70	5.86	-2.30
		MQC (200)	97.83	2.52	-2.17	96.32	4.17	-3.68
		HQC (450)	96.94	2.09	-3.06	97.73	3.99	-2.27
	DOPAC	LQC (10)	98.56	3.29	-1.44	97.79	6.56	-2.21
		MQC (200)	98.23	2.48	-1.77	98.83	4.36	-1.17
		HQC (450)	97.24	1.90	-2.76	96.51	4.34	-3.49

[a] Accuracy given in bias (%) = [(predicted concentration - nominal concentration)/nominal concentration × 100].

Table 3.14. Results of Stability Studies in Rat Plasma and Brain Homogenate

Method	Analyte	QC levels (ng/mL)	Accuracy (Bias, %) (n = 3)						
			BTS [a]		FTS (3 cycles) [b]			PPS [c] (4°C for 24 h)	LTS [d] (-80°C for 20 days)
			25°C for 4 h	Wet ice for 24 h	1	2	3		
Plasma	LD	LQC (25)	-8.22	-7.40	-8.64	-9.45	-9.47	-6.22	-10.82
		HQC (1800)	-3.73	-1.03	-3.61	-4.03	-5.38	-0.34	-6.15
	CD	LQC (25)	-4.33	-2.45	-4.11	-7.64	-6.46	-3.52	-5.80
		HQC (1800)	-4.37	-2.75	-3.23	-4.03	-4.14	-3.68	-5.36
	3-OMD	LQC (50)	-4.81	-5.75	-4.07	-7.35	-4.07	-3.31	-6.02
		HQC (3600)	-2.39	-2.18	-2.38	-4.10	-3.53	-0.97	-5.90
Brain homogenate	LD	LQC (20)	-6.73	-3.42	-6.87	-8.31	-5.51	-4.75	-8.40
		HQC (900)	-5.89	-3.80	-5.12	-5.22	-4.79	-3.21	-7.68
	3-OMD	LQC (40)	-6.83	-5.51	-5.62	-6.83	-4.07	-4.73	-9.41
		HQC (1800)	-4.10	-3.10	-4.57	-6.77	-2.80	-3.38	-6.92
	DA	LQC (10)	-6.36	-5.70	-5.49	-7.16	-5.18	-4.66	-7.79
		HQC (450)	-4.98	-0.79	-4.36	-6.59	-2.65	-2.60	-3.50
	DOPAC	LQC (10)	-8.28	-4.44	-6.29	-8.28	-4.42	-4.78	-8.87
		HQC (450)	-4.99	-0.64	-5.90	-6.84	-5.49	-2.24	-5.72

[a] Bench top stability study
[b] Freeze thaw stability study
[c] Post preparative stability study
[d] Long term stability study

3.5.8. Applications of Developed Method

The validated method was successfully applied to study oral pharmacokinetic of LD and CD in rats (Chapter 6). Further, method developed for estimation of analytes in brain homogenate was applied in estimation of 3-OMD, DA and DOPAC in lesioned rats during pharmacodynamic study (Chapter 6). However, method failed to determine the concentration of LD in brain which can be attributed to less sensitivity of method for estimation of LD.

3.1. Conclusions

Developed HPTLC methods were precise, accurate, specific and stability indicating. Statistical analysis confirmed that the developed methods are reproducible and selective for estimation of drug from their pharmaceutical formulation. Forced degradation studies, confirmed that methods were efficient in separating drug peaks from their degradation product. Therefore, it can be employed for stability studies.

The proposed HPLC method was found to be simple, rapid, accurate, precise and inexpensive and can be used for routine analysis of LD and CD in bulk, pharmaceutical formulations and in vitro release samples. The method had required linearity, precision, accuracy, selectivity, detection and quantitation limits needed for the simultaneous estimation of LD and CD. The sample recoveries in all formulations were in good agreement with their respective label claims and thus suggested non-interference of formulations excipients in the estimation precluding use of any organic solvents for extraction of LD and CD from the formulation.

The separate method for estimation of LD, CD, 3-OMD in rat plasma and LD, 3-OMD, DA, DOPAC in rat brain have been developed. Analytes were stabilized with sodium metabisulfite during sample processing and analysis. In addition, proposed method was validated as per the guidelines. Proposed plasma method uses faster protein precipitation procedure and does not require evaporation to dryness and reconstitution in mobile phase as reported in previous methods (Lucarelli et al., 1990). Developed methods in rat plasma and brain were found to be simple, rapid, accurate, precise, specific, fit for the purpose and successfully used in pharmacokinetic and pharmacodynamic study in rats. All

developed methods were used for different analytical and pharmacokinetic studies of the present research work.

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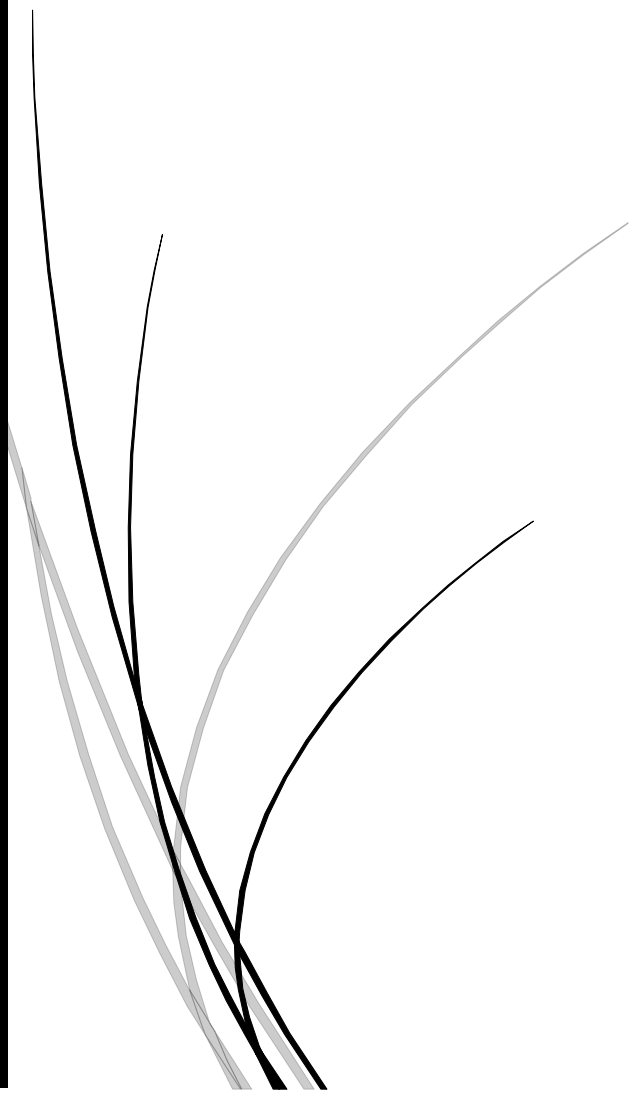
4 PREFORMULATION STUDIES



4.1. Introduction

A successful formulation design takes into account the prior information of physical, chemical and biological properties of the drug to produce an effective, stable, safe marketable product (Charman et al., 1997; Carstensen, 1998; Gibson, 2009). Frequently, this prior information minimizes the efforts in the later stage of product design and development by reducing cost and time to reach the market. Preformulation studies are designed to investigate the specific drug characteristics, which address the identity, purity, strength of drug substance and quality of the drug product. Further, the preformulation data confirm the presence or absence of potential barriers to the development of optimally bioavailable and stable formulation for a drug substance, thereby helping in developing a clinically effective formulation.

An adequate understanding of these properties of the drug substance minimizes the problems in formulation stages and helps in selection of compatible excipients and development of appropriate dosage form for the drug substance (Ravin and Radebaugh, 1985; Fiese and Hagen, 1986; Wadke et al., 1989). For drugs with poor and erratic oral availability, variability in the bioavailability in most of the cases can be traced down to their physicochemical properties (D'Incalci et al., 1982; Harvey et al., 1985). This exploratory activity at early stage of product development cycle aid in establishing a correlation between physicochemical properties of a drug substance and the biopharmaceutical parameters, such as prediction of the in vivo performance of the drug product.

Although Food and Drug Administration has not released any specific guidelines for pellets, these pharmaceutical and analytical investigations were carried out as per the standard product development guidelines. Typically, a standard pharmaceutical product development study includes comprehensive drug characterization such as determination of the dissociation constant, partition coefficient, solubility, polymorphism and hydrates, powder properties, thermal behavior, molecular spectroscopic profile, drug-excipient compatibility studies, stability studies etc. (Monkhouse, 1984; Charman et al., 1997; Ghosh et al., 1998; Serajuddin et al., 1999; Bruni et al., 2002). Thorough understanding of stability of drug in pure form and in physical mixture with proposed excipients under various conditions of temperature, light and humidity is important for identification of potential

drug excipient incompatibility problems. However, considering the scope of pellet formulations, method were selected to investigate product specific questions, which offer a rational basis for pellet formulation design and development strategies.

Although LD and CD combination is used widely in several research investigations and clinical applications, the information about its physicochemical properties is scarce in literature. Therefore, broad goal of the study was to evaluate physicochemical properties, determine the drug excipient compatibility, investigate the drug stability and characterize drug substance in a manner that would regulate the subsequent formulation development event.

4.2. Experimental

4.2.1. Materials

Levodopa (purity > 99.0%) and Carbidopa (purity > 99.0%) were purchased from Shaanxi Hygethy Biotechnology Co., Ltd. Xi'an, China and Smruthi Organics Pvt. Ltd., Solapur, India, respectively. Deionized ultrapure water (18.2 MΩ·cm at 25°C) was obtained using Milli-Q water purification system (Millipore®, MA). Hydrochloric acid (HCl, purity = 37.00%), orthophosphoric acid (purity ≥ 85%), potassium dihydrogen orthophosphate (purity > 99.5%) and sodium chloride (purity ≥ 98.00%) were purchased from Merck, Mumbai, India. Microcrystalline cellulose (MCC, Avicel® PH101) was purchased from FMC BioPolymer, USA. Povidone (PVPK30) was supplied as a gift sample by BASF Ludwigshafen, Germany. Hydroxy Propyl Methyl cellulose E5 (HPMC 5cps) and ethyl cellulose (EC, standard 10 premium) were gifted by Colorcon Asia Pvt. Ltd. Goa, India. Sodium metabisulfite ($Na_2S_2O_5$, purity ≥ 99.00%), spectroscopy grade potassium bromide (purity ≥ 99.00%), sodium hydroxide (purity ≥ 98.00%) were purchased from Sigma-Aldrich® Corporation, Mumbai, India. All chemicals were of analytical grade and used as received without any modification.

4.2.2. Instruments and Equipment

A digital pH meter (pH Tutor, Eutech instruments, Singapore) equipped with glass electrode and automatic thermal compensation probe was used for measurement of pH. A five digit analytical balance (AG 135, Mettler Toledo, Switzerland) with a sensitivity of ±

0.01 mg was used for all weighing purposes. A humidity chamber (MAC Instruments, India) was used to maintain accelerated conditions [temperature: 40 ± 2°C/relative humidity (RH): 75 ± 5%]. A frost-free-200 L refrigerator (Godrej, India) was used for stability studies at refrigerated conditions. Ultrasonicator (1201, Systronics Instruments, India) and vortex mixer (Spinix, India) were used for sample preparation and analysis. All other analytical instruments were of standard grade and used after calibration.

4.2.3. Methods

Various analytical methods are required to perform the preformulation studies. For present study, analysis of the drug was carried out using either HPTLC (Section 3.3) or HPLC (Section 3.4) method as discussed in Chapter 3. A fourier transform infrared spectrophotometer (IR Prestige - 21, Shimadzu, Japan) equipped with a diffuse reflectance attachment was used to record infrared absorption spectrum of all the samples. The individual samples were suitably mixed with moisture free spectral grade potassium bromide. The infrared absorption spectra were recorded in the range of 400 to 4000 cm^{-1} with resolution of 4 cm^{-1} using a high energy ceramic source, CsI beam splitter and DLATGS detector. The diffuse reflectance FTIR spectra were acquired using a Labsolutions® workstation (CreonLab Control, Japan) and the data was transformed using Kubelka-Munk conversion before all the interpretations.

Thermal behavior of LD and CD were studied by using differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) to investigate the interaction between LD and CD. The DSC analysis was carried out using DSC 4000 (PerkinElmer, MA, USA) equipped with Pyris analyzer. For all the measurements, 2 ± 0.2 mg of individual sample was loaded into the aluminium pan and covered by crimping the lid. Considering the melting point of sample, each component was suitably scanned between 30 to 400°C at 10°C/min heating rate. Inert environment was ensured by purging nitrogen gas at 30 mL/min flow rate. The thermograms were acquired using a Pyris analyzer and melting temperatures (Tm) were recorded. Prior to actual analysis, DSC instrument was calibrated using a pure indium standard (melting point 156.6°C) with verification of performance using a zinc standard (melting point 419.5°C).

The TGA analysis was carried out using TGA 4000 (PerkinElmer, MA, USA). A sample of 2 ± 1 mg was loaded into open pan attached to microbalance and experiments were performed in triplicate for each drug and the mixtures. Samples were scanned in the range of 30 to 400°C at 10°C/min heating rate under dry nitrogen purge. The weight of the loaded sample was monitored throughout the experiment by the microbalance. Temperature control system was calibrated using ferromagnetic standard.

For statistical analysis the concentration of LD and CD in stability samples were plotted as a function of time. The order and the reaction rate constant were determined after modeling the obtained data using a linear and non-linear regression analysis using the statistical package S-Plus®.

4.2.4. Bulk Characterization

The LD and CD are listed in the various pharmacopoeias including United States Pharmacopoeia, British Pharmacopoeia etc. However, few in house tests were performed to establish identification and characterization of LD and CD.

4.2.4.1. Assay and Purity

For assay and percent purity purpose, in house stability indicating HPTLC methods were used as described in Chapter 3.

4.2.4.2. pH

The aqueous suspensions of LD and CD were prepared in deionized water individually each having concentration of 1%, w/v and pH were recorded at 25°C.

4.2.4.3. Spectral Analysis

a. Ultraviolet Absorption Spectrum

The solutions of LD and CD were prepared in 0.1N HCl individually each having concentration of 50 µg/mL and Ultraviolet (UV) spectrums were recorded in the range of 400 to 200 nm using UV - Visible spectrophotometer (UV - 3600, Shimadzu, Japan).

b. Fourier Transform Infrared Absorption Spectrum

The FTIR spectrum for pure LD and CD were recorded separately using diffused reflectance spectroscopy as described earlier (Section 4.2.3).

4.2.4.4. Thermal Analysis

Thermal behavior of the pure LD and CD were studied using a previously calibrated DSC and TGA as described earlier (Section 4.2.3).

4.2.5. Solubility Analysis

In the present work, solubility studies of LD and CD were carried out in selected aqueous media with varying pH using shake flask method. The selection of various aqueous pH media was done according to product development needs and in vivo physiological conditions. Saturation solubility of LD and CD was determined separately in buffered and unbuffered media at selected pH conditions. In addition, the solubilities of LD and CD were also estimated in deionized water. For pH solubility profile, unbuffered solutions were prepared by adjusting pH of pure water using hydrochloric acid or sodium hydroxide solution and ionic strength was adjusted using 0.5%, w/v of sodium chloride solution. The HCl solution (0.1 N) was used as buffer solution of pH 1.2. The buffer solutions of pH ranging from 2 to 4.5 were prepared by adjusting pH of 10 mM potassium dihydrogen phosphate solution using 0.1 M orthophosphoric acid solution. Further, saturation solubility of LD and CD in phosphate buffer pH 3.0 were also evaluated in presence of polysorbate 20 (0.05%, w/v). In the shake flask method, an excess of drug was added to 5 mL of each solvent measured into a glass flask and mixture was vortex mixed for 5 min. The samples were agitated in an orbital shaker maintained at 37 ± 2°C for 30 min. All the samples were protected from light using amber colored glassware during the study in order to avoid photodegradation. The samples were filtered through 0.22 µm Millipore® syringe filters (Millex® Syringe Filter, Millipore, USA) and diluted at isothermal conditions to ensure they were free from particulate matter before analysis. The processed samples were further diluted appropriately and analyzed by HPTLC method (Section 3.3) discussed in Chapter 3. All solubility experiments were performed in triplicate and average solubility of LD and CD in various pH media was calculated.

4.2.6. Stability Analysis

In order to investigate the integrity of the drug molecule under various pH conditions during the product formulation development and in vivo biological environment, stability studies were performed in both liquid and solid state.

4.2.6.1. Liquid State Stability

The solution state stability of LD and CD was established in media of varying pH (1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0). Individual stock solutions of the LD (1 mg/mL) and CD (0.25 mg/mL) were prepared in deionized water and protected from the light. Appropriate amount of prepared stock solutions were transferred individually in each pH solution. Samples were prepared in triplicate. Prepared samples were stored at controlled room temperature (CRT: 25 ± 2°C). Samples from each solution were withdrawn at predetermined time interval (0, 1, 2, 3, 4, 6, 8, 12, 18, 24 and 48 h) and analyzed using HPTLC methods (Section 3.3) proposed in Chapter 3.

4.2.6.2. Solid State Stability

For solid state stability studies, pure LD and pure CD were exposed to the various conditions of temperature and humidity. Pure drugs were stored in clear glass vial separately at various storage conditions selected as per the ICH guidelines (ICHQ1A(R2), 2003). Selected storage conditions were accelerated [accelerated temperature (AT): 40 ± 2°C/RH: 75 ± 5%] and long term (ambient) conditions [controlled room temperature (CRT): 25 ± 2°C/RH: 60 ± 5%]. Control samples of LD and CD were kept in refrigerator (5 ± 3°C). At predetermined time interval [for accelerated condition: 0, 1, 2, 3 and 6 months, for long term (ambient) condition: 0, 1, 2, 3, 6, 9 and 12 months and for refrigerator 0, 1, 2, 3, 6, 9, 12, 18 and 24 months] samples were withdrawn, processed and analyzed independently by using HPTLC methods (Section 3.3) discussed in Chapter 3.

The amount of drug remaining was plotted as a function of time. The order of degradation kinetics and the rate constant were determined. In addition, all stability samples were analyzed for physical (DSC) and chemical (HPTLC and FTIR) integrity on completion of the stability period (12 months).

4.2.7. Drug Excipient Stability Study

The solid state interaction between drug and various excipients were studied by subjecting physical admixture of drug and individual excipient to different stress conditions. The prepared mixtures were studied for physical observation (color, odor and physical state), drug content (assay degradation products), thermal analysis (DSC) and spectroscopic analysis (FTIR) etc.

The drug and excipient were weighed accurately and mixed pharmaceutically (1:1 w/w proportion) by sieving (60 #) and blending process. The prepared physical admixtures were stored in amber colored glass vials separately and each vial was kept at controlled (CRT: 25 ± 2°C/RH: 60 ± 5%) or accelerated (AT: 40 ± 2°C/RH: 75 ± 5%) storage condition. The pure drug stability data obtained at each condition was used as a control. At predetermined time interval, samples were withdrawn in triplicates and physical observations, drug content and impurity analysis were performed. The drug content results were plotted as a function of the time and data was fitted to determine order of the reaction. The degradation kinetics of both LD and CD were studied and degradation rate constants were expressed as $T_{90\%}$ which indicated the time duration required to retain 90% of drug potency, in presence of individual excipient at respective stress condition. At the end of stability period (12 months), thermal, HPTLC and spectroscopic analysis were performed to investigate possible physical and chemical interactions.

4.3. Results and Discussion

4.3.1. Bulk Characterization

4.3.1.1. Assay and Purity

The analyzed samples indicated that LD and CD are 99.76% and 99.91% pure, respectively. The observed purity values were in good agreement with the labeled purity.

4.3.1.2. pH

Aqueous suspensions of LD (1%, w/v) and CD (1%, w/v) showed pH of 4.86 ± 0.12 and 4.29 ± 0.02, respectively at 25°C.

4.3.1.3. Spectral Analysis

a. Ultraviolet Absorption Spectrum

The UV - Vis spectroscopic study demonstrated that, both LD (50 µg/mL) and CD (50 µg/mL) showed maximum absorption at 280 nm in 0.1N HCl. The UV spectrum of LD and CD are represented in Figure 4.1a and Figure 4.1b, respectively.

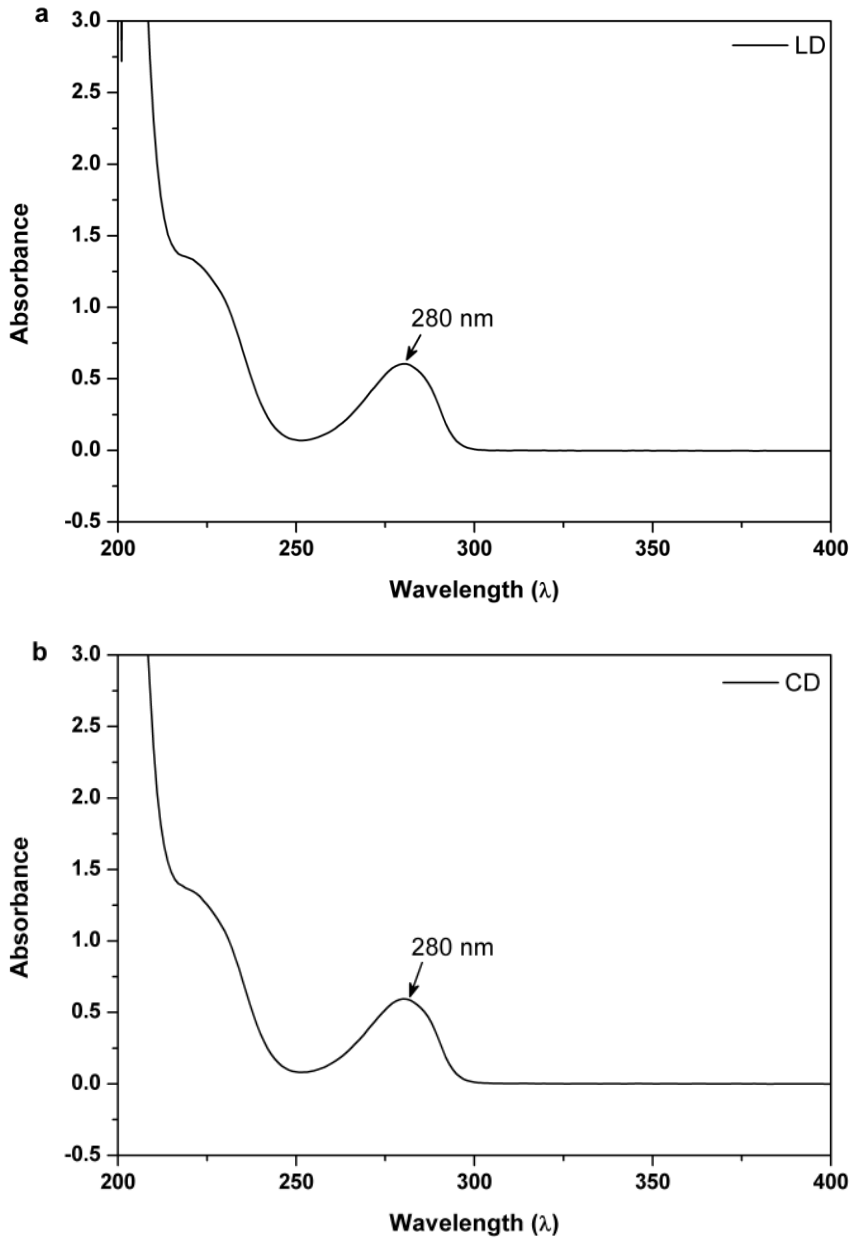


Figure 4.1. Representative Ultra-Violet spectra of (a) LD (50 µg/mL) and (b) CD (50 µg/mL) in 0.1 N HCl.

Both LD and CD showed superimposable UV spectrum at same concentration (Figure 4.2), which makes simultaneous estimation of LD and CD difficult by UV spectroscopy.

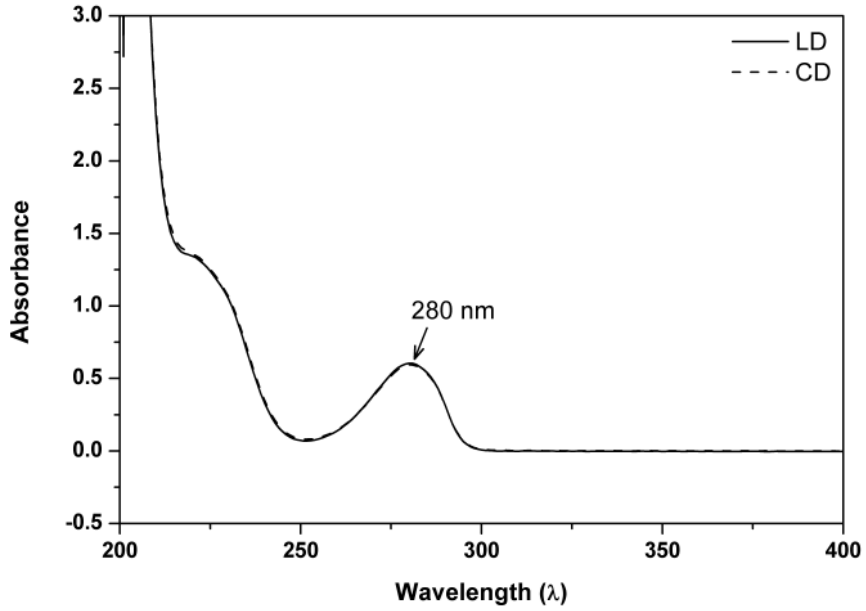


Figure 4.2. Representative overlaid Ultra-Violet spectra of LD (50 µg/mL) and CD (50 µg/mL) in 0.1N HCl.

b. Fourier Transform Infrared Absorption Spectrum

The FTIR spectroscopic analysis of pure drugs revealed, the peaks specific to the functional groups of LD and CD which were in agreement with reported data (Edwin and Hubert Joe, 2013). The representative FTIR spectra's for pure LD and pure CD are represented in Figure 4.3 and Figure 4.4 respectively. The infrared peaks observed for primary amine, -CH2- stretching, aromatic -C=C- peak, phenolic O-H stretching, O-H stretching of carboxylic acid in case of LD and CD are summarized in Table 4.1 and Table 4.2, respectively.

4.3.1.4. Thermal Analysis

The thermal behavior of pure LD, pure CD and physical mixture of LD and CD were studied by DSC and TGA techniques. The representative DSC thermograms of pure LD

and pure CD are represented in Figure 4.5 and Figure 4.6, respectively. DSC thermogram for combination of LD and CD (4:1) was also recorded (Figure 4.7).

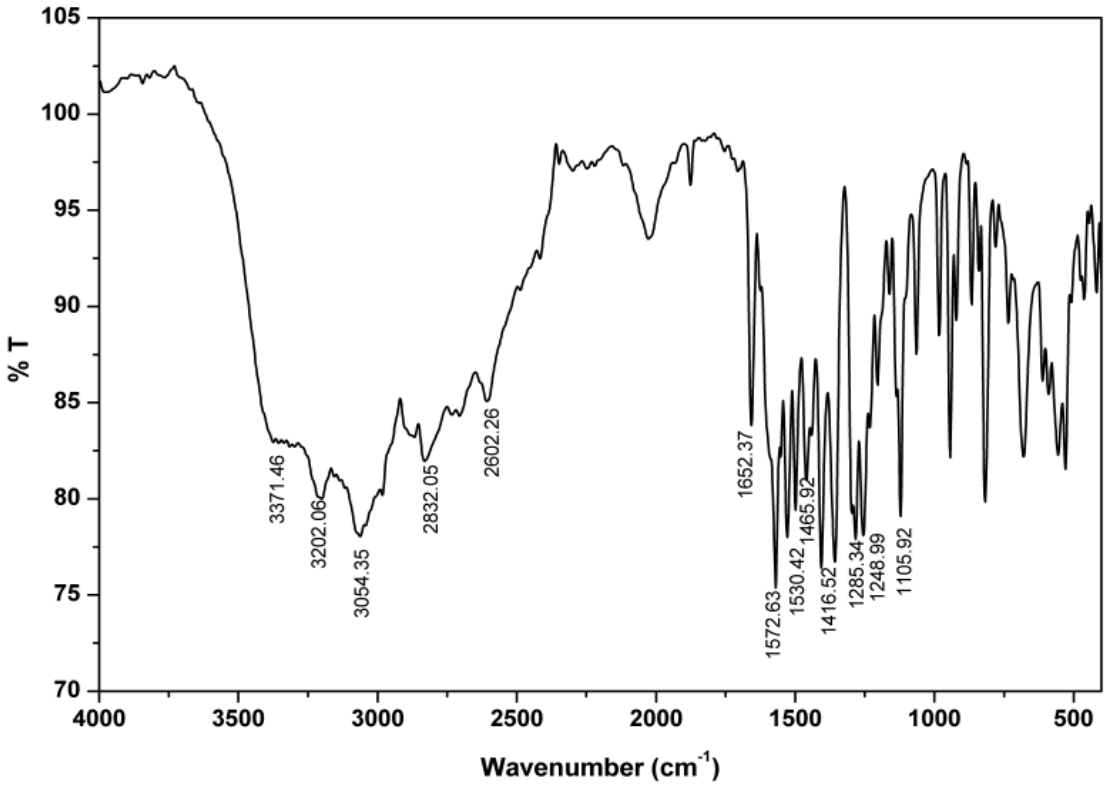


Figure 4.3. Representative FTIR spectrum of LD in potassium bromide.

Table 4.1. Wavelength Attribution of IR Spectrums of LD in Potassium Bromide

Wavelength (cm^{-1})	Attribution
3200 - 3500	Phenolic O-H stretching
3346 - 3360	Primary amine (two peaks)
2500 - 3300	Broad peak centered at 3000 cm^{-1} for O-H stretching of carboxylic acid
2829 - 2830	-CH2- stretching
1580 - 1650	N-H bending
1400 - 1600	Multiple peaks for aromatic -C=C-
1000 - 1300	C-O stretching for carboxylic acid

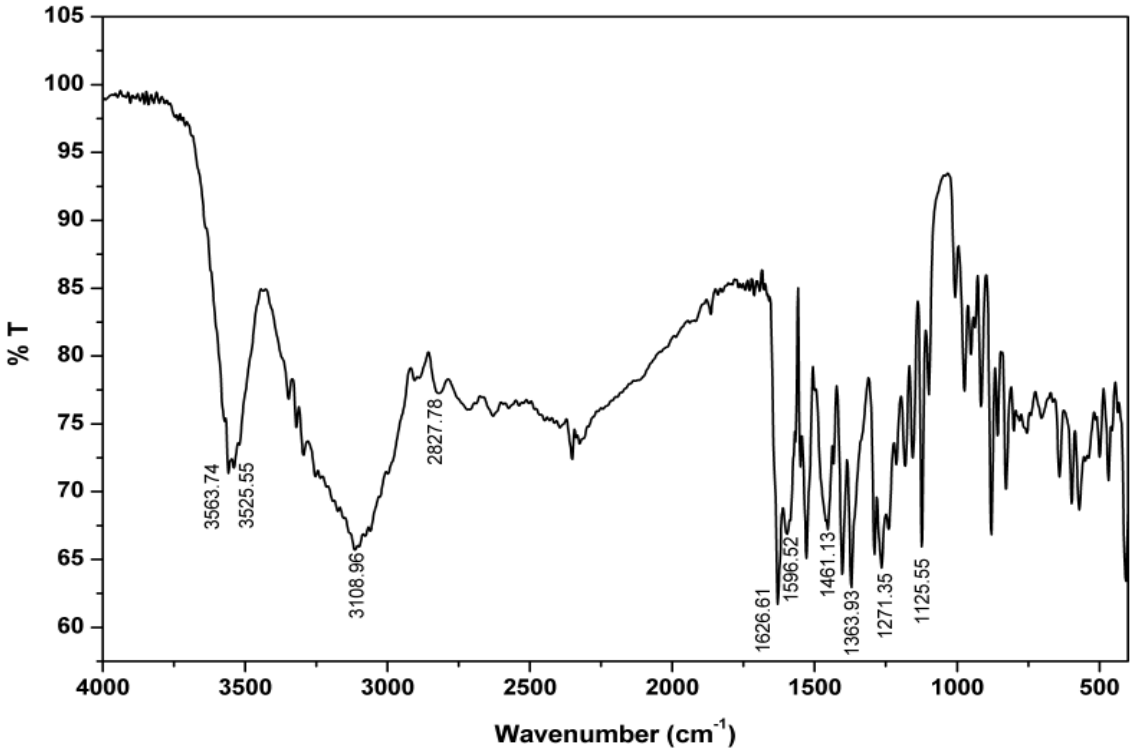


Figure 4.4. Representative FTIR spectrum of CD in potassium bromide.

Table 4.2. Wavelength Attribution of IR Spectrums of CD in Potassium Bromide

Wavelength (cm^{-1})	Attribution
3300 - 3500	Primary amine (two peaks) and secondary amine (single)
3200 - 3500	Phenolic O-H stretching
2500 - 3300	Broad peak centered at 3000 cm^{-1} for O-H stretching of carboxylic acid
2829 - 2830	-CH2- stretching
1580 - 1650	N-H bending
1400 - 1600	Multiple peaks for aromatic -C=C-
1458.18	Methyl and secondary amine group
1000 - 1300	C-O stretching for carboxylic acid

The DSC thermogram of pure LD demonstrated a sharp endothermic peak onset (T_s) at 265.08°C and average melting temperature (T_m) was found to be 297°C (Figure 4.5). Interpretation of thermograms provided melting enthalpy (ΔH) of -346.89 J/g and drug was found to decompose at its melting point. The repeated measurement of same sample did

not demonstrate any endothermic event between 275 to 300°C indicating complete degradation of LD.

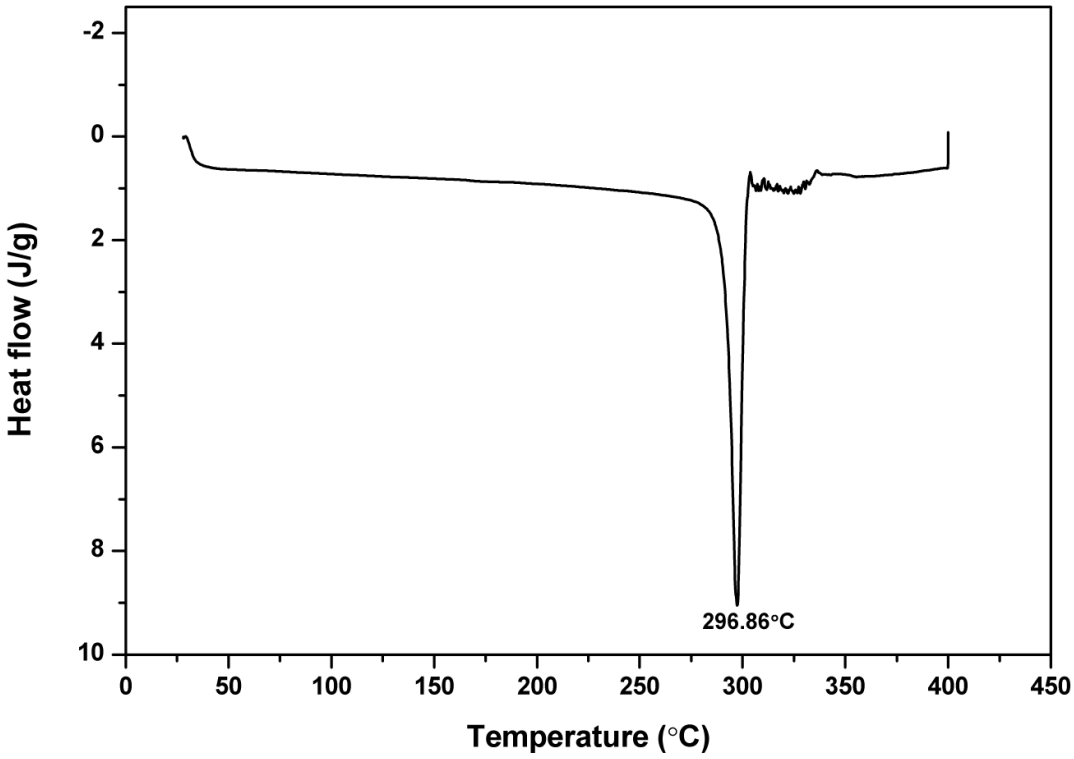


Figure 4.5. Representative DSC thermal profile of pure LD.

A broad endotherm with peak temperature approximately 104.95°C (ΔH = -333.26 J/g) owing to dehydration of the sample was observed in DSC thermogram of CD (Figure 4.6). The dehydration event was followed by endothermic and exothermic peaks. The endothermic peak was observed approximately at 198.71°C corresponding to the melting point of CD (ΔH = -42.32 J/g) whereas exothermic peak at 203.10°C (ΔH = 295.35 J/g) can be ascribed to decomposition of CD. The sample of CD was analyzed repeatedly in order to confirm decomposition. The absence of endothermic and exothermic event within temperature range of 175 to 225°C indicated complete decomposition of CD immediately after melting.

Further, DSC thermogram of physical mixture of LD and CD (4:1) was also recorded and representative thermogram is shown in Figure 4.7. DSC thermogram of physical mixture of LD and CD indicated broad endotherm at 99.31°C with ΔH of -28.69 J/g which might be

due to dehydration of CD. The broad endotherm was followed by endothermic and exothermic event at 194.91°C (ΔH = -3.34 J/g) and 200.60°C (ΔH = 21.59 J/g) which may probably related to decomposition and melting of CD respectively. In addition, a broad peak at 274.47°C (ΔH = -282.36 J/g) was observed corresponding to the melting/ decomposition of LD (Table 4.3).

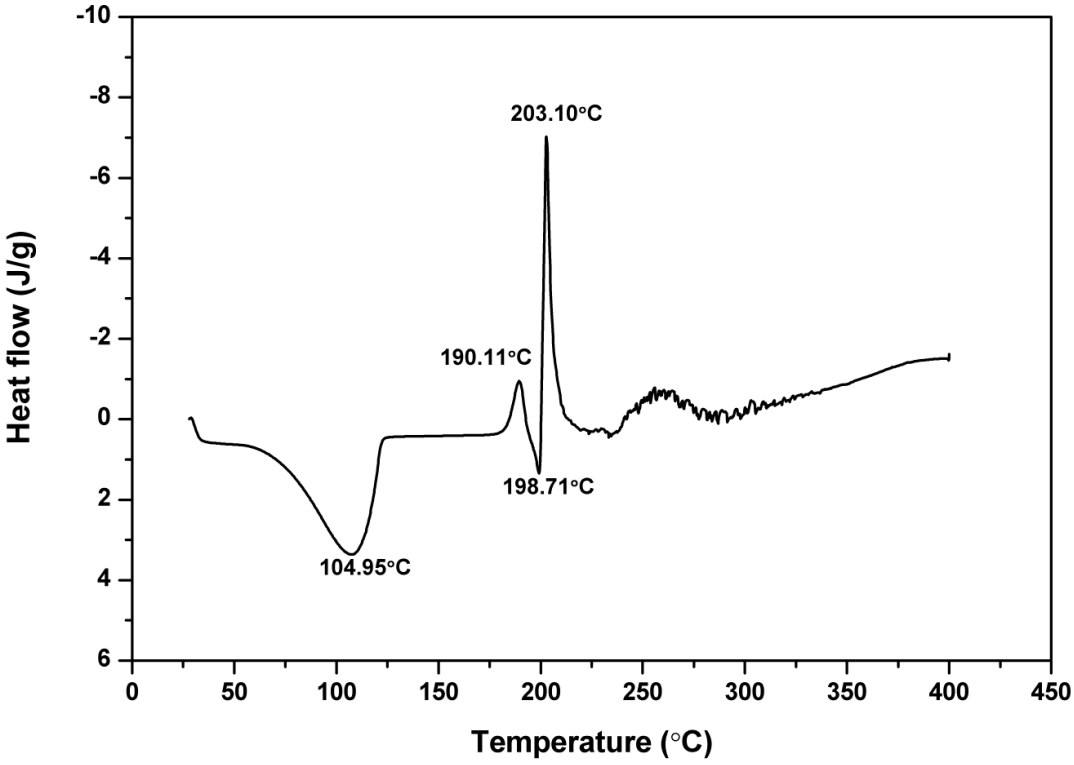


Figure 4.6. Representative DSC thermal profile of pure CD.

The melting transition of CD in DSC thermogram of physical mixture was less prominent in comparison to DSC thermogram of pure CD. This may be due to five fold dilution of CD in physical mixture of LD and CD (4:1, w/w). In physical mixture, broadening of melting/decomposition peak of LD was observed with reduction in melting/decomposition temperature by almost 19°C. This can be attributed to faster melting/decomposition of LD in molten CD and also due to lowering of purity of LD when mixed with CD. Further, dehydration behavior of CD and melting and decomposition events of both LD and CD were studied by TGA.

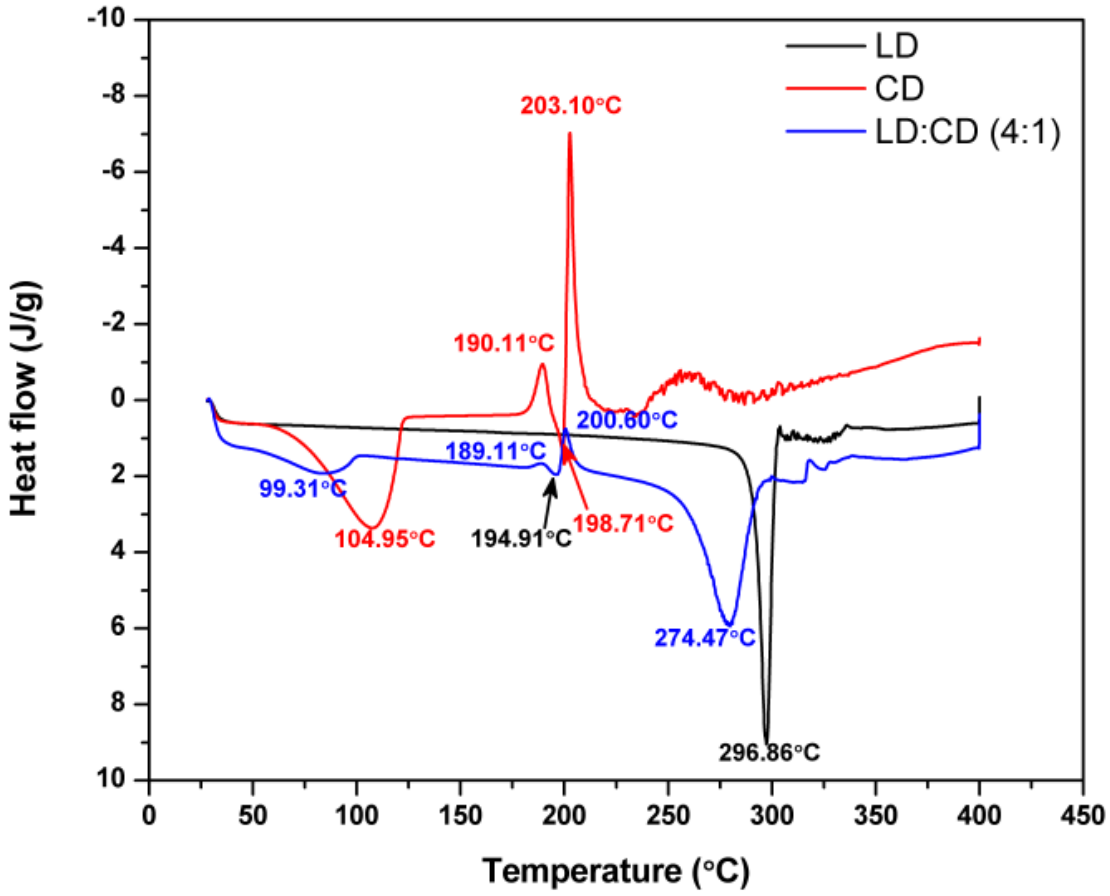


Figure 4.7. Overlaid DSC thermograms of pure LD, pure CD and physical mixture of LD and CD (4:1, w/w).

The relationships between temperature and weight loss for pure LD, pure CD and physical mixture of LD and CD in the ratio of 4:1 (w/w) was generated with the help of TGA. The TGA thermogram of pure LD is represented in Figure 4.8. The weight loss event was observed at 280°C for the LD owing to decomposition of sample. The representative TGA thermogram of pure CD is shown in Figure 4.9. The loss of weight between 75°C to 140°C has been observed in TGA profile of CD. This loss of weight (approximately 4 to 5%, w/w) could be due to removal of molecule of water present in the CD during heating. For stoichiometric hydrate, weight loss would be approximately 3.88%. Further, the loss in weight (approximately 44%, w/w) was observed between temperatures in the range of 180°C to 260°C. This change in the weight may be attributed to the decomposition of the CD.

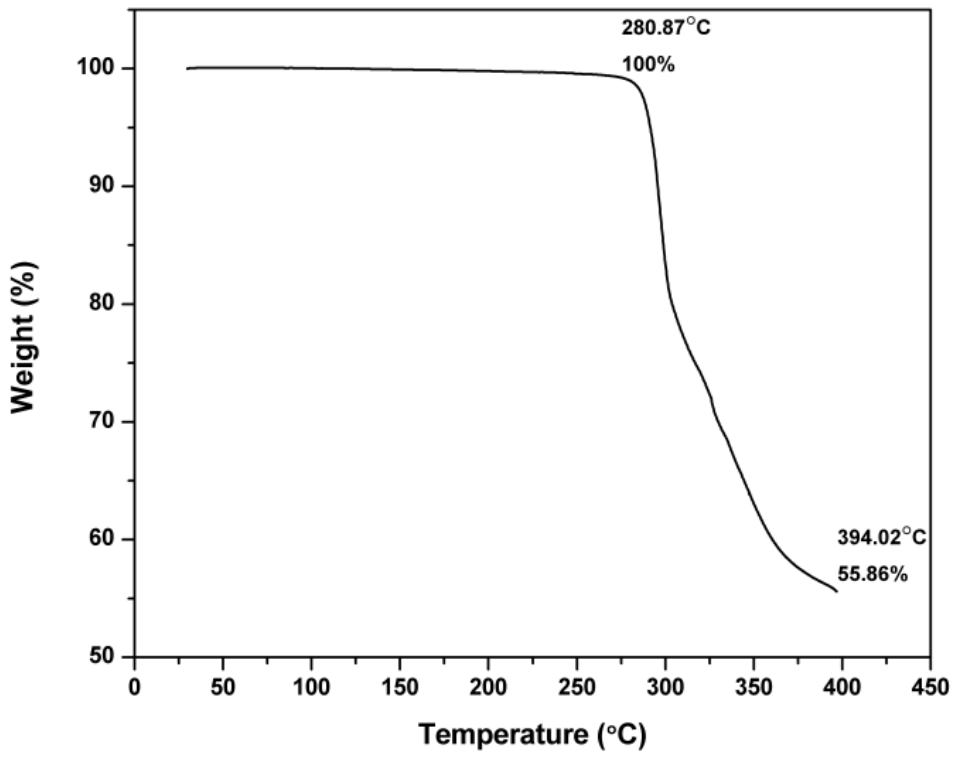


Figure 4.8. Representative TGA profile of pure LD.

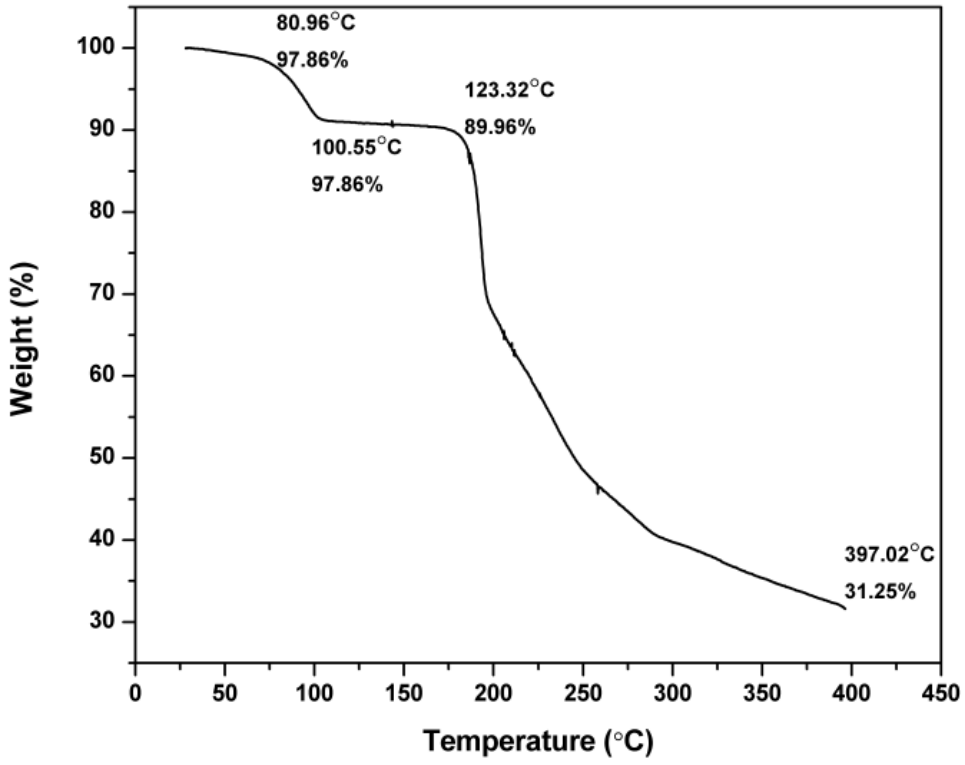


Figure 4.9. Representative TGA profile of pure CD.

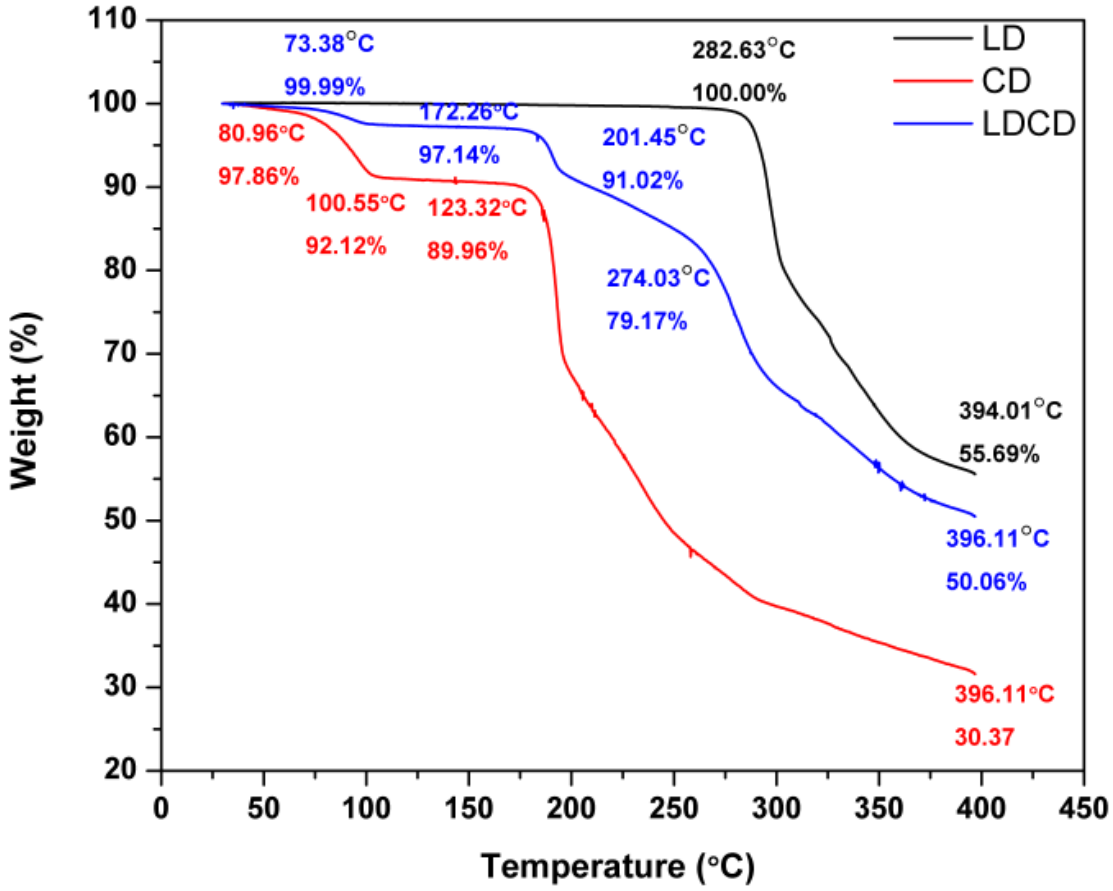


Figure 4.10. Overlaid TGA thermal profiles of pure LD, pure CD and physical mixture of LD and CD (4:1, w/w).

The overlaid TGA profiles of pure LD, pure CD and combination of LD and CD in the ratio of 4:1 (w/w) is represented in Figure 4.10. When mixture of LD and CD (LD:CD = 4:1, w/w) was heated, initial 4% reduction in weight is related with initial loss of water of crystallization of CD was followed by further weight reduction after 180°C due to decomposition of CD. In physical mixture of LD and CD, weight reduction event for LD was observed at temperature lower than the pure LD. The results obtained with DSC were in agreement with the results obtained with TGA suggesting both LD and CD undergo melting with decomposition (Figure 4.11). Furthermore, physical mixtures of LD and CD with different excipient were also evaluated by DSC.

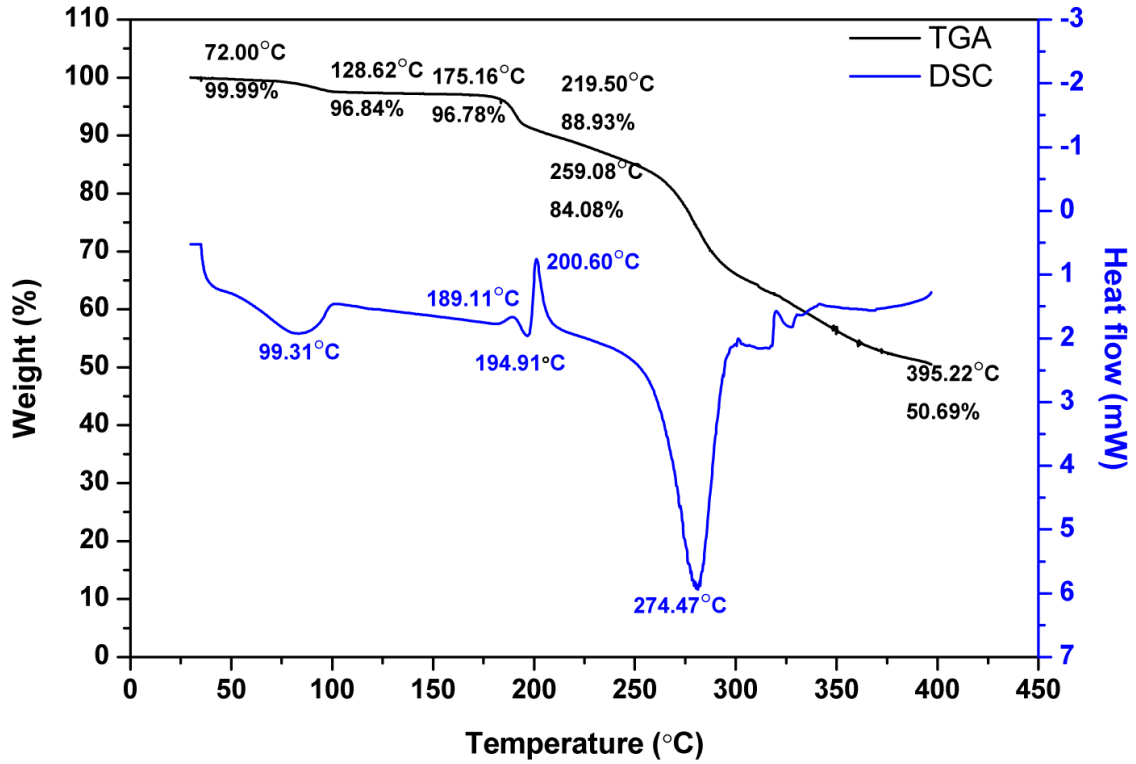


Figure 4.11. Overlaid DSC and TGA thermograms of physical mixture of LD and CD (4:1, w/w).

In case of pure PVPK30, a broad endothermic peak was observed at 73.51°C (Figure 4.12) which can be attributed to the loss of the water molecules (Li et al., 2011; Yu et al., 2011). In DSC thermogram of physical mixture of LDCD and PVPK30, melting/decomposition peaks for LD and CD were well preserved with little change in enthalpy indicating lack of interaction between drug and PVPK30 (Table 4.3). In DSC thermogram of pure MCC (Avicel® PH101) (Figure 4.13) peak was observed at 333.36°C owing to thermal decomposition of MCC (Uesu et al., 2000; Picker and Hoag, 2002). For physical mixtures, melting/decomposition of drug was well preserved (Figure 4.13) with little or no change in enthalpy value of drug (Table 4.3) indicating good compatibility.

The DSC thermograms of pure EC 10cps and pure HPMC 5cps are represented in Figure 4.14 and Figure 4.15, respectively. In DSC thermograms of pure HPMC 5cps and EC 10cps, no peak was observed. For physical mixtures of LD and CD with HPMC 5cps and EC 10cps, melting/decomposition endotherm of LD and exotherm of CD were well

preserved (Figure 4.14 and Figure 4.15) with little change in enthalpy values (Table 4.3) indicating compatibility.

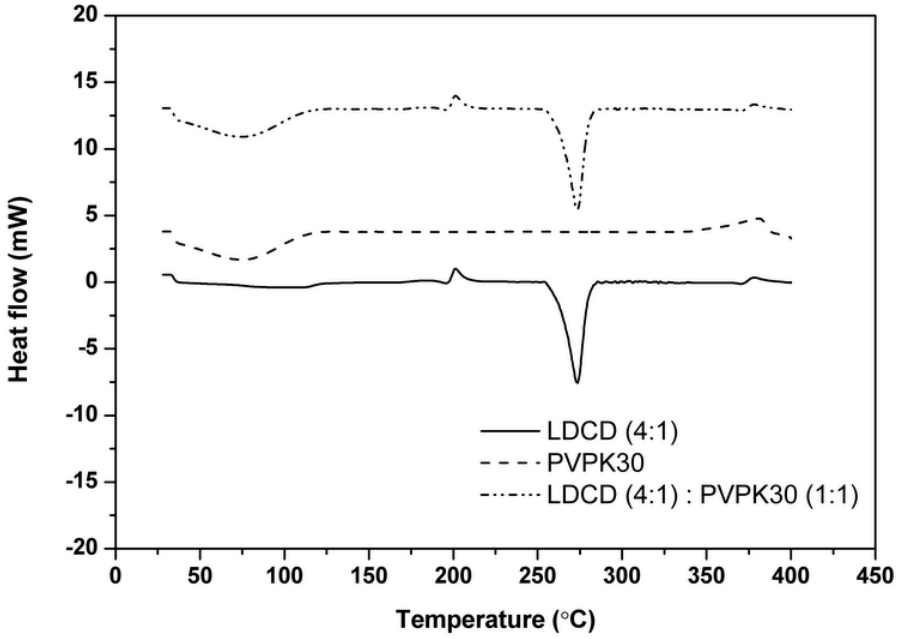


Figure 4.12. Overlaid DSC thermograms of physical mixture of LD and CD (4:1, w/w), pure PVPK30 and 1:1 (w/w) physical mixture of LD:CD (4:1, w/w) and PVPK30.

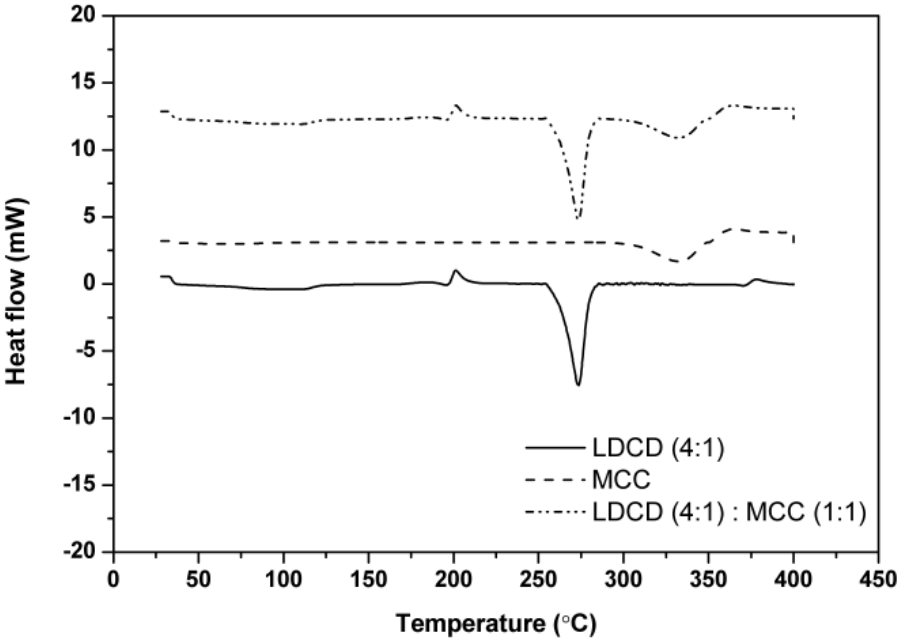


Figure 4.13. Overlaid DSC thermograms of physical mixture of LD and CD (4:1, w/w), pure MCC (Avicel® PH101) and 1:1 (w/w) physical mixture of LD:CD (4:1, w/w) and MCC.

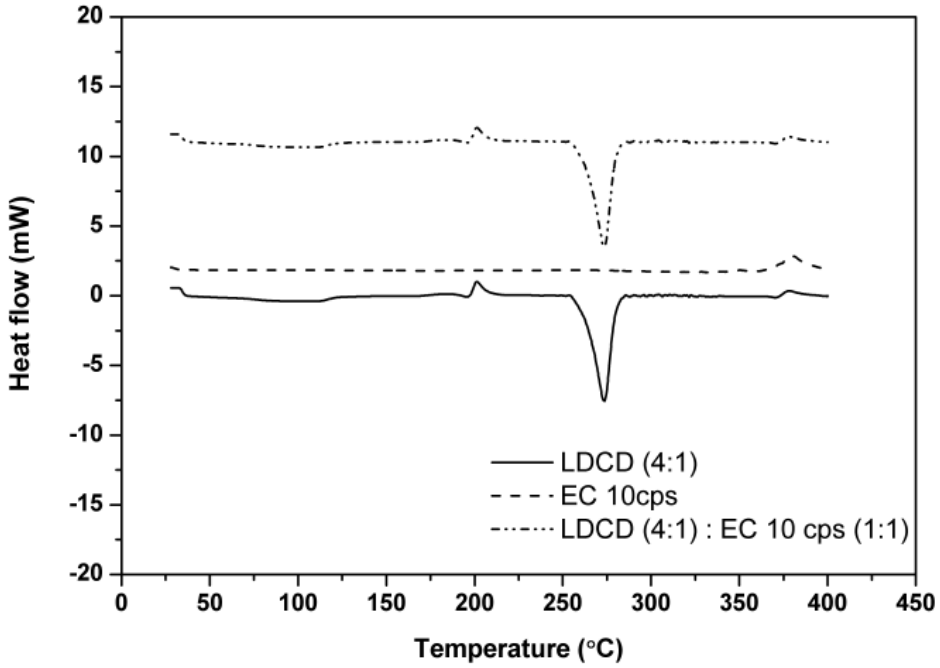


Figure 4.14. Overlaid DSC thermograms of physical mixture of LD and CD (4:1, w/w), pure EC and 1:1 (w/w) physical mixture of LD:CD (4:1, w/w) and EC 10cps.

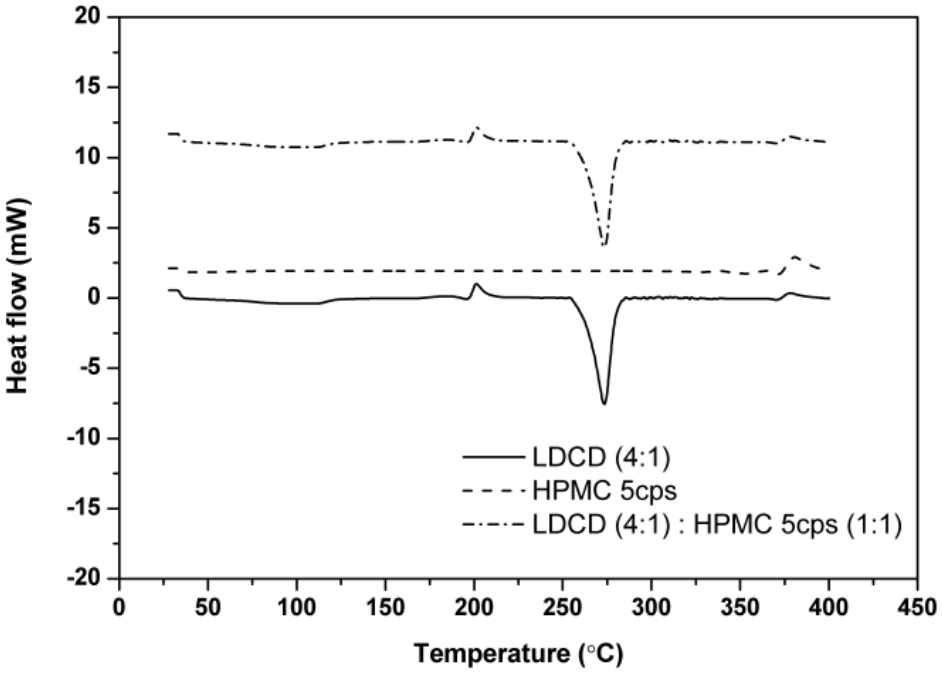


Figure 4.15. Overlaid DSC thermograms of combination of LD and CD (4:1, w/w), pure HPMC 5cps and 1:1 w/w physical mixture of LD:CD (4:1, w/w) and HPMC 5cps.

Table 4.3. Thermal Properties of Pure Drug Alone, Excipient Alone and 1:1 (w/w) Physical Mixture of LD and CD (4:1, w/w) with Different Excipient

Sample	Peak	Onset (°C)	Peak (°C)	Endset (°C)	Heat (J/g)
Pure LD	Endothermic	265.08	296.86	303.26	-346.89
Pure CD	Endothermic	64.77	104.95	120.54	-333.26
	Endothermic	189.71	198.71	201.11	-42.32
	Exothermic	201.11	203.10	217.50	295.35
LDCD combination	Endothermic	70.70	99.31	118.15	-28.69
	Endothermic	191.05	194.91	197.16	-3.34
	Exothermic	196.66	200.60	206.68	21.59
	Endothermic	259.88	274.47	284.18	-282.36
PVPK30	Endothermic	45.65	73.51	108.62	-176.77
LDCD + PVPK30	Endothermic	38.48	74.38	120.95	-301.23
	Endothermic	190.92	196.05	198.98	-4.21
	Exothermic	198.98	200.63	211.62	23.42
	Endothermic	258.54	275.65	286.32	-273.26
Avicel® PH101	Endothermic	309.18	333.36	347.71	-393.54
LDCD + Avicel® PH101	Endothermic	76.77	96.55	118.91	-32.52
	Endothermic	189.27	195.13	198.06	-5.26
	Exothermic	198.06	202.09	209.79	35.26
	Endothermic	254.86	274.47	288.21	-275.36
	Endothermic	306.71	333.10	364.43	-398.78
EC 10cps	---	---	---	---	---
LDCD + EC 10cps	Endothermic	75.67	100.77	119.46	-29.52
	Endothermic	187.62	195.31	198.61	-3.12
	Exothermic	198.61	200.81	210.70	22.12
	Endothermic	256.88	273.37	286.56	-279.85
HPMC 5cps	---	---	---	---	---
LDCD + HPMC 5cps	Endothermic	73.10	100.59	120.37	-31.28
	Endothermic	190.73	196.23	199.53	-4.25
	Exothermic	199.53	200.63	212.72	27.85
	Endothermic	253.39	273.19	286.19	-271.52

In all the cases, endothermic/exothermic peaks in DSC thermogram at a temperature corresponding to the melting/decomposition temperature of LD and CD were found to be retained (Figure 4.12 to 4.15). Further, little or no change in the enthalpy values at respective exothermic and endothermic peak demonstrated compatibility of LD and CD with selected excipient (Table 4.3).

4.3.2. Solubility Analysis

LD has four pKa values (2.3, 8.7, 9.7 and 13.4) and shows a highly pH-dependent solubility profile, especially in the pH range that exists across the upper GIT (pH 1.2 to 4.5). The solubilities of LD and CD at 37 ± 2°C in deionized water and various buffered and unbuffered solutions of pH ranging from 1.2 to 4.5 are summarized in Table 4.4. The pH solubility profiles (in both buffered and unbuffered systems) of LD and CD are represented in Figure 4.16 and Figure 4.17, respectively. LD showed maximum solubility of 17.73 and 16.52 mg/mL at acidic pH of 1.2 in buffered and unbuffered solutions respectively. Similar solubility trend was observed in case of CD with maximum solubility of 12.07 and 11.89 mg/mL at acidic pH of 1.2 in buffered and unbuffered solutions respectively.

In general with increasing pH, solubility decreased in both cases. The solubility profiles of LD and CD in case of both buffered and unbuffered systems were found to be almost identical. It can be said that solubilities of LD and CD are dependent of pH with a significant reduction in solubility as pH increases from 1.2 to 4.5. In case of LD, small increment in pH from 1.2 to 2.0 resulted in the drastic reduction in the solubility of the drug (17.73 to 5.55 mg/mL). This might be due to one of the pKa value of LD, which is 2.3. Hence, at pH 1.2 the proportion of the ionized form (protonated species) was much higher than that of at pH 2.0 (according to Henderson's Hasselbach equation). Similarly, decrease in solubility values were observed from pH 2.0 to 4.5 due to the same concept of ionization. However, no significant difference was observed in the solubility from pH 2.0 to 4.5. Similarly, for CD decrease in solubility was observed with increasing pH in case of both buffered and unbuffered system. The solubility values were found to be higher in buffered solutions than in unbuffered solution for both LD and CD.

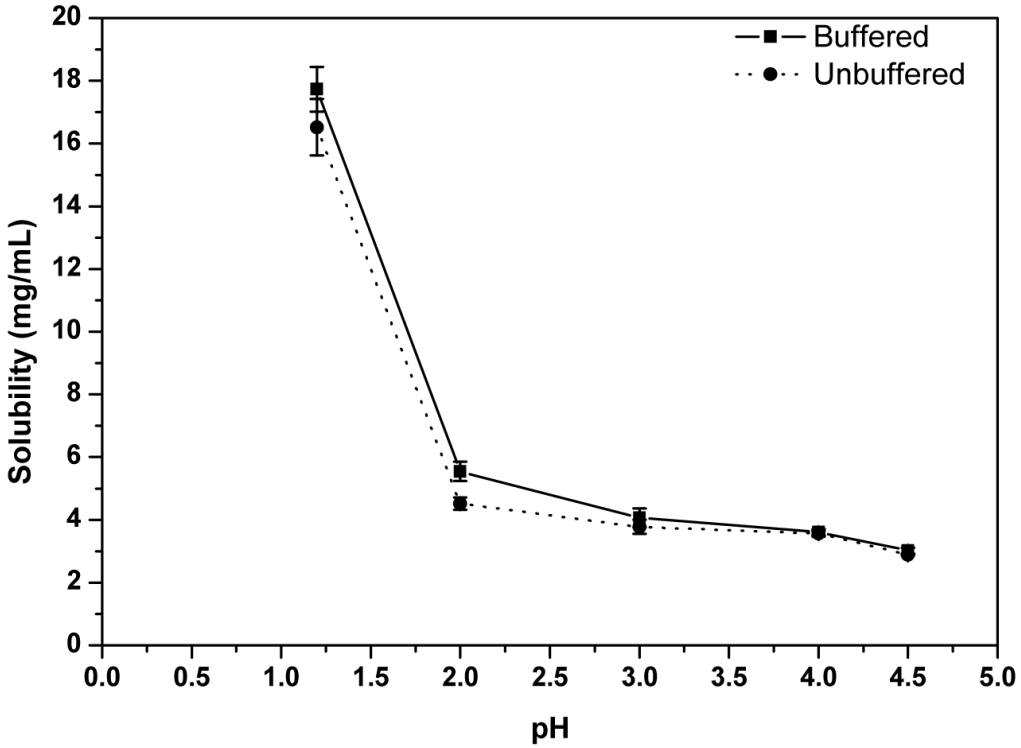


Figure 4.16. Solubility profile of LD in various buffered solutions of varying pH at 37 ± 2°C. (Each point represents mean of three independent determinations with standard deviation)

In presence of 0.05%, w/v of polysorbate 20, solubilities of LD and CD were found to be increased to 6.23 and 3.12 mg/mL, respectively in pH 3.0 phosphate buffer at 37 ± 2°C. This result shows that pH 3.0 phosphate buffer with 0.05%, w/v of polysorbate 20 can be used as in vitro release media for evaluating drug release pattern from the designed drug delivery systems of LD and CD combination.

The poor aqueous solubility of drugs has already been reported as one of the reason for poor and erratic absorption of drug with large inter and intra subject variations in blood levels (Hörter and Dressman, 2001). Moreover, the poor solubility of LD has also been reported as one of the reason for poor and erratic bioavailability of LD (Müller, 2009). Therefore, solubility studies before formulation development play important role in selection of dosage form.

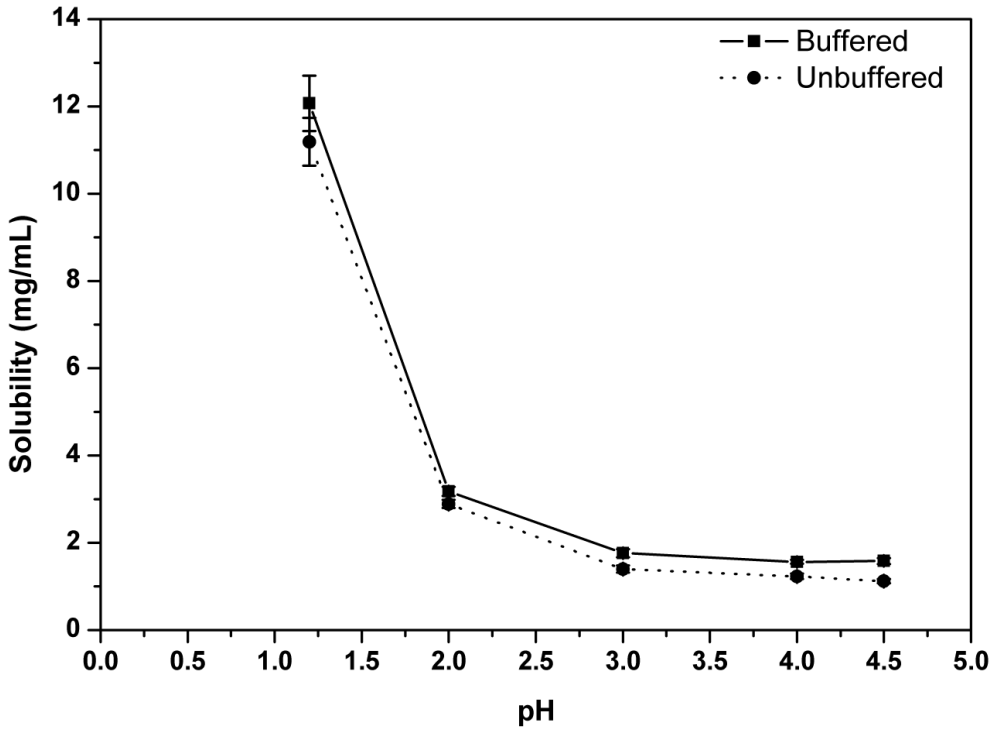


Figure 4.17. Solubility profile of CD in various buffered solutions of varying pH at 37 ± 2°C. (Each point represents mean of three independent determinations with standard deviation).

4.3.3. Stability Analysis

a. Liquid State Stability

LD and CD were found to be sensitive towards various pH conditions with similar degradation kinetics (Figure 4.18 and Figure 4.19). First order degradation rate constant (K*d*) was calculated from the slope obtained by plotting percentage of unchanged drug as a function of time on semi logarithmic scale. The obtained degradation data could be well described by first order degradation kinetic with high correlation coefficient and low akaike information criterion (AIC). The shelf life is the storage period of the drug without significant loss of the potency. The shelf life was expressed as $T_{90\%}$ (days). The relationship between first order degradation rate constant and the shelf life for LD and CD are represented in Figure 4.20 and Figure 4.21 respectively.

The first order degradation over pH range indicated that both drug exhibit comparatively less degradation rate in acidic conditions while basic condition were found to be

detrimental on stability of both. For LD, first order degradation rate constant in acidic condition was 2.88×10^{-2} day^{-1} and $T_{90\%}$ of 3.65 days on the other hand in basic condition degradation rate constant was found to be 171.12×10^{-2} day^{-1} and $T_{90\%}$ of 0.06 days. For CD, fast degradation was observed in basic condition (143.25×10^{-2} day^{-1} with $T_{90\%}$ of 0.07 days) (Table 4.5).

The rapid degradation at basic pH condition may be due to hydroxide ion catalyzed oxidation of LD and CD. The catechol ring of LD and CD undergoes oxidation to the open chain quinone (Madrakian et al., 2004). The degradation rate constant increased from pH 1.0 to 9.0 for both LD and CD, which may be due to increasing concentration of hydroxide ion resulting in faster degradation of drugs. Thus, it can be concluded that the LD and CD need to be handled at acidic pH conditions in liquid state and prolonged exposure to alkaline conditions may lead to significant degradation of the drug.

Table 4.4. Solubility of LD and CD in Deionized Water and Various Buffered and Unbuffered Solutions of Varying pH at 37 ± 2°C

Analyte	Media/pH	Solubility (mg/mL) (Mean ± SD) (n = 3)		
		Deionized water	Buffered system	Unbuffered system
LD	Deionized water	3.01 ± 0.05	-	-
	1.0	-	17.73 ± 0.71	16.52 ± 0.90
	2.0	-	5.55 ± 0.30	4.52 ± 0.20
	3.0	-	4.07 ± 0.30	3.78 ± 0.23
	4.0	-	3.61 ± 0.10	3.56 ± 0.09
	4.5	-	3.02 ± 0.10	2.89 ± 0.02
CD	Deionized water	1.63 ± 0.02	-	-
	1.0	-	12.07 ± 0.64	11.89 ± 0.55
	2.0	-	3.18 ± 0.10	2.89 ± 0.09
	3.0	-	1.76 ± 0.09	1.10 ± 0.08
	4.0	-	1.56 ± 0.04	0.98 ± 0.06
	4.5	-	1.58 ± 0.07	0.89 ± 0.05

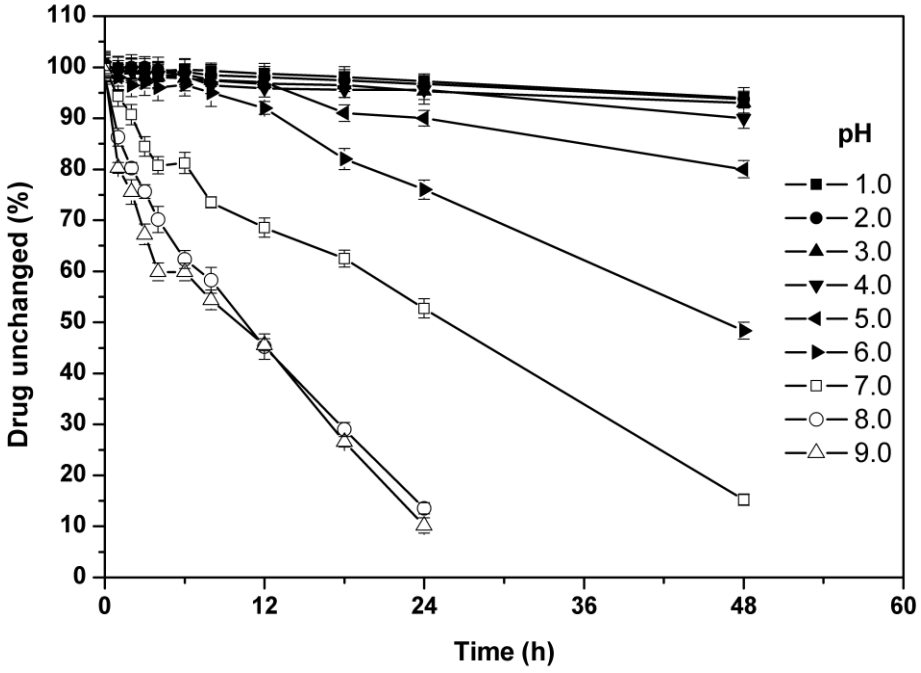


Figure 4.18. Solution state stability of LD in different pH stored at 25 ± 2°C. (Each point represents mean of three independent determinations with standard deviation)

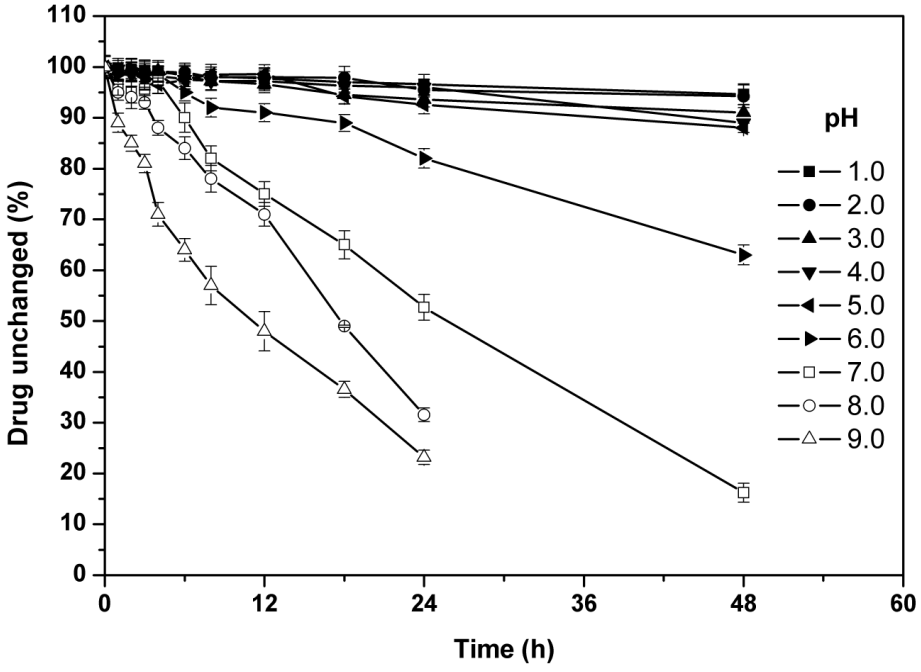


Figure 4.19. Solution state stability of CD in different pH stored at 25 ± 2°C. (Each point represents mean of three independent determinations with standard deviation)

b. Solid State Stability

The solid state stability data suggested that the LD and CD were stable at refrigerated temperature with first order degradation rate constants of 11.58 × 10^{-4} and 11.11 × 10^{-4} $month^{-1}$, respectively (Table 4.6). In addition both LD and CD found to be stable at controlled room temperature with first order degradation rate constants of 28.73 × 10^{-4} and 27.51 × 10^{-4} $month^{-1}$, respectively (Table 4.7). Similarly, LD and CD also showed stability at accelerated temperature with first order degradation rate constants of 46.65 × 10^{-4} and 36.47 × 10^{-4} $month^{-1}$, respectively (Table 4.7). Moreover, FTIR studies revealed that there is no change in the infrared absorption peaks even at the end of 12 months. In addition, thermal analysis carried out using DSC suggested that there is no evidence of the significant change in the melting temperature (onset, peak and endset), enthalpy and degradation pattern indicating stability of both LD and CD (Figure 4.12 to 4.15) (Table 4.3) (data discussed in thermal analysis section). Thus, solid state stability studies for drug content (HPTLC), chemical (FTIR) and physical (DSC) analysis confirmed the stability of LD and CD at studied storage conditions.

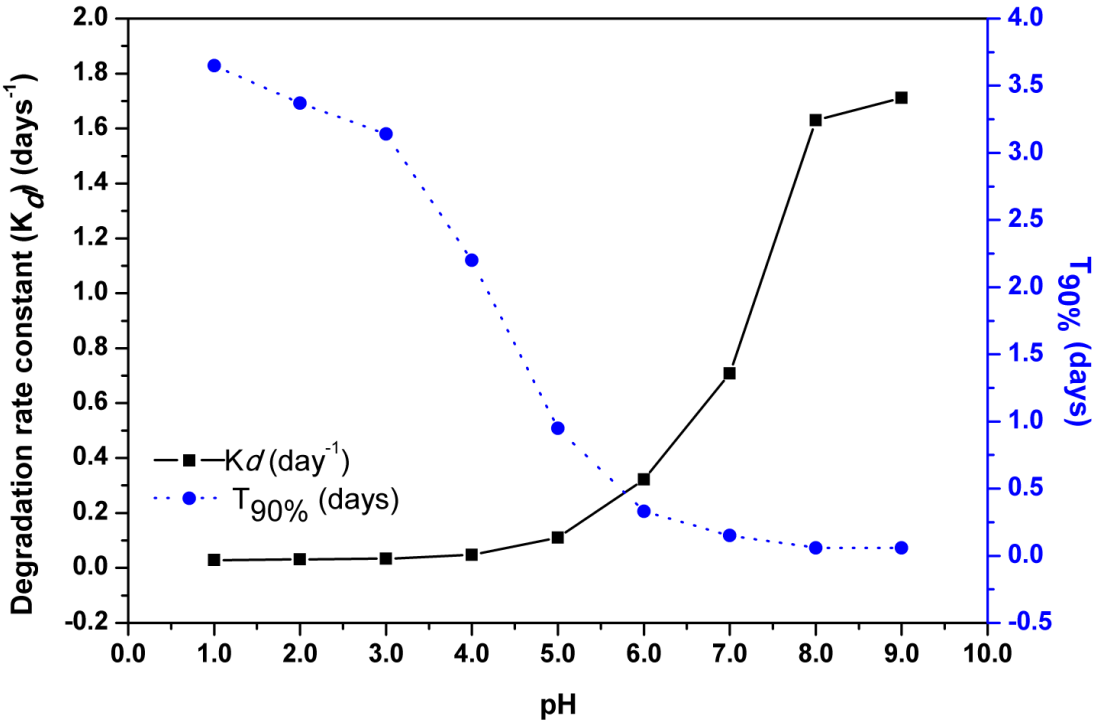


Figure 4.20. First order degradation rate constant (K*d*) versus pH profile of LD in buffered media at 25 ± 2°C.

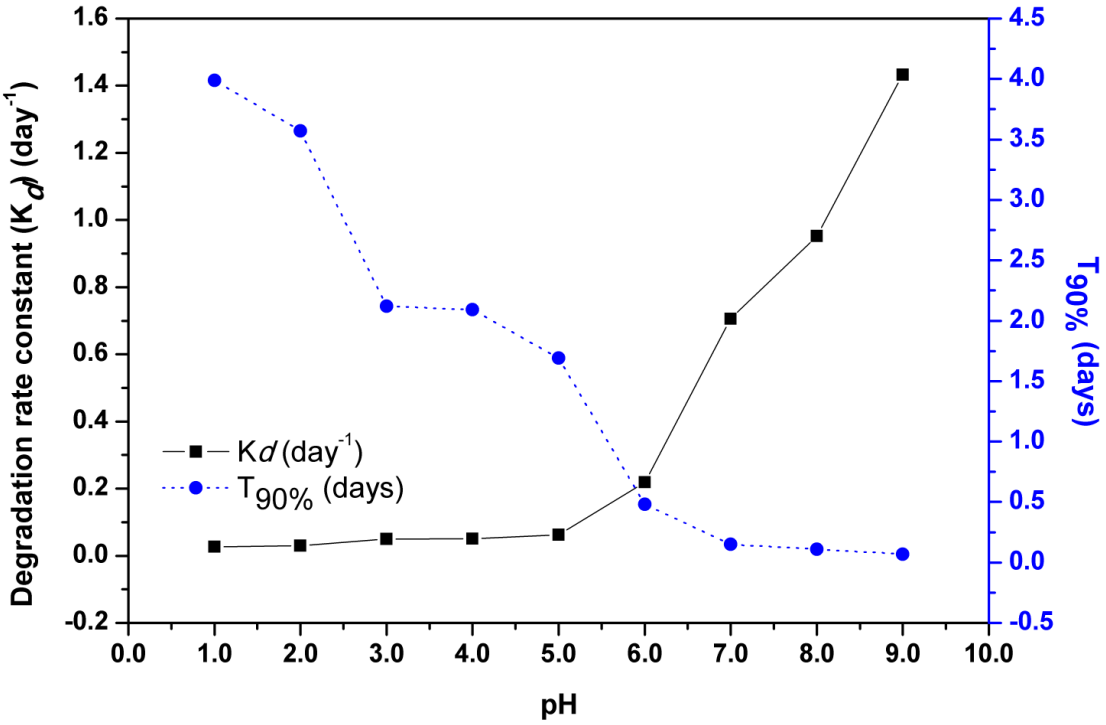


Figure 4.21. First order degradation rate constant (K*d*) versus pH profile of CD in buffered media at 25 ± 2°C.

Table 4.5. First Order Degradation Kinetics of LD and CD in Buffered Media of Varying pH at 25 ± 2°C

Treatment	LD				CD			
	$K_d \times 10^2$ (day^{-1})[a]	$T_{90\%}$ (days)	R^b	AIC[c]	$K_d \times 10^2$ (day^{-1})[a]	$T_{90\%}$ (days)	R^b	AIC[c]
pH-1.0	2.88	3.65	0.9850	-3.72	2.64	3.99	0.9491	7.17
pH-2.0	3.12	3.37	0.9799	1.369	2.95	3.57	0.9289	12.76
pH-3.0	3.36	3.14	0.9247	16.93	4.97	2.12	0.9413	21.23
pH-4.0	4.80	2.20	0.9379	22.36	5.05	2.09	0.9414	22.20
pH-5.0	11.04	0.95	0.9728	31.10	6.24	1.69	0.9176	30.98
pH-6.0	32.16	0.33	0.9648	54.63	21.84	0.48	0.9808	39.68
pH-7.0	70.80	0.15	0.9574	61.45	70.58	0.15	0.9683	60.97
pH-8.0	162.96	0.06	0.9759	47.63	95.21	0.11	0.9580	54.98
pH-9.0	171.12	0.06	0.9433	61.06	143.25	0.07	0.9899	46.55

[a]First order degradation rate constant
[b]Regression coefficient
[c]Akaike information criterion

Table 4.6. First Order Reaction Kinetics of Pure LD, Pure CD and Physical Mixture (4:1) Stored at 5 ± 3°C

Sample	LD			CD		
	$K_d \times 10^4$ (month^{-1})[a]	$T_{90\%}$ (months)	R^2[b]	$K_d \times 10^4$ (month^{-1})[a]	$T_{90\%}$ (months)	R^2[b]
Pure LD	11.48	91.44	0.9745	-	-	-
Pure CD	-	-	-	10.82	97.05	0.9968
Physical mixture of LDCD (4:1)	11.58	90.69	0.9951	11.11	94.55	0.9984

[a] First order degradation rate constant
[b] Regression coefficient

4.3.4. Drug Excipient Stability Study

In this study, LD and CD compatibility with the various excipients were studied at different storage conditions. The first order degradation rate constant (month^{-1}) and $T_{90\%}$ (months) results of the compatibility study after 6 months and 12 months are summarized in Table 4.8. The assay values for LD were 98.96 to 100.78% and 98.21 to 99.82% for accelerated and long term conditions respectively, whereas for CD assay values for accelerated condition were in the range of 98.56 to 100.93% and for long term condition were in the range of 98.48 to 99.85%. The absence of variation between samples taken at different time intervals and control sample for both LD and CD indicated stability of analytes. The consistent repeatable R_f values for LD ($R_f = 0.37 \pm 0.02$) and CD ($R_f = 0.62 \pm 0.02$) further demonstrated stability of LD and CD with excipient selected for compatibility studies. Moreover, absence of degradation product peak further confirmed compatibility of both LD and CD with selected excipients. In addition, drug excipient compatibility was also studied and confirmed by FTIR. In all stability samples, structure defining IR peaks were observed for both LD and CD. Thus, drug excipient compatibility study did not revealed any unfavorable chemical interaction between drugs and selected excipients. From the study results, it can be concluded that the LD and CD were found to be compatible with the selected excipients at accelerated and long term storage conditions.

Table 4.7. First Order Reaction Kinetics of Incompatibility Study of Physical Mixture of LD and CD with Different Excipients

Drug	Sample	CRT (T: 25 ± 2°C/ RH: 60 ± 5%)			AT (T: 40 ± 2°C/ RH: 75 ± 5%)		
		$K_d \times 10^4$ (month^{-1})[a]	$T_{90\%}$ (months)	R^b	$K_d \times 10^4$ (month^{-1})[a]	$T_{90\%}$ (months)	R^b
LD	Pure LD	28.73	36.54	0.9591	46.65	22.51	0.9997
	LD + Avicel PH101	32.31	32.49	0.9820	78.85	13.32	0.9840
	LD + Povidone	27.77	37.80	0.9976	69.57	15.09	0.9749
	LD + HPMC5cps	24.50	42.85	0.9879	76.77	13.68	0.9870
	LD + EC10cps	30.91	33.97	0.9720	64.86	16.19	0.9949
	LD + Talc	29.79	35.25	0.9952	80.88	12.98	0.9808
CD	Pure CD	27.51	38.17	0.9849	36.47	28.79	0.9778
	CD + Avicel PH101	30.25	34.71	0.9692	67.26	15.61	0.9858
	CD + Povidone	32.18	32.63	0.9648	53.66	19.57	0.9854
	CD + HPMC5cps	33.18	31.64	0.9825	51.01	20.59	0.9897
	CD + EC10cps	28.89	36.35	0.9507	59.61	17.61	0.9815
	CD + Talc	29.62	35.45	0.9873	49.57	21.18	0.9771

[a] First order degradation rate constant
[b] Regression coefficient

Table 4.8. First Order Reaction Kinetics of Incompatibility Study of Physical Mixture of LD and CD (4:1) with Different Excipients

Storage condition	Sample	LD			CD		
		$K_d \times 10^4$ (month^{-1})[a]	$T_{90\%}$ (months)	R^b	$K_d \times 10^4$ (month^{-1})[a]	$T_{90\%}$ (months)	R^b
CRT (T: 25 ± 2°C/ RH: 60 ± 5%)	LDCD	30.25	34.72	0.9963	22.49	46.68	0.9819
	LDCD + Avicel PH101	36.34	28.90	0.9932	25.50	41.18	0.9542
	LDCD + Povidone	36.74	28.58	0.9881	34.24	30.67	0.9785
	LDCD + HPMC 5cps	29.60	35.45	0.9905	40.42	25.98	0.9944
	LDCD + EC 10cps	29.33	35.80	0.9885	30.73	34.17	0.9594
	LDCD + Talc	28.54	36.79	0.9925	28.54	36.79	0.9933
AT (T: 40 ± 2°C/ RH: 75 ± 5%)	LDCD	46.12	22.77	0.9890	41.19	25.49	0.9962
	LDCD + Avicel PH101	58.07	18.08	0.9868	56.77	18.50	0.9774
	LDCD + Povidone	62.39	16.83	0.9952	63.36	16.57	0.9970
	LDCD + HPMC 5cps	56.37	18.63	0.9860	72.36	14.51	0.9950
	LDCD + EC 10cps	76.23	13.77	0.9926	68.09	15.42	0.9882
	LDCD + Talc	71.37	14.71	0.9731	65.07	16.14	0.9931

[a] First order degradation rate constant
[b] Regression coefficient

4.4. Conclusions

LD and CD showed pH dependent solubility profile with high solubility in acidic environment and low solubility in alkaline environment. Liquid state stability studies revealed that both LD and CD follow first order degradation kinetics. The stability studies indicated that both LD and CD are sensitive for basic environment as degradation constant were found to be high in basic pH conditions. LD and CD were found to be comparatively more stable at pH 1.0. The solid state stability studies confirmed that LD and CD are stable at refrigerated and ambient temperature.

The drug-excipient compatibility studies indicated that LD and CD are stable in presence of various excipients at ambient as well as at accelerated storage conditions. In addition, there was no polymorphic transformation observed during the study. Thus, drug excipient compatibility study supports the rational for the selection of various excipients and can justify the product life span.

Although poor aqueous solubility of drug has been known to cause bioavailability related problems, frequently these challenges can simply be overcome either by manufacture of prodrug (Djaldetti et al., 2002; Müller, 2009) or by delivering drug through different routes (Kao et al., 2000). Conversely, non-solubility related pharmacokinetic challenges need to be addressed specifically after detailed in vivo investigation. Considering the solubility data of the LD and CD, it can be predicted that the drug solubilization process would not be major a rate limiting step in absorption upon oral dosing. Thus, possibility of poor absorption and subsequent therapeutic failure due to poor solubility may be excluded and LD and CD related problems may be attributed to the pharmacokinetics of the LD and CD. These pharmacokinetic challenges may be overcome by designing the floating drug delivery system. Thus preformulation data provided the strategic input to the design and optimization of the formulation.

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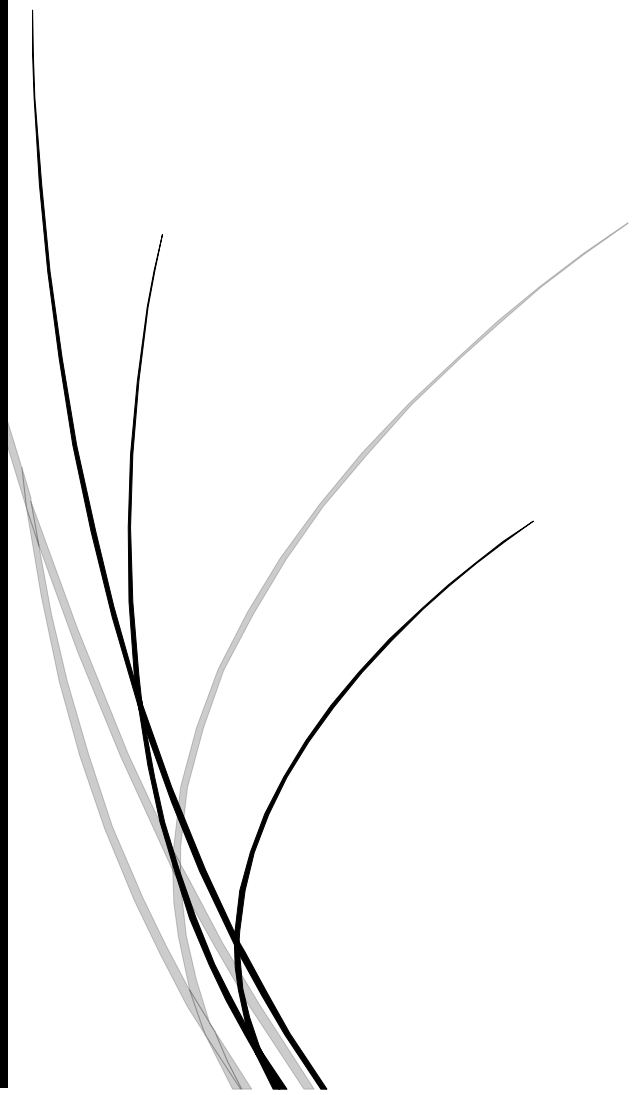
5 FORMULATION DESIGN AND DEVELOPMENT



5.1. Introduction

In this chapter, studies involving development and evaluation of modified release gastroretentive multi unit particulate formulation for combination of LD and CD have been presented. Among the various methods of pelletization, extrusion-spheronization technique was used for the manufacturing of LD and CD loaded pellets in current study. Extrusion-spheronization has ability to produce pellets with a high drug loading (Reynolds, 1970.). Microcrystalline cellulose (MCC) has been reported as a spheronization aid in most of the formulations manufactured by extrusion spheronization technology (Chamsai and Sriamornsak, 2013; Lau et al., 2014; Bryan et al., 2015). MCC provides appropriate rheological properties to the wet mass for smooth extrusion and spheronization (Shah et al., 1995; Newton, 2002). It exhibit good binding properties which provides cohesiveness to a wet mass, therefore it is considered as gold standard for extrusion spheronization (Dukić-Ott et al., 2009). Further, due to larger surface area and internal porosity (Sonaglio et al., 1995), MCC can absorb and hold large quantity of water and thereby facilitating smooth extrusion, by improving plasticity of wet mass. It has already been reported that due to water holding ability, it controls the movement of water through plastic mass and thus it prevents phase separation during extrusion or spheronization (Fielden et al., 1992) which is a common problem associated with other types of spheronization aid. Due to these properties, pellets produced via extrusion spheronization using MCC exhibit good sphericity, low friability, high density and smooth surface properties. Therefore, Avicel® PH101, 102, 302 and 105 were investigated as spheronization aid for manufacturing of LD and CD loaded pellets in current study. Povidone (PVPK30) was used as binder in order to provide additional hardness to final pellet formulation and to enhance the usable yield.

Floating pellets were designed by coating gas generating agents and gas entrapment polymer on LD and CD loaded core pellets. Upon contact of coated pellets with dissolution medium gas generating agent generates carbon dioxide gas due to neutralization reaction. Evolved gas entraps in outer gas entrapment polymer film thus it would reduce the density of pellets resulting in floatation of pellets. Designed core pellet were coated by Wurster coating technique using fluidized bed processor. Gas generating agents like sodium carbonate, tartaric acid were studied alone or in combination. Further, various gas entrapment polymers were studied. Selection of ideal polymer for gas entrapment depends

on its physicochemical properties. Ideal polymer material used as an entrapment membrane should exhibit high permeability for water or dissolution medium so that CO_2 gas would form rapidly to aid in floating. Moreover, in order to maintain longer floating after hydration such coating material should be impermeable to the formed CO_2 gas (Krögel and Bodmeier, 1999). Mechanically, in wet state gas entrapped polymeric membrane should exhibit sufficient flexibility, to avoid rupturing and withstand the pressure exerted by the generated CO_2 gas. Cellulosic polymers are not ideal polymers for gas entrapment (Krögel and Bodmeier, 1999). Further, cellulose acetate also fail to expand sufficiently and ruptures immediately due to lack of flexibility. Ethyl cellulose membrane ruptures immediately upon CO_2 formation due to poor flexibility and gas bubbles release rapidly after bursting of coating. Therefore, polymers having higher flexibility (Eudragit® RL30D, Eudragit® RS30D, Eudragit® NE30D, Eudragit® RL100 and combinations of Eudragit® RL30D and Eudragit® RS30D) were investigated as gas entrapment polymeric material in current study.

The effect of polymer type, polymer proportion and polymer combination on in vitro drug release and floating behavior were studied. In addition, effect of spheronization aid used in core pellet manufacturing and plasticizer type on floating and in vitro drug release properties were also studied. Various quality control tests were performed for evaluation of designed formulations. Further, stability of developed formulations was assessed at various conditions of temperature and humidity. Furthermore, batch reproducibility studies and robustness for optimized formulations were also performed. Robustness of formulations were evaluated by studying the effect of agitation speed, dissolution media pH, spheronization aid, type of plasticizer used on in vitro floating and release behavior of LD and CD.

5.2. Experimental

5.2.1. Materials

Levodopa (purity > 98%) and Carbidopa (purity > 98%) were obtained from Shaanxi Hygethy Biotechnology Corporation, Ltd., China and Smruthi Organics Pvt. Ltd., India, respectively. Cellulose microcrystalline (Avicel® PH101, Avicel® PH102 and Avicel® PH105) was obtained from FMC BioPolymer, USA. Hydroxy propyl methyl cellulose E5

(HPMC) and ethyl cellulose (EC) (standard 10 premium) were provided as a gift sample by Colorcon Asia Pvt. Ltd., India. Povidone (PVPK30) was gifted by BASF, Germany. Polyethylene glycol (PEG, Mol. wt. = 6000), tartaric acid (TA) and sodium bicarbonate ($NaHCO_3$) anhydrous were obtained from Sigma-Aldrich® Corporation, India. Eudragit® RL100, Eudragit® RL30D, Eudragit® RS30D and Eudragit® NE30D was gifted by Evonik India Pvt. Ltd., India. Triethyl citrate (TEC), dibutyl phthalate (DBP), diethyl phthalate (DEP) was purchased from Sigma-Aldrich® Corporation, India. Hydroxy propyl cellulose Klucel™ EXF (HPC) was obtained as gift sample from Ashland Aqualon Functional Ingredients (Wilmington, DE). Ultra-pure deionized water (18.2 MΩ·cm at 25°C) was obtained using a Milli-Q water purification system (Millipore®, USA). Potassium dihydrogen orthophosphate (purity > 99.5%) and orthophosphoric acid (purity ≥ 85%) were obtained from Merck, India. HPLC grade methanol (purity > 99.7%) was purchased from Merck, India. All chemicals were of analytical or HPLC grade and were used as received without any modification.

5.2.2. Manufacture of Floating Sustained Release Pellets

5.2.2.1. Manufacture of Core Pellets

The drug loaded pellets were manufactured using the extrusion spheronization technique. LD, CD, PVPK30 and MCC were screened through 60 # and mixed uniformly in a planetary mixer (Kenwood Chef Classic, UK) at 160 rpm for 20 min. Deionized water was added slowly as a granulating liquid during mixing to obtain a wet mass of suitable consistency. The wet mass was then extruded through a 1 mm screen at 80 rpm using axial screw extruder (Sun Sai Pvt. Ltd. India). The manufactured extrudates were spheronized using spheronizer (crosshatch plate, 2 mm) (Sun Sai Pvt. Ltd. India) at a rotation speed of 1500 rpm for about 10 min to obtain spherical pellets. The resulting drug loaded pellets were dried at 40°C for 24 h in a tray dryer. Finally, dried pellets were screened and 16 - 20 # pellets were selected for the further coating process and formulation development. The composition of the formulations is summarized in Table 5.1.

5.2.2.2. Coating of the Core Pellets

Modified release floating pellets were manufactured by coating protective layer/sustained release layer, gas generating (either single gas generating agent or two gas generating agents) and gas entrapped polymeric layers on drug loaded core pellets. In all cases coating was carried out using a fluidized bed coater (Umang Minilab Coater, India) by Wurster coating technique. Two type of formulations were manufactured viz. formulation coated with single gas generating agent and formulations coated with two gas generating agents.

In case of formulations manufactured by using single gas generating agent ($NaHCO_3$), HPMC was used as binder for coating of $NaHCO_3$. The floating properties of the designed pellets was optimized using design of experiments. The composition of effervescent layer (HPMC:$NaHCO_3$) and its impact on floating properties of the designed pellets were optimized using response surface methodology. A 3^2 type face-centered factorial design was used to investigate the effect of composition of effervescent layer (A) at constant coating level of 12%, w/w and different coating levels of outer gas entrapped polymeric film (B) (Eudragit® RL30D) on floating lag time of designed pellets and pellets floating at 20 h (%). The influence of independent variables (A: ratio of HPMC:$NaHCO_3$ and B: coating level of Eudragit® RL30D) on the responses were modeled using second order polynomial equation (equation 5.1) represented below.

$$Y: b_0 + b_1A + b_2B + b_{11}A^2 + b_{22}B^2 + b_{12}AB \qquad (5.1)$$

Mathematical modeling, validation of model and response surface modeling were carried out using Design Expert® software (version 8.0.7.1, Stat-Ease Inc., Minneapolis, MN, USA). Floating lag time was considered as time required for pellets to float. Pellets floating at 20 h (%) was determined visually (using method discussed in in vitro floating study). The studied factors (independent variables), selected levels and responses (dependent variables) are summarized in Table 5.2 and 5.3. Composition of the formulations manufactured using single effervescent agent coating are summarized in Table 5.4 and Table 5.5.

Formulations manufactured by using two gas generating agents are listed in Table 5.6. In this type of formulations, $NaHCO_3$ and TA were used as gas generating agents. HPC was used as binder for both $NaHCO_3$ and TA. Various sustained release polymers, gas generating agents and gas entrapment polymers were investigated during formulation development. The compositions of coating solutions used for the coating of the pellets including significance of each ingredient used in coating solution preparation are represented in Table 5.7 to 5.9. Further, coating process parameters set during coating of the pellets and solid content in the coating solutions are summarized in Table 5.10. Finally, after completion of coating process, 0.5%, w/w of colloidal silica (Aerosil® 200) was mixed with pellets in case of all types of formulations to avoid sticking. Detailed justification for using pore forming agents, different coating solutions and compositions, processing conditions (Table 5.7 to 5.10) kept during coating process has been presented in result discussion section (Section 5.3).

5.2.3. Evaluation of the Manufactured Pellets

5.2.3.1. Size Analysis

The size of designed core pellets was evaluated by sieve analysis technique using standard set of sieves (595 - 2000 µm). The pellets were separated in to various size fractions using sieve shaker (Electromagnetic sieve shaker, EMS-8, Electrolab, India) vibrating at 1 mm amplitude for 20 min and weight distribution of core pellets was determined (Koo and Heng, 2001; Sinha et al., 2005; Sungthongjeen et al., 2006). The pellet size and size distribution was characterized by calculating the geometric mean diameter and span. The obtained results were processed using log-probability plot according to Martin (Martin et al., 1991). The geometric mean diameter on weight basis (d_g) was calculated from log-probability plot (d_g is considered as a particle size equivalent to 50% on log-probability scale). Further, span of pellet size distribution was calculated (equation 5.2) by dividing difference obtained between pellets diameter at 90 percentile and at 10 percentile by the pellet diameter at 50 percentile (Vertomrnen and Kinget, 1997; Heng et al., 2002).

$$\text{Span} = \frac{90^{\text{th}}\ \text{percentile} - 10^{\text{th}}\text{percentile}}{50^{\text{th}}\ \text{percentile}} \qquad (5.2)$$

Table 5.1. Composition of Core Pellets Manufactured by Extrusion Spheronization Technique

Formulation code	Formulation composition (%, w/w)						
	LD	CD	Microcrystalline cellulose grades				Povidone (PVPK30)
			Avicel® PH101	Avicel® PH102	Avicel® PH302	Avicel® PH105	
LC1	48.00	12.00	40.00	-	-	-	-
LC2	48.00	12.00	-	40.00	-	-	-
LC3	48.00	12.00	-	-	40.00	-	-
LC4	48.00	12.00	39.50	-	-	-	0.50
LC5	48.00	12.00	-	39.50	-	-	0.50
LC6	48.00	12.00	39.00	-	-	-	1.00
LC7	48.00	12.00	-	39.00	-	-	1.00
LC8	48.00	12.00	-		-	40.00	-
LC9	48.00	12.00	-	-	-	39.00	1.00

Table 5.2. Experimental Design: Factors and Responses

Factors (Independent variables)	**Levels used**			**Responses (dependent variable)**
	-1	**0**	**1**	
A= ratio of HPMC:$NaHCO_3$	4:1	2:2	1:4	Floating lag time (Y_1, min)
B= coating level of Eudragit® RL30D (%)	10	15	20	Pellets floating at 20 h (Y_2, %)

Table 5.3. Composition of Experimental Formulations (Runs)

Run	**Independent variable**		**Dependent variables (Mean ± SD) (n = 3)**	
	A= HPMC:$NaHCO_3$	**B= Eudragit® RL30D**	**Floating lag time (min)**	**Pellets floating at 20 h (%)**
1 (HPMC/ERL/1)	4:1 (-1)	10 (-1)	30.34 ± 2.12	18.00 ± 1.00
2 (HPMC/ERL/2)	4:1 (-1)	15 (0)	46.52 ± 3.45	23.00 ± 3.00
3 (HPMC/ERL/3)	4:1 (-1)	20 (1)	58.56 ± 3.12	30.00 ± 2.00
4 (HPMC/ERL/4)	2:2 (0)	10 (-1)	11.55 ± 1.25	45.00 ± 2.65
5 (HPMC/ERL/5)	2:2 (0)	15 (0)	18.57 ± 2.15	47.00 ± 2.00
6 (HPMC/ERL/6)	2:2 (0)	20 (1)	25.36 ± 1.75	61.00 ± 2.64
7 (HPMC/ERL/7)	1:4 (1)	10 (-1)	3.25 ± 2.15	81.00 ± 3.61
8 (HPMC/ERL/8)	1:4 (1)	15 (0)	6.12 ± 1.25	85.00 ± 1.00
9 (HPMC/ERL/9)	1:4 (1)	20 (1)	8.66 ± 1.25	91.00 ± 2.00

Table 5.4. Composition of Modified Release Floating Pellet Formulations Coated with Single Gas Generating Layer (Sodium Bicarbonate Layer Alone) and Gas Entrapment Layer

Formulation code	Formulation composition [weight gain (%, w/w)]					
	HPMC	**EC**	**HPMC:$NaHCO_3$[b]**	**Eudragit® RL30D**	**Eudragit® RS30D**	**Eudragit® NE30D**
HPMC/ERL/1	5.00	-	4:1	10.00	-	-
HPMC/ERL/2	5.00	-	4:1	15.00	-	-
HPMC/ERL/3	5.00	-	4:1	20.00	-	-
HPMC/ERL/4	5.00	-	2:2	10.00	-	-
HPMC/ERL/5	5.00	-	2:2	15.00	-	-
HPMC/ERL/6	5.00	-	2:2	20.00	-	-
HPMC/ERL/7	5.00	-	1:4	10.00	-	-
HPMC/ERL/8	5.00	-	1:4	15.00	-	-
HPMC/ERL/9	5.00	-	1:4	20.00	-	-
EC/ERL/1		2.00	1:4	20.00	-	-
EC/ERL/2	-	3.00	1:4	20.00	-	-
EC/ERL/3	-	4.46	1:4	20.00	-	-
EC/ERL/4	-	6.00	1:4	20.00	-	-
EC/ERL/5 [a]	-	3.00	-	20.00	-	-
EC/ERL/6	-	3.00	-	-	-	-
EC/ERS/1	-	2.00	1:4	-	5.00	-
EC/ERS/2	-	2.00	1:4	-	10.00	-
EC/ERS/3	-	2.00	1:4	-	15.00	-
EC/ENE/1	-	2.00	1:4	-	-	5.00
EC/ENE/2	-	2.00	1:4	-	-	10.00
EC/ENE/3	-	2.00	1:4	-	-	15.00

[a] Formulation coated with HPMC E5 (12%, w/w) instead of effervescent layer
[b] Fixed weight gain of effervescent layer coating (12%, w/w)

Table 5.5. Composition of Modified Release Floating Pellet Formulations Coated with Single Gas Generating Layer (Sodium Bicarbonate Layer Alone) and Gas Entrapment Layer Containing Pore Forming Agent

Formulation code	Formulation composition [weight gain (%, w/w)]						
	HPMC	EC	HPMC: $NaHCO_3$	Eudragit® RL30D:Eudragit® RS30D		Eudragit® NE30D:PEG 6000	
				Ratio	Coating level (%, w/w)	Ratio	Coating level (%, w/w)
EC/ERLRS/1	-	2.00	1:4	80:20	10.00	-	-
EC/ERLRS/2	-	2.00	1:4	80:20	15.00	-	-
EC/ERLRS/3	-	2.00	1:4	80:20	20.00	-	-
EC/ERLRS/4	-	2.00	1:4	60:40	10.00	-	-
EC/ERLRS/5	-	2.00	1:4	60:40	15.00	-	-
EC/ERLRS/6	-	2.00	1:4	60:40	20.00	-	-
EC/ERLRS/7	-	2.00	1:4	40:60	10.00	-	-
EC/ERLRS/8	-	2.00	1:4	40:60	15.00	-	-
EC/ERLRS/9	-	2.00	1:4	40:60	20.00	-	-
HPMC/ENEPEG/1	5.00	-	1:4	-	-	90:10	10.00
HPMC/ENEPEG/2	5.00	-	1:4	-	-	80:20	10.00
HPMC/ENEPEG/3	5.00	-	1:4	-	-	70:30	10.00

Table 5.6. Composition of Modified Release Floating Pellet Formulations Coated with Two Gas Generating Layers (Tartaric Acid and Sodium Bicarbonate) and Gas Entrapment Layer

Formulation code	Formulation composition [weight gain (%, w/w)]				
	EC	Kollicoat® SR30D	HPC:TA (1:4)	HPC:$NaHCO_3$ (1:4)	Eudragit® RL100
ERL100/1	-	-	14.00	8.00	5.00
ERL100/2	-	-	10.00	10.00	5.00
ERL100/3	-	-	8.00	14.00	5.00
ERL100/4	-	-	14.00	8.00	10.00
ERL100/5	-	-	10.00	10.00	10.00
ERL100/6	-	-	8.00	14.00	10.00
ERL100/7	-	-	14.00	8.00	15.00
ERL100/8	-	-	10.00	10.00	15.00
ERL100/9	-	-	8.00	14.00	15.00
EC/ERL100/1	2.00	-	8.00	14.00	15.00
EC/ERL100/2	3.00	-	8.00	14.00	15.00
EC/ERL100/3	4.50	-	8.00	14.00	15.00
KSR/ERL100/1	-	4.00	8.00	14.00	15.00
KSR/ERL100/2	-	6.00	8.00	14.00	15.00
KSR/ERL100/3	-	10.00	8.00	14.00	15.00
EC/ERL100P/1	3.00	-	8.00	14.00	15.00 (TEC)
EC/ERL100P/2	3.00	-	8.00	14.00	15.00 (DEP)
EC/ERL100P/3	3.00	-	8.00	14.00	15.00 (DBP)
LC6/EC/ERL100	3.00	-	8.00	14.00	15.00 (Avicel® PH101)
LC9/EC/ERL100	3.00	-	8.00	14.00	15.00 (Avicel® PH105)

Table 5.7. Composition of Solutions Used for Coating of Protective and Sustained Release Layer

Ingredient	Quantity (g)			Function
	HPMC E5	EC (10 cps)	Kollicoat® SR30D	
Ethyl cellulose (EC)	-	3.50	-	Polymer
Hydroxy propyl methyl cellulose (HPMC E5)	5.00	-	-	Polymer
Kollicoat® SR30D (KSR)	-	-	100	Polymer
Povidone (PVPK30)	-	1.50	-	Pore former
Acetyl triethyl citrate	-	0.45	-	Plasticizer
Triethyl citrate	-	-	3.41	Plasticizer
Polyethylene glycol (PEG 6000)	0.50	-	-	Plasticizer
Talc	1.00	-	12.00	Anti-tacking
Water	93.50	-	250.00	Diluent
Ethanol:Water (95:5)	-	94.55 (115.72)	-	Diluent

Table 5.8. Composition of Solutions Used for Coating of Effervescence Layers

Ingredient	Quantity(g)			Function
	$NaHCO_3$ (Aqueous)	**$NaHCO_3$ (organic)**	**TA (organic)**	
Hydroxy propyl methyl cellulose (HPMC E5)	4.00	-	-	Binder
Hydroxy propyl cellulose (HPC)	-	8.00	8.00	Binder
Sodium bicarbonate ($NaHCO_3$)	16.00	32.00	-	Effervescent agent
Tartaric acid	-	-	32.00	Effervescent agent
Polyethylene glycol (PEG 6000)	0.40	-	-	Plasticizer
Talc	0.40	0.40	0.40	Anti-tacking
Water	250.00	-	-	Diluent
Isopropyl alcohol	39.25	235.50	235.50	Diluent

Table 5.9. Composition of Solutions Used for Coating of Gas Entrapped Polymeric Layer

Ingredient	Quantity (g)						Function
	Eudragit® RL30D	**Eudragit® RS30D**	**Eudragit® NE30D**	**Eudragit® RL100**	**Eudragit® RL30D: Eudragit® RS30D**	**Eudragit® NE30D: PEG 6000**	
Eudragit® RL30D	100.00	-	-	-	80.00	-	Polymer
Eudragit® RS30D	-	100.00	-	-	20.00	-	Polymer
Eudragit® NE30D	-	-	100.00	-	-	100.00	Polymer
Eudragit® RL100	-	-	-	30.00	-	-	Polymer
PEG 6000	-	-	-	-	-	12.00	Plasticizer
Triethyl citrate	3.00	3.00	-	3.00	3.00	-	Plasticizer
Talc	7.50	7.50	12.00	10.50	7.50	12.00	Anti-tacking
Water	100.00	100.00	300.00	-	100.00	300.00	Diluent
Isopropyl alcohol	-	-	-	120.00	-	-	Diluent
Acetone	-	-	-	120.00	-	-	Diluent

Table 5.10. Process Parameters for the Coating of the Drug Loaded Core Pellets

Coatings	Coating parameters						
	Inlet Air temp. (°C)	**Product temp. (°C)**	**Exhaust air temp. (°C)**	**Spray rate (g/min)**	**Atomization air pressure (bar)**	**Final drying**	**Solid content (%, w/w)**
HPMC E5	52 - 55	39 - 40	38 - 39	0.70 - 1.20	1.20	45°C for 30 min	6.00
EC (10 cps)	40 - 42	35 - 36	34 - 36	1.10 - 1.89	1.00	45°C for 30 min	5.00
HPMC:$NaHCO_3$	52 - 55	39 - 40	38 - 39	0.60 - 1.30	1.20	45°C for 30 min	7.19
Eudragit® RL30D	45 - 47	35 - 36	35 - 36	0.50 - 1.00	0.90	45°C for 30 min	18.75
Eudragit® RS30D	45	32 - 34	34 - 35	0.50 - 1.10	0.90	45°C for 30 min	18.75
Eudragit® RL30D: Eudragit® RS30D	45 - 47	30 - 36	33 - 36	0.50 - 1.00	0.90	45°C for 30 min	18.75
Eudragit® NE30D	28 - 30	25	24 - 26	0.40 - 0.80	1.10	40°C for 24 h	14.00
Eudragit® NE30D:PEG	28 - 30	20 - 25	20 - 25	0.50 - 0.90	1.10	40°C for 24 h	18.00
Kollicoat® SR30D	50 - 55	38 - 42	42 - 44	0.60 - 1.30	1.00	45°C for 30 min	16.80
HPC:TA	48 - 52	40 - 42	38 - 40	0.90 - 1.30	1.20	45°C for 15 min	17.15
HPC:$NaHCO_3$	46 - 50	36 - 40	35 - 37	0.90 - 1.30	1.20	45°C for 15 min	17.15
Eudragit® RL100	40 - 42	29 - 30	28 - 30	0.90 - 1.40	1.00	45°C for 30 min	13.50

5.2.3.2. Usable Yield (% Theoretical Yield)

The size distribution of pellets was performed by sieve analysis as discussed in section 5.2.3.1. The fraction of the pellets passed from sieve 16 # and retained on sieve 20 # was considered as usable yield (Howard et al., 2006; Pund et al., 2011).

5.2.3.3. Shape Analysis

The modal class fractions obtained from the sieve analysis was considered for the shape analysis for each batch. The modal class was considered as class wherein maximum fraction of pellets were retained. Pellet sample (50 pellets) from modal class of each batch were randomly selected for shape evaluation. The images of the pellets were drawn manually on graph paper with the help of light microscope coupled with Camera Lucida. The area of images, maximum and minimum redii were calculated. Further, various shape factors which includes roundness, elongation and rectang were calculated (Koo and Heng, 2001; Sinha et al., 2005) using equation 5.3, 5.4 and 5.5 respectively.

$$\text{Roundness} = \frac{\text{Area}}{\pi \times (\text{Max. radius})^2} \tag{5.3}$$

$$\text{Elongation} = \frac{\text{Max. radius}}{\text{Min. radius}} \tag{5.4}$$

$$\text{Rectang} = \frac{\text{Area}}{4 \times \text{Max.radius} \times \text{Min.radius}} \tag{5.5}$$

The shape factor, roundness measures the sphericity or circularity of pellets, elongation measures the oblongated shape of pellet and rectang measures, rectangular shape of pellets.

5.2.3.4. Hausner's Ratio and Carr's Index

The bulk and tapped densities of the pellets from each batch were calculated in order to estimate Hausner's ratio and Carr's index. Pellets were filled in to graduated cylinder using glass funnel. The weight of pellets required to fill the cylinder volume up to 20 mL mark was calculated. The cylinder was then tapped from a height of 2 cm until there was no more

change in the volume. Bulk density (ρb) was considered as ratio of weight of pellets filled in cylinder to the volume of cylinder occupied by pellets before tapping. Tapped density (ρt) was considered as ratio of weight of pellets filled in cylinder to the volume of cylinder occupied by pellets after tapping. Hausner's ratio and Carr's index were calculated as per the equation 5.6 and 5.7 respectively. All estimations were carried out in triplicates.

$$\text{Hausner's ratio} = \frac{\rho t}{\rho b} \tag{5.6}$$

$$\text{Carr's index} = \frac{\rho t - \rho b}{\rho t} \tag{5.7}$$

5.2.3.5. Flow Properties

Designed pellets were also evaluated for the flow properties which are represented in terms of angle of repose and flow rate. Angle of repose for pellets was determined by pouring pellets gently through the wall of funnel positioned so that its end of the tip was at height of 2 cm above from the horizontal hard surface. The pellets were continuously poured through the funnel till upper tip of pile surface touches the lower tip of the funnel. The height of the pile is measured and radius of the pile base was also calculated. Then angle of repose was calculated by estimating $\tan^{-1}$ of the (height of pile/radius of its base). Further, flow rate of designed pellets was considered as time taken (measured in seconds) by pellets (10 g) to flow through the funnel having internal diameter of 5 mm. All estimations were carried out in triplicates.

5.2.3.6. Friability

The mechanical strength of pellets was estimated by using USP friability testing apparatus. The friability of the designed floating pellet formulations was represented as the percentage weight loss after 200 revolutions of 10 g of the core pellets in friabilator (Roche friabilator, Campbell Electronics, Mumbai, India) (Kim et al., 2007; Amrutkar et al., 2012). Friability was expressed in terms of % weight loss. Weight loss (%) was calculated using equation 5.8.

$$\text{Weight loss (\%)} = \frac{M_1 - M_2}{M_1} \times 100 \quad (5.8)$$

Where, M_1: Mass of pellets before test in grams (10 g) and M_2: Mass of pellets after test in grams.

5.2.3.7. Crushing Strength

The crushing strength of the designed formulations (25 pellets from each batch) was evaluated using texture analyzer (Stable Micro Systems, UK). The probe (P20 type) was used for crushing of the pellets. The probe was lowered towards pellet at 0.1 mm/s. Upon contact with pellet, force [Newton, (N)] required to crush the pellet was measured when the probe goes below 50% of the height of the pellet. Crushing strength of pellet was represented as arithmetic mean of the force.

5.2.3.8. Residual Moisture Content

The residual moisture present in the pellet (core pellet batches) after drying was estimated using infrared (IR) moisture balance (Adair Dutt, Kolkata). The equipment was pre-calibrated and standardized with starch. Pellets (2 g) were placed on IR moisture balance and dried at 105°C until two successive readings matches each other. Each batch was analyzed in triplicate.

5.2.3.9. Estimation of Active Agent Content

Pellets (1 g) were weighed accurately and powdered using mortar and pestle. The resulting powder (10 mg) was weighed and transferred to 100 mL calibrated flask. The volume was made up with 0.1%, v/v orthophosphoric acid/water containing 0.1%, w/v sodium metabisulfite. Sample was sonicated for 10 min for complete extraction of LD and CD. After sonication samples were filtered through 0.22 µm Millipore® syringe filters (Millex® Syringe Filter, Millipore). The resulting samples were subsequently diluted with mobile phase appropriately and analyzed using HPLC method discussed in Chapter 3 (section 3.4) (Raut and Charde, 2014).

5.2.3.10. Microscopic Studies

The micromorphological studies of designed floating pellets were carried out by using scanning electron microscope (SEM) (Hitachi S-3400N, Hitachi High-Technologies Corporation, Tokyo, Japan), fluorescence microscope under optical microscopy mode (Olympus IX 53, Olympus Corporation, Tokyo, Japan) and stereo zoom microscope (Leica MZ16, GmbH, Germany). The morphology of surface and the internal layers of pellets were examined and photographed using SEM. Pellets were cut in to two hemispheres in order to view and examine internal morphology. To study the morphology of floating pellet, pellets were charged in the dissolution medium then floating pellets were taken out of dissolution media carefully and dried at 40°C for 12 h. The processed pellet samples were fixed on the surface of stubs using adhesive tape and hole was made in the outer cover. Photographs was taken by using stereo zoom microscope equipped with 3.0 megapixel camera then fixed pellets samples were sputter coated with gold palladium under argon atmosphere using ion sputter coater (Hitachi E-1010, Hitachi High-Technologies Corporation, Tokyo, Japan). The sputter coated pellets were examined using SEM at 15.0 kV accelerating voltage. To investigate the floating process of designed pellets, microphotographs of pellets were taken at different time intervals after charging in 0.1 N HCl with the help of fluorescence microscope under optical microscopy mode equipped with 5.0 megapixel camera under 10X objective lens.

5.2.3.11. In Vitro Floating Study of Designed Pellets

The % of floating pellets at different time interval of the designed formulation was estimated by using the procedure suggested by (Ichikawa et al., 1991). Pellets were charged in a beaker (100 mL) containing 70 mL of dissolution medium (0.1N HCl/phosphate buffer pH 3.0) maintained at 37 ± 0.5°C. Beaker was kept on horizontal shaking at speed of 100 cycles/min in the incubator at 37 ± 0.5°C. Total numbers of pellets floating on the surface of dissolution medium was measured by photographing the surface of dissolution medium in beaker and counting numbers of floating pellets in the picture. Before catching the picture it was ensured that neither sticking of the pellets nor submerging of the pellet beneath the other pellets occurred. In case of pellets trapped beneath the other pellets, beaker was softly shaken in order to get a monolayer of floating pellets on the dissolution

medium surface before taking picture. Total numbers of pellets floating on the surface of dissolution medium were counted at different time intervals and percentage of floating pellets was estimated by using following equation 5.9:

$$\text{Floating Pellets (\%)} = \frac{F_t}{F} \text{ X } 100 \qquad (5.9)$$

Where, F_t = total number of floating pellets at measured time t, F = total numbers of pellets in beaker (total number of pellets charged for study). Analysis of floating pellets was carried out in triplicates.

5.2.3.12. In Vitro Drug Release Studies

In vitro drug release studies for designed floating pellets were carried out using USP type II dissolution test apparatus (TDT-08L, Electrolab, India). Drug release study was conducted in 900 mL 0.1 N HCl maintained at 37.0 ± 0.5°C. The paddle rotation speed employed was 50 rpm. Pellets equivalent to 100 mg of LD (and 25 mg of CD) were charged in dissolution medium. At predetermined time intervals samples were withdrawn and analyzed using HPLC method discussed in Chapter 3 (section 3.4) (Raut and Charde, 2014). In order to evaluate the impact of the paddle rotation speed on the in vitro release of LD and CD from the optimized formulations, dissolution study was also carried out keeping paddle rotation speed at 100 rpm. Further, influence of the pH on the LD and CD release from the optimized formulations were investigated by evaluating dissolution of the formulation in 0.1 N HCl and potassium dihydrogen phosphate buffer (10 mM) pH = 3.0.

(a) Model Independent Approach

In order to facilitate the interpretation and comparison of the dissolution profiles of designed formulations, model-independent approach [Mean dissolution time (MDT), dissimilarity (f_1) and similarity (f_2) factors] was used.

Mean dissolution time (MDT) is the arithmetic mean of dissolution profile (Podczeck, 1993; Yuksel et al., 2000) and was calculated using equation 5.10:

$$MDT = \frac{\sum_{j=1}^{n} t_{mid}\ \Delta M}{\sum_{j=1}^{n} \Delta M} \quad (5.10)$$

Where, j is the dissolution sample number, n is the number of sampling time points, t_{mid} is the time at the midpoint between t_j and t_{j-1} and ΔM is the amount of drug dissolved between t_j and t_{j-1} .

Dissimilarity (f_1) *and similarity* (f_2) *factors* were used to statistically compare the dissolution pattern of the samples (Moore and Flanner, 1996). Dissimilarity (f_1) is defined as the measure of percent error between two dissolution profiles. Similarity (f_2) is a logarithmic transformation of the sum-squared error of the difference between the percentage dissolved of test and reference product over all time points. Furthermore, similarity factor (f_2) value was calculated for assessing the similarity of dissolution profiles as recommended in the FDA Guidance for Industry (FDA, 1997; Shah et al., 1998). These values were calculated using equation 5.11 and 5.12:

$$f_1 = \left\{\frac{[\sum_{t=1}^{n}|R_t - T_t|]}{\sum_{t=1}^{n} R_t}\right\} \times 100 \quad (5.11)$$

$$f_2 = 50 \log\left\{\left[1 + (1/n)\sum_{t=1}^{n}(R_t - T_t)^2\right]^{-0.5} \times 100\right\} \quad (5.12)$$

Where n is number of sampling points, R_t and T_t are the cumulative drug release from reference and test sample at each sampling point t, respectively.

The f_1 factor assumes value of 0 in case of identical dissolution profiles of the test and reference. The value of f_1 increases with increase in the dissimilarity. The f_2 value of 100 indicates the identical dissolution profile of the test and reference. Higher f_2 value, in the range of 50 and 100 indicates similar dissolution profile and values of f_2 lesser than 50 signify dissimilarity. The dissolution data used for comparison was obtained under the same test conditions and their dissolution time points were the same.

(b) Model Dependent Approach

The kinetics of drug release from the formulation can be understood by comparing drug release data either with theoretically proven kinetic mechanisms such as zero order, first order or with empirical equations (Hixson-Crowell, Weibull, Higuchi, Korsemeyer-Peppas, etc.) suggested by various scientist for variety of formulation based on single unit or multiple unit, film formation or based on shape of formulations (Ritger and Peppas, 1987b, a; Costa and Lobo, 2001; Siepmann and Peppas, 2001; Siepmann and Peppas, 2012). Therefore, data obtained in vitro dissolution study was fitted to different mathematical models to assess the drug release kinetics (zero order and first order model) and release mechanism (Higuchi and Korsemeyer-Peppas model). The cumulative percentage drug release data was fitted to the power law equation (equation 5.13) given by Korsemeyer et al.,(Korsmeyer et al., 1983) and Ritger and Peppas (Ritger and Peppas, 1987a; Siepmann and Peppas, 2012).

$$\frac{M_t}{M_\infty} = K_{kp} t^n \qquad (5.13)$$

Where, M_t/M_∞ is the percentage of drug released at any time t; K is release rate constant incorporating the structural and geometric characteristics of the polymeric system and drug; and n is the diffusion exponent indicative of the release mechanism of the drug. The value of n for a sphere is 0.43 for fickian release (diffusion controlled), $0.43 < n < 1.00$ for anomalous non-fickian release (diffusion and polymer relaxation), 1.00 for zero order release (relaxation and erosion) for non-swellable spherical controlled release systems (Ritger and Peppas, 1987a).

The values of the coefficient were calculated using linear regression analysis between log M_t/M_∞ and log t data obtained from drug release studies on MS Office Excel 2013 software. The value of n was obtained as slope of the regression equation, and K_{kp} was calculated as antilog of the intercept value. The $t_{80\%}$ (time required for 80% drug release) was calculated using equation 5.14.

$$t_{80\%} = \text{anti}\log\left\{\frac{\left(\log 90 - \log K_{kp}\right)}{n}\right\} \quad (5.14)$$

Further, the goodness of fit test for each drug release kinetic model was carried out by fitting data of amount of drug dissolved as a function of time in model equations. In addition, regression analysis was performed for model fitting between observed dissolution profile and theoretical model dissolution profile. The model with highest regression coefficient and lowest akaike information criteria (AIC) values was considered to be best fit model for obtained dissolution data.

5.2.3.13. Batch Reproducibility

To study batch reproducibility, batches of optimized formulation were manufactured in triplicates keeping similar processing conditions. Triplicate samples from each batch were evaluated for all quality parameters discussed earlier.

5.2.4. Stability Studies

To investigate the stability of optimized batches of pellets, formulations showing desired release and floating behavior were packed in cellophane packets placed in hermetically sealed vials under refrigerated temperature (FT: 5 ± 2°C), controlled room temperature (CRT: 25 ± 2°C/ 60 ± 5% RH) for 12 months and accelerated condition (AT: 40 ± 2°C/75 ± 5% RH) for 6 months as per ICH guidelines (ICH, 2003). Samples in triplicate were withdrawn from each batch at predetermined time intervals (0, 0.5, 1, 3 and 6 months for AT condition; 0, 1, 3, 6 and 12 months for CRT condition and FT condition). All the quality control tests were carried out on aged samples to assess stability of developed formulations. Drug content of aged formulations was determined using analytical method discussed in analytical methods section (Chapter 3). The results of quality control tests of aged samples were compared with zero time results. The percentage drug remaining to be degraded (% RTD) was plotted against time in order to calculate degradation rate constant (K_d) and $T_{90\%}$ value at different storage conditions for all the formulations.

5.3. Results and Discussion

5.3.1. Formulation Development Strategy

The literature review and preformulation studies, suggested that the modified release floating pellets could be better option for the efficient drug delivery of LD and CD combination for the treatment of PD. In current study, extrusion spheronization technique was used for the manufacture of the LD and CD loaded core pellets using MCC as spheronization aid and PVPK30 as binder. Further, modified release floating pellets was designed on the principle of gas formation technique. Two strategies were investigated to design modified release floating pellets viz. pellets designed by using single gas generating agent ($NaHCO_3$ alone) and floating pellets designed using two gas generating agents ($NaHCO_3$ and TA). The manufacturing process of modified release floating pellets is represented in Figure 5.1.

The modified release floating pellet designed using single effervescent layer consisted three coatings viz. protective/sustained release layer, effervescent layer and gas entrapped polymeric layer (Figure 5.2). When designed pellets were exposed to dissolution fluid, $NaHCO_3$ reacts with acidic dissolution medium and liberates carbon dioxide (CO_2) gas. The liberated CO_2 gas gets entrapped around the core of the pellets by flexible polymeric membrane, reducing the density of pellet (< 1.0 g/mL) and resulting in floating of pellets over the surface of dissolution medium. During initial formulation development, the color of core pellets turned blackish, upon direct application of $NaHCO_3$ coating probably due to degradation of LD and CD. This could be because of the basic pH (approximately 9.8) of the effervescent layer coating solution. LD and CD are not stable at basic pH (Nahata et al., 1999; Kankkunen et al., 2002). Therefore in this study, protective layer (HPMC or EC) was applied in order to avoid direct contact of drug loaded pellet surface with the gas forming agent present in coating solution of effervescent layer (Table 5.8). The pellets coated with protective and effervescent layers were assayed by using HPLC method (Chapter 3). Assay studies confirmed the absence of degradation of LD and CD in pellets coated with the protective layer. In gas forming layer solution, low viscosity grade HPMC E5 was used as a binder in order to increase stickiness of $NaHCO_3$ on the surface of pellets (Table 5.8).

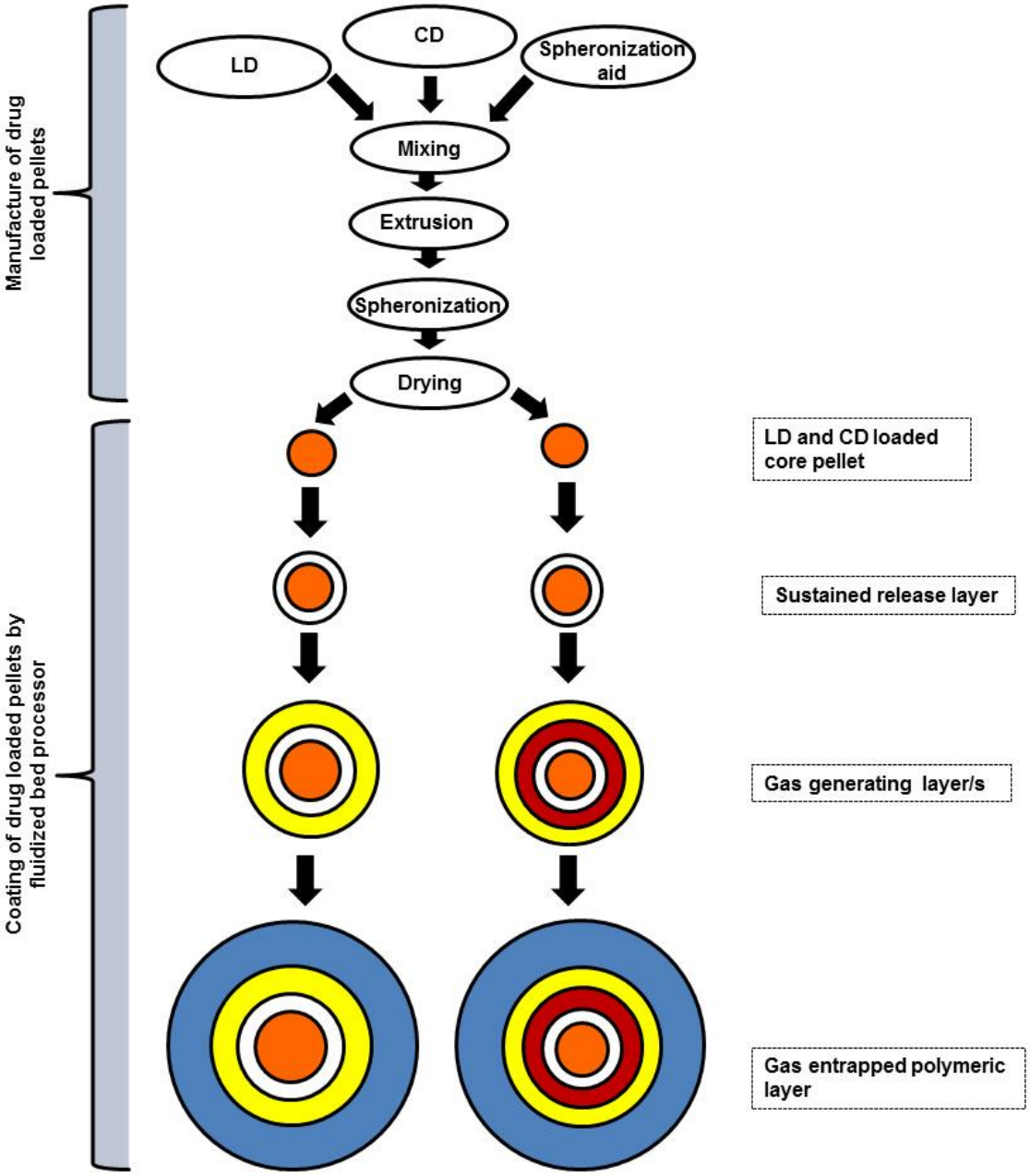


Figure 5.1. Manufacturing process of modified release floating pellets

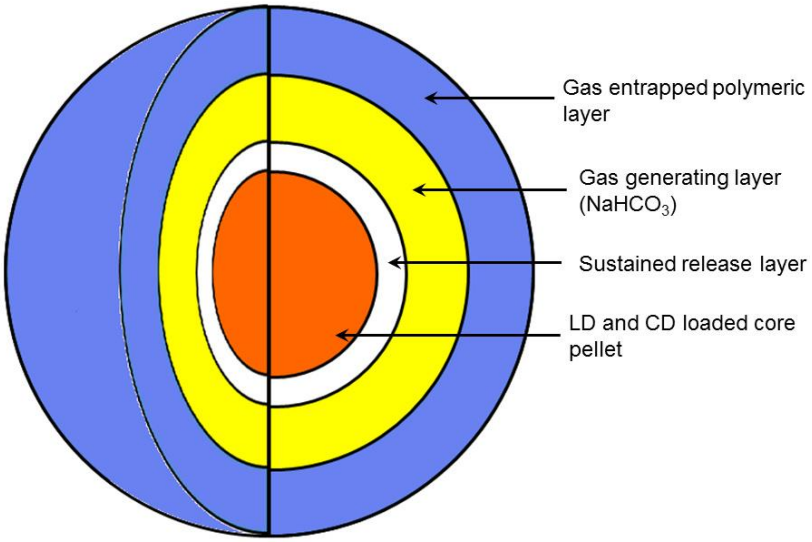


Figure 5.2. Representative design of modified release floating pellet manufactured using single gas generating agent ($NaHCO_3$)

In another technique, core pellets were coated with four successive layers viz. sustained release/protective layer, TA layer, $NaHCO_3$ layer and gas entrapped polymeric layer. Representative design of pellets coated with four successive layers is shown in Figure 5.3. Initially, core pellets were coated with TA layer thereby contact of $NaHCO_3$ with core pellet and resulting drug degradation was avoided. It was postulated that, permeation of dissolution medium through outermost membrane would results in evolution of CO_2 gas due to reaction between TA and $NaHCO_3$. The evolved gas would get entrapped around the core pellet due to flexible swollen polymeric membrane. This would results in the reduction of density (increased buoyancy) and hence would aid in floatation of pellets. A low viscosity grade HPC was used as a binder for coating of TA and $NaHCO_3$ layer on the surface of the core pellets (Table 5.8).

In vitro dissolution studies of initial formulation batches (HPMC/ERL/1 to HPMC/ERL/9 and ERL100/1 to ERL100/9) demonstrated that, gas entrapment polymer alone was not efficient to control drug release in case of the both techniques. Therefore, it was decided to apply sustained release layer in order to control drug release (Figure 5.2 and Figure 5.3).

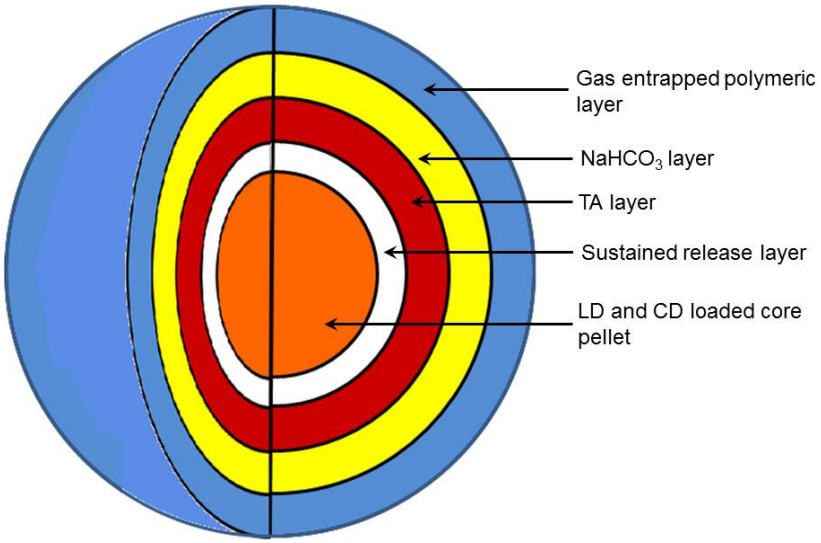


Figure 5.3. Representative design of modified release floating pellet manufactured using two gas generating agents ($NaHCO_3$ and TA).

5.3.2. Physical Characterization

5.3.2.1. Size Analysis

The results of distribution of size fraction based on sieve analysis is shown in Table 5.11. Variation in mean pellet diameter was not significant among the studied Avicel® grades. Core pellets obtained by extrusion spheronization process showed narrow size distribution and dominant size fraction was observed between 16 to 20 #. The narrow size distribution may be attributed to the fact that Avicel® acts as molecular sponge (Ek and Newton, 1998; Dukić-Ott et al., 2009) and suppresses the variation in pellet size induced by presence of LD and CD. According to sponge model, extrusion and subsequent spheronization properties of MCC is decided by the microfibers of it and the void spaces between them. The water present in pores of the fibers as well as water present in the void space provides adequate rheological properties to wet mass for smooth extrusion and spheronization to produce spherical pellets. LD and CD are small molecules present in the wet mass, they could well resides in these void spaces and fiber would be able to retain their own properties. Further, span was also calculated as it is a measure of the width (breadth) of a distribution. The observed lower span values (Table 5.11) further demonstrated narrow pellet size distribution.

5.3.2.2. Usable Yield (% Theoretical Yield)

For successful extrusion spheronization process and formulation, a high percentage of pellets should be produced within expected size range with sufficient mechanical robustness (Howard et al., 2006; Pund et al., 2011). Our findings demonstrated that the LD and CD loaded core pellets were manufactured with high usable yield ranging from 81.56 to 90.00% (Table 5.11). The observed high usable yield values (except for LC3) indicated that selected extrusion spheronization process parameters for manufacturing of core pellets were optimum at selected level of excipients. Formulation LC3 showed comparatively lower usable yield (46.00 %). This may be attributed to the lower compactibility and high density of Avicel® PH 302 compared to Avicel® PH 101, Avicel® PH 102 and Avicel® PH 105.

Table 5.11. Summary of Size Analysis Parameters and Usable Yield

Formulation code	Arithmetic mean diameter (mm)	Geometric mean diameter (d_g, mm)	Span	Usable yield (%)
LC1	1.015	1.014	0.673	84.23
LC2	1.015	1.007	0.672	81.56
LC3	1.300	0.805	0.616	46.00
LC4	1.015	1.010	0.674	90.00
LC5	1.015	1.008	0.683	87.23
LC6	1.015	1.011	0.687	86.23
LC7	1.015	1.010	0.680	87.56
LC8	1.015	1.002	0.667	89.00
LC9	1.015	1.011	0.686	88.34

5.3.2.3. Shape Analysis

The shape of pellets affects other properties such as flowability and coating performance. Therefore, circularity is important pellet characterization parameter. Current findings demonstrated that circularity parameters differed among different Avicel® grades. The results of shape parameters are summarized in Table 5.12. The observed results were in agreement with the reports published by (Koo and Heng, 2001; Pund et al., 2011). Formulations manufactured using Avicel® PH101 and Avicel® PH105 gave round pellets whereas Avicel® PH102 gave fairly round pellets. In contrast, Avicel® PH302 produced dumbbell shaped pellets. Variation in pellets roundness might be attributed to the density

difference of the Avicel® powders. Avicel® PH101 has lower bulk density and compactibility compared to the Avicel® PH102 and Avicel® PH302. Further, Avicel® PH102 has lower bulk density and compactibility compared to Avicel® PH302. Moreover, Avicel® PH105 has lowest density and highest compactibility among the all Avicel® grades studied. Further, Sinha et al (Sinha et al., 2005) observed good correlation between circularity values and log bulk density of the Avicel® powders. High density MCC grades exhibit low void volume and therefore are poorly compactibility. Thus, during extrusion spheronization process high density MCC grades are more resistance to deformation and spheronization even at high attrition and rounding forces (Koo and Heng, 2001; Pund et al., 2011). In contrast, low density MCC grades deform more quickly into spherical pellets at low rounding forces. Thus, formulations prepared using Avicel® PH101 (LC1, LC4 and LC6) and Avicel® PH105 (LC8 and LC9) showed low elongation and rectang values. It has already been observed that, roundness vales of pellets increases with increase in sphericity of pellets (Koo and Heng, 2001). In contrast elongation and rectang values decreases with increase in sphericity of the pellets (Koo and Heng, 2001). Lower values of elongation and rectang demonstrated Avicel® PH101 and Avicel® PH105 were satisfactory spheronization aid in terms of pellet properties for the selected drug candidates. The circularity value of 0.88 and above was accepted as round by Helle´n and coworkers (Hellén and Yliruusi, 1993; Hellén et al., 1993), while Wan and coworkers considered values above 0.93 (Wan et al., 1993). However, circularity values were found to be dependent on the numbers of pellets considered for evaluation (Podczeck et al., 1999). The observed values for circularity in case of formulations prepared with Avicel® PH101 and Avicel® PH105 were found to be in good agreement with previously reported values for sphericity. Among the different Avicel® grades studied, Avicel® PH101 and Avicel® PH105 were found to produce spherical core pellets with acceptable circularity values. Therefore, LD and CD loaded pellets produced using Avicel® PH101 and Avicel® PH105 as spheronization aid were used for further studies.

5.3.2.4. Flow Properties, Hausner's Ratio and Carr's Index

The quality of designed pellets depends on the physical properties of the pellets like size, shape and surface geometry etc. The flow properties of pellets were confirmed by

evaluating bulk density, tapped density and Hausner's ratio. The designed core pellets and modified release floating pellet formulations containing LD and CD showed very good flow properties. The results of flow properties, Hausner's ratio and Carr's index for core pellets are presented in Table 5.12 and Table 5.13. The results of flow and physical properties for coated pellets (after application of all coating layers) are presented in Table 5.14a, Table 5.14b and Table 5.14c. Bulk density and tapped density of the designed core pellets was found to be in the range of 0.80 to 0.83 g/mL and 0.83 to 0.86 g/mL, respectively whereas for coated pellets bulk and tapped densities were found to be in the range of 0.77 to 0.84 g/mL and 0.80 to 0.90 g/mL respectively. The observed values of Hausner's ratio were in the range of 1.01 to 1.13 which confirmed good flow properties of the designed pellets. In addition, Carr's index values were found to be ≤ 0.11 which demonstrated good compressibility of designed formulations. Angle of repose values were found to be ≤ 37.67 for uncoated core pellets formulations (Table 5.12) and ≤ 30.95 for coated formulations which indicated very good flowability of pellets (Table 5.14a, 5.14b and 5.14c). In addition, faster flow rate for designed core pellets, further demonstrated good roundness of the designed pellets.

5.3.2.5. Friability, Crushing Strength and Residual Moisture Content

Comparatively higher friability values were observed for the core pellet batch LC1 and LC2 compared to the batches from LC3 to LC9, which might be attributed to the absence of PVPK30 in LC1 and LC2. However, all formulations showed acceptable friability values (≤ 1%, w/w) (Table 5.13). Friability values observed for coated pellets from all batches were ≤ 0.38%, w/w which suggested that the coating parameters selected for coating process were optimum. Further, friability values of LC4 to LC7 and LC9 were comparatively lower than LC1 to LC4 due to use of PVPK30 as binder. In addition, mechanical crushing strength for core pellets was observed in the range of 9.56 to 15.33 N. Further, for coated pellets formulations mechanical crushing strength values were observed in the range of 13.04 to 17.20 N. The observed crushing strength values confirmed adequate mechanical strength of designed pellets for withstanding the stress generated during successive coating and further handling.

Table 5.12. Summary of Flow Properties, Shape Analysis Parameters of Various Batches of Core Pellets Manufactured by Extrusion Spheronization Technique

Formulation code	Flow properties		Shape analysis parameters		
	Angle of repose (°) (Mean ± SD) (n = 3)	Flow rate (g/s) (Mean ± SD) (n = 3)	Circularity (Mean ± SD) (n = 3)	Elongation (Mean ± SD) (n = 3)	Rectang (Mean ± SD) (n = 3)
LC1	28.80 ± 0.78	1.37 ± 0.10	0.933 ± 0.015	1.125± 0.131	0.824 ± 0.088
LC2	30.64 ± 2.63	1.37 ± 0.10	0.865 ± 0.075	1.278 ± 0.079	0.865± 0.033
LC3	37.67 ± 0.57	1.04 ± 0.06	0.629 ± 0.054	1.590 ± 0.079	0.785 ± 0.080
LC4	26.33 ± 1.11	1.59 ± 0.14	0.927 ±0.052	1.105 ± 0.048	0.802 ± 0.019
LC5	25.67 ± 1.01	1.70 ± 0.29	0.835± 0.098	1.128 ± 0.097	0.737 ± 0.085
LC6	29.45 ± 0.45	1.89 ± 0.19	0.874 ± 0.065	1.108 ± 0.128	0.758 ± 0.076
LC7	27.42 ± 0.92	1.78 ± 0.19	0.902 ± 0.037	1.114 ± 0.037	0.788 ± 0.029
LC8	29.88 ± 2.04	1.81 ± 0.33	0.926 ±0.052	1.112 ± 0.040	0.809 ± 0.018
LC9	28.80 ± 0.78	1.89 ± 0.19	0.887± 0.159	1.114 ± 0.168	0.762 ± 0.017

Table 5.13. Summary of Drug Content, Mechanical Strength, Hausner Ratio and Carr's Index of Various Batches of Core Pellets Manufactured by Extrusion Spheronization Technique

Formulation code	Mechanical strength		Hausner ratio (Mean ± SD) (n = 3)	Carr's index (Mean ± SD) (n = 3)	Assay (Mean ± SD) (n = 3)	
	Friability (%)	Crushing strength (N) (Mean ± SD) (n = 25)			LD	CD
LC1	0.40	10.91 ± 0.48	1.04 ± 0.01	0.037 ± 0.010	98.56 ± 1.56	97.23 ± 1.32
LC2	0.43	10.40 ± 0.72	1.02 ± 0.00	0.020 ± 0.004	97.56 ± 1.12	96.52 ± 1.89
LC3	0.47	9.56 ± 0.64	1.03 ± 0.01	0.028 ± 0.010	96.25 ± 1.65	97.59 ± 0.98
LC4	0.36	12.50 ± 0.51	1.03 ± 0.01	0.029 ± 0.006	98.25 ± 0.78	98.82 ± 1.24
LC5	0.28	13.42 ± 1.47	1.03 ± 0.00	0.026 ± 0.002	97.82 ± 0.96	96.52 ± 1.26
LC6	0.30	14.36 ± 0.60	1.02 ± 0.01	0.020 ± 0.004	99.52 ± 1.45	98.52 ± 1.01
LC7	0.26	14.73 ± 1.31	1.04 ± 0.01	0.037 ± 0.010	98.56 ± 1.86	97.45 ± 1.02
LC8	0.20	15.33 ± 0.52	1.04 ± 0.00	0.037 ± 0.009	99.01 ± 2.32	97.25 ± 1.03
LC9	0.25	15.23 ± 0.35	1.03 ± 0.01	0.025 ± 0.003	97.56 ± 1.23	98.12 ± 0.96

Table 5.14a. Angle of Repose, Hausner Ratio, Carr's Index, Friability, Crushing Strength and Assay for Designed Formulations (Coated Pellets)

Formulation code	Angle of repose (Mean ± SD) (n = 3)	Hausner ratio (Mean ± SD) (n = 3)	Carr's index (Mean ± SD) (n = 3)	Friability (%)	Crushing strength (N) (Mean ± SD) (n = 25)	Assay (Mean ± SD) (n = 3)	
						LD	CD
HPMC/ERL/1	30.07 ± 0.89	1.06 ± 0.05	0.055 ± 0.016	0.24	14.07 ± 1.00	96.45 ± 1.23	95.26 ± 1.23
HPMC/ERL/2	29.23 ± 0.84	1.03 ± 0.02	0.032 ± 0.016	0.28	15.44 ± 0.55	95.56 ± 0.85	95.86 ± 0.96
HPMC/ERL/3	28.10 ± 2.81	1.04 ± 0.00	0.035 ± 0.002	0.23	15.84 ± 0.42	97.23 ± 0.68	96.52 ± 1.85
HPMC/ERL/4	28.22 ± 1.67	1.05 ± 0.03	0.044 ± 0.008	0.26	13.73 ± 0.65	96.56 ± 1.28	95.56 ± 1.26
HPMC/ERL/5	26.84 ± 2.36	1.05 ± 0.01	0.045 ± 0.012	0.23	15.21 ± 1.09	95.26 ± 1.35	96.52 ± 1.28
HPMC/ERL/6	27.17 ± 1.06	1.05 ± 0.01	0.044 ± 0.009	0.22	15.89 ± 0.49	96.25 ± 1.85	97.53 ± 0.98
HPMC/ERL/7	27.35 ± 1.36	1.05 ± 0.02	0.043 ± 0.016	0.33	13.19 ± 0.97	97.58 ± 0.96	96.59 ± 0.99
HPMC/ERL/8	29.39 ± 1.08	1.06 ± 0.01	0.061 ± 0.011	0.20	14.73 ± 0.42	96.25 ± 0.91	97.56 ± 0.89
HPMC/ERL/9	30.38 ± 1.39	1.09 ± 0.01	0.083 ± 0.012	0.20	15.80 ± 0.42	97.58 ± 1.23	95.25 ± 1.79
EC/ERL/1	28.47 ± 1.06	1.06 ± 0.02	0.056 ± 0.019	0.24	16.45 ± 0.80	98.25 ± 1.11	97.45 ± 1.21
EC/ERL/2	28.33 ± 0.36	1.02 ± 0.00	0.020 ± 0.004	0.25	14.05 ± 0.89	95.89 ± 1.12	96.52 ± 1.21
EC/ERL/3	29.50 ± 0.39	1.06 ± 0.02	0.056 ± 0.015	0.20	14.72 ± 0.75	96.58 ± 1.11	97.58 ± 1.02
EC/ERL/4	29.12 ± 1.71	1.04 ± 0.01	0.043 ± 0.005	0.20	14.19 ± 0.84	97.85 ± 1.12	96.25 ± 1.23
EC/ERL/5	30.65 ± 0.88	1.05 ± 0.01	0.047 ± 0.007	0.22	15.44 ± 0.68	96.25 ±1.56	95.25 ± 1.25
EC/ERL/6	30.33 ± 0.32	1.05 ± 0.00	0.051 ± 0.003	0.25	17.20 ± 0.98	97.58 ± 1.93	96.12 ± 1.23
EC/ERS/1	29.60 ± 1.00	1.06 ± 0.01	0.059 ± 0.011	0.25	14.75 ± 0.70	97.58 ± 0.96	96.25 ± 1.85
EC/ERS/2	29.61 ± 1.44	1.08 ± 0.01	0.071 ± 0.007	0.33	14.43 ± 0.65	96.25 ± 1.28	95.25 ± 0.98
EC/ERS/3	30.95 ± 1.45	1.08 ± 0.01	0.078 ± 0.009	0.25	14.57 ± 1.61	96.12 ± 1.12	96.25 ± 1.21
EC/ENE/1	29.55 ± 0.33	1.07 ± 0.01	0.064 ± 0.008	0.25	15.21 ± 1.00	96.25 ± 0.92	97.85 ± 1.01
EC/ENE/2	28.77 ± 0.54	1.09 ± 0.00	0.085 ± 0.003	0.22	15.18 ± 1.06	97.23 ± 1.89	98.56 ± 1.01
EC/ENE/3	29.75 ± 0.82	1.09 ± 0.02	0.079 ± 0.014	0.18	15.00 ± 1.60	97.45 ± 0.78	97.58 ± 1.02

Table 5.14b. Angle of Repose, Hausner Ratio, Carr's Index, Friability, Crushing Strength and Assay for Designed Formulations (Coated Pellets) Cont…

Formulation code	Angle of repose (Mean ± SD) (n = 3)	Hausner ratio (Mean ± SD) (n = 3)	Carr's index (Mean ± SD) (n = 3)	Friability (%)	Crushing strength (N) (Mean ± SD) (n = 25)	Assay (Mean ± SD) (n = 3)	
						LD	CD
EC/ERLRS/1	29.95 ± 0.88	1.09 ± 0.01	0.081 ± 0.007	0.24	16.92 ± 1.46	95.26 ± 0.89	96.25 ± 1.02
EC/ERLRS/2	29.16 ± 1.09	1.07 ± 0.01	0.063 ± 0.009	0.24	15.05 ± 1.63	96.89 ± 0.91	95.62 ± 1.85
EC/ERLRS/3	30.07 ± 0.67	1.02 ± 0.00	0.020 ± 0.004	0.25	14.98 ± 0.60	97.56 ± 1.14	96.25 ± 1.23
EC/ERLRS/4	29.75 ± 0.82	1.07 ± 0.01	0.066 ± 0.007	0.25	15.37 ± 0.93	96.34 ± 1.12	96.25 ± 1.52
EC/ERLRS/5	28.21 ± 1.05	1.08 ± 0.01	0.074 ± 0.006	0.26	16.20 ± 1.03	97.58 ± 0.89	97.85 ± 1.36
EC/ERLRS/6	29.35 ± 1.19	1.07 ± 0.01	0.066 ± 0.013	0.36	14.49 ± 1.61	98.56 ± 1.23	97.58 ± 1.26
EC/ERLRS/7	29.60 ± 1.00	1.07 ± 0.01	0.061 ± 0.011	0.24	14.18 ± 1.18	97.25 ± 0.89	96.25 ± 1.32
EC/ERLRS/8	30.07 ± 0.67	1.07 ± 0.00	0.061 ± 0.002	0.24	14.29 ± 0.56	98.56 ± 1.35	97.58 ± 1.20
EC/ERLRS/9	30.33 ± 1.09	1.06 ± 0.01	0.054 ± 0.008	0.25	14.48 ± 1.62	96.25 ± 1.10	95.12 ± 1.02
HPMC/ENEPEG/1	29.86 ± 1.01	1.05 ± 0.00	0.046 ± 0.000	0.25	14.83 ± 0.55	96.12 ± 0.89	95.14 ± 1.63
HPMC/ENEPEG/2	28.77 ± 1.05	1.06 ± 0.01	0.059 ± 0.009	0.25	15.14 ± 0.98	97.45 ± 1.45	96.12 ± 1.85
HPMC/ENEPEG/3	30.33 ± 1.09	1.07 ± 0.01	0.061 ± 0.007	0.34	14.84 ± 0.55	96.85 ± 2.31	95.99 ± 0.96

Table 5.14c. Angle of Repose, Hausner Ratio, Carr's Index, Friability, Crushing Strength and Assay for Designed Formulations (Coated Pellets)

Formulation code	Angle of repose (Mean ± SD) (n = 3)	Hausner ratio (Mean ± SD) (n = 3)	Carr's index (Mean ± SD) (n = 3)	Friability (%)	Crushing strength (N) (Mean ± SD) (n = 25)	Assay (Mean ± SD) (n = 3)	
						LD	CD
ERL100/1	30.24 ± 0.93	1.06 ± 0.01	0.054 ± 0.007	0.35	13.04 ± 0.89	96.72 ± 1.19	97.58 ± 1.23
ERL100/2	29.56 ± 1.06	1.08 ± 0.03	0.077 ± 0.023	0.38	15.38 ± 0.96	97.24 ± 1.93	97.56 ± 1.22
ERL100/3	30.43 ± 0.69	1.06 ± 0.01	0.061 ± 0.009	0.17	15.44 ± 0.68	96.56 ± 0.97	96.23 ± 1.52
ERL100/4	30.21 ± 0.72	1.06 ± 0.01	0.060 ± 0.008	0.38	16.28 ± 0.24	97.12 ± 0.89	97.56 ± 1.26
ERL100/5	29.82 ± 1.08	1.09 ± 0.01	0.081 ± 0.007	0.38	16.05 ± 0.34	96.19 ± 1.12	96.25 ± 1.23
ERL100/6	28.45 ± 1.08	1.08 ± 0.01	0.073 ± 0.008	0.27	15.78 ± 0.73	97.54 ± 0.89	96.25 ± 1.52
ERL100/7	30.53 ± 0.54	1.08 ± 0.02	0.072 ± 0.016	0.27	13.12 ± 1.60	97.25 ± 0.89	97.45 ± 1.02
ERL100/8	26.08 ± 1.11	1.10 ± 0.01	0.091 ± 0.005	0.35	13.95 ± 0.66	96.12 ± 1.11	96.25 ± 1.32
ERL100/9	23.97 ± 1.07	1.08 ± 0.01	0.073 ± 0.011	0.35	16.55 ± 0.58	97.75 ± 1.89	95.98 ± 0.96
EC/ERL100/1	26.76 ± 1.05	1.08 ± 0.01	0.075 ± 0.007	0.25	15.27 ± 0.97	97.96 ± 1.03	96.23 ± 1.96
EC/ERL100/2	29.60 ± 1.00	1.08 ± 0.01	0.075 ± 0.007	0.24	16.15 ± 0.80	95.25 ± 1.89	97.58 ± 0.23
EC/ERL100/3	28.20 ± 1.07	1.07 ± 0.01	0.063 ± 0.013	0.21	16.45 ± 0.41	96.92 ± 1.21	95.96 ± 0.96
KSR/ERL100/1	28.45 ± 1.08	1.09 ± 0.02	0.084 ± 0.018	0.24	16.47 ± 1.07	97.75 ± 0.89	97.58 ± 0.63
KSR/ERL100/2	30.34 ± 0.86	1.02 ± 0.00	0.020 ± 0.004	0.20	15.91 ± 1.49	97.32 ± 1.01	98.56 ± 0.98
KSR/ERL100/3	27.02 ± 1.07	1.08 ± 0.01	0.077 ± 0.007	0.17	14.88 ± 0.56	97.45 ± 1.35	99.56 ± 1.23
EC/ERL100P/1	26.82 ±1.23	1.06 ± 0.02	0.062 ± 0.007	0.20	15.23 ± 1.56	96.25 ± 1.23	96.25 ± 1.03
EC/ERL100P/2	27.12±1.63	1.07 ± 0.01	0.061 ± 0.006	0.24	16.23 ± 1.24	95.25 ± 1.23	97.56 ± 1.03
EC/ERL100P/3	29.03±1.52	1.04 ± 0.02	0.043 ± 0.002	0.25	14.21 ± 1.02	97.56 ± 1.20	96.23 ± 1.02
LC6/EC/ERL100	28.59±1.23	1.06 ± 1.08	0.061 ± 0.003	0.26	15.23 ± 1.20	96.52 ± 1.20	96.42 ± 1.23
LC9/EC/ERL100	27.45±1.25	1.08 ± 0.01	0.074 ± 0.004	0.20	14.53 ± 1.63	97.56 ± 1.02	96.25 ± 1.20

Moreover, the residual moisture in case of all batches of core pellets and coated pellets was found to be ≤ 1.02% which indicated that the selected condition for drying of core pellets and coated pellets were optimum.

5.3.2.6.Active Agent Content

Designed core pellets contained LD and CD in the range of 96.25 to 99.52 % and 96.52 to 98.82% respectively which indicated that the uniform mixing was achieved during the formulation of core pellets (Table 5.13). Protective layer was used to protect LD and CD present in the core pellets from $NaHCO_3$. Therefore, drug content was estimated in uncoated (Table 5.13) as well as coated pellets (Table 5.14a, 5.14b and 5.14c). The assay values of coated and uncoated pellets did not differ significantly which indicated stability of LD and CD during manufacturing process. In addition, absence of degradation product peak in the chromatograms of coated and uncoated pellets further confirmed the stability of both the drugs.

Pellets produced using Avicel® PH101 and Avicel® PH105 showed good sphericity, sufficient mechanical strength and low residual moisture and high drug loading. Therefore, Avicel® PH101 was used for the formulation development and optimization. Moreover, impact of Avicel® PH101 and Avicel® PH105 on in vitro release and floating properties of optimized formulation was also evaluated.

5.3.3. Core Pellet

The in vitro release profiles of LD and CD from the pellets prepared using different MCC (Avicel®) grade are represented in Figure 5.4a and Figure 5.4b, respectively. From uncoated pellet formulations, LD as well as CD were found to be released immediately (drug release > 80% within 1 h). Faster release from core pellet can be attributed to the hydrophilic nature of the spheronization aid used for core pellet manufacturing. Pellets manufactured using Avicel® PH105 showed little slower drug release which may attributed to the lower particle size and porosity of the Avicel® PH105. Previous reports suggested, that pellets manufactured using different Avicel grades showed different release rate (Alvarez et al., 2002; Sinha et al., 2005).

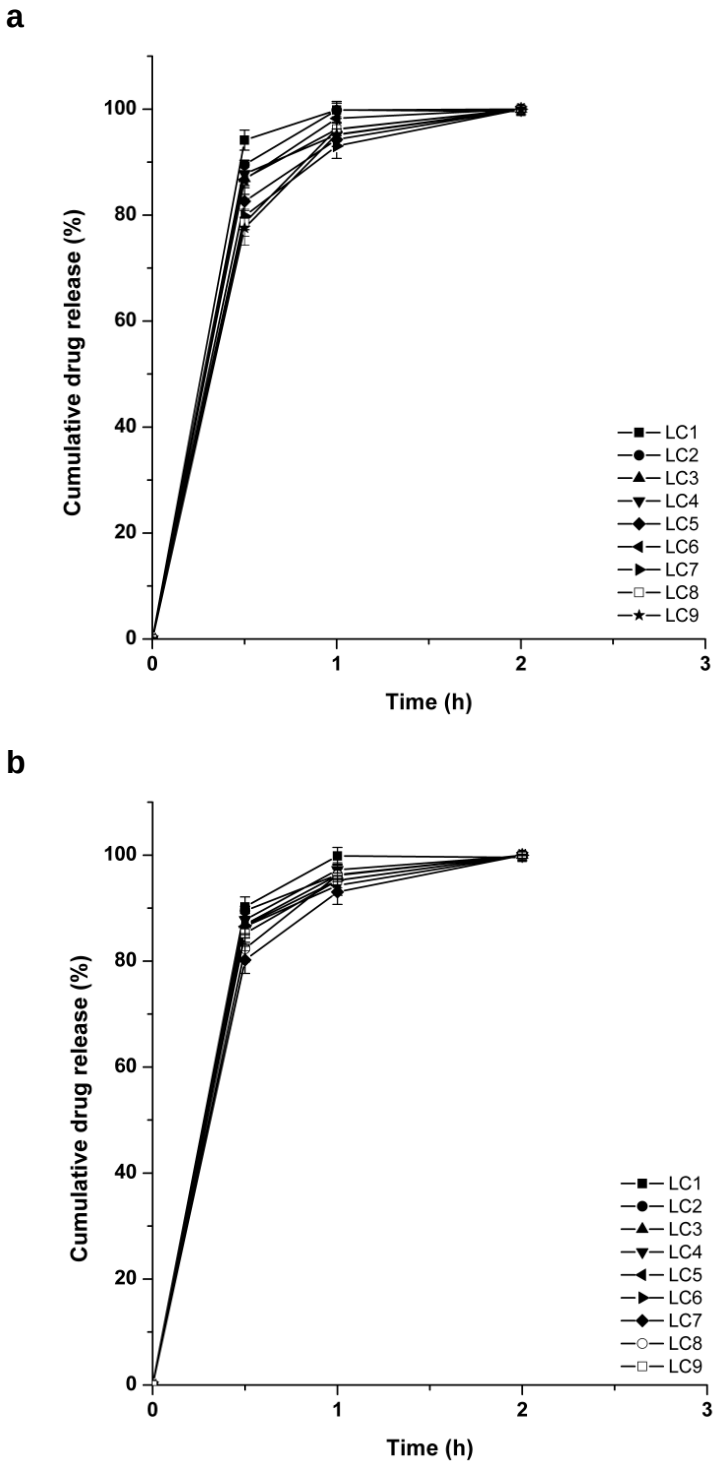


Figure 5.4. In vitro release profiles of (a) LD and (b) CD from uncoated core pellets in 0.1N HCl. (Each value represents mean of three independent determinations with standard deviation).

Sinha and coworkers, concluded that among various factors, porosity and particle size of the MCC could be responsible for different release rate (Sinha et al., 2005). However, in present study similarity in dissolution profiles of LD (Figure 5.4a) and CD (Figure 5.4b) demonstrated insignificant impact of studied Avicel® grades on the drug release. Similarity in release profiles of LD and CD can be attributed to the hydrophilic nature of the drugs and use of lower amount of MCC as spheronization aid. Results observed in current study were different than the previous report (Sinha et al., 2005). This could be because of the fact that the amount of Avicel® grades and physicochemical properties of drugs used in present study were different than the previous reports (Sinha et al., 2005). Further, use of PVPK30 also did not show any influence on drug release. This could be because of lower amount used and hydrophilic nature of PVPK30.

5.3.4. Pellets Coated with Sodium Bicarbonate as Gas Generating Layer

5.3.4.1. Optimization of Floating Properties

Designed system was expected to float in few minutes after getting into contact with the gastric fluid to avoid transfer into the small intestine along with food. (Iannuccelli et al., 1998). The composition of effervescent layer and level of gas entrapped polymeric membrane plays a key role in floating of the pellets. Therefore, composition of effervescent layer and coating level of gas entrapped polymeric membrane were optimized by design of experiments. Floating lag time was considered as the time taken by pellets to start floating. Not only time to float but percentage of pellets floating for longer duration also plays important role in the success of designed formulation. Gastric emptying time ranges from 15 min to 3 h depending on gastric content, thus floating system should float within 15 min (Hung et al., 2014). Based on these considerations, the constraint for floating lag time was considered as $Y_1 < 10$ min. Further, literature suggested that, formulation should float more than 20 h (Iannuccelli et al., 1998; Sungthongjeen et al., 2006). Therefore, another constraints for floating pellets (%) were considered as $Y_2 > 85\%$ floating pellets at 20 h. In all formulations, coating level of effervescent layer was fixed at 12%, w/w. Increase in effervescent layer coating level up to 14%, w/w did not show significant variation in the floating lag time and pellets floating at 20 h (%). Therefore, composition of HPMC and $NaHCO_3$ in effervescent layer was optimized at constant coating level of 12%, w/w.

Among the different dependent variables, floating lag time (Y_1) and floating pellets at 20 h (%) (Y_2) were selected as most representative dependent variables (Table 5.2). The constraints used for the responses were: $Y_1 < 10$ min and $Y_2 > 85\%$. The experimental runs, observed floating lag time and pellets floating at 20 h (%) are summarized in Table 5.3. In order to investigate the effect of independent variables on the dependent variables, mathematical relationship was generated between them by using statistical software Design Expert® (version 8.0.7.1, Stat-Ease Inc., Minneapolis, MN, USA). Least square second - order polynomial regression equations obtained for floating lag time (Y_1) and pellets floating at 20 h (%) (Y_2) are represented in Table 5.15. These equations, demonstrated the quantitative effect of independent variables (A and B) on the floating lag time and pellets floating at 20 h (%). The effect of independent variables on each response is shown in three dimensional response surface plots (Figure 5.5).

Interaction between factors was suggested by coefficient with more than one factor whereas coefficients with second order represent quadratic nature. The obtained Prob > F values less than 0.05 suggested significant influence of independent variable on the response. Factors A (< 0.0001), B (< 0.0001), AB (< 0.0001), and A^2 (< 0.0001) influence floating lag time while A (< 0.0001), B (0.0002) and A^2 (0.0065) influence pellets floating at 20 h (%) significantly (Table 5.15). The values of A and B were substituted in respective equation to estimate predicted values of Y_1 and Y_2. Predicted values were found to be in good agreement with observed values demonstrating validity of obtained equations for Y_1 and Y_2 (Table 5.16) (Figure 5.6). In addition, analysis of variance (ANOVA) test confirmed that selected regression models were significant (5% level of significance) and valid for each considered response (F-value for $Y_1 = 844.34$ and $Y_2 = 255.15$) (Table 5.15).

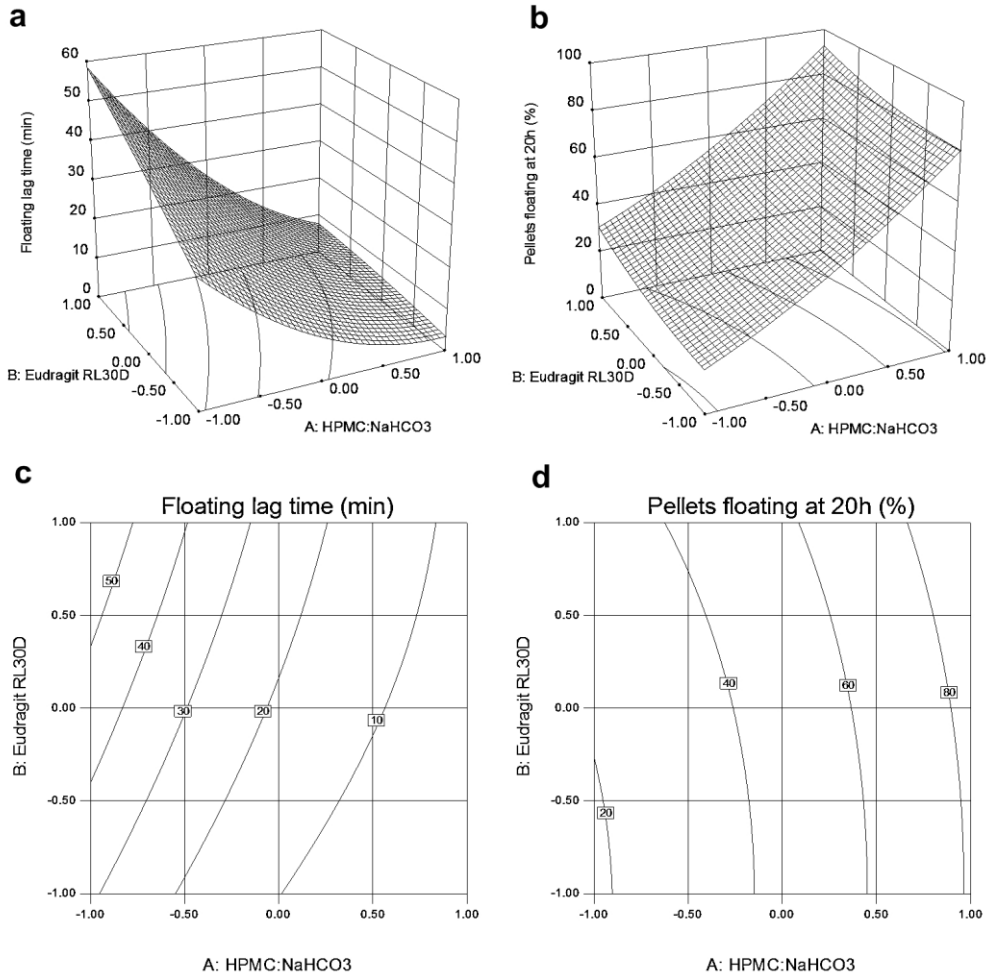


Figure 5.5. Response surface plots for (a) Y_1 response (floating lag time, min); (b) Y_2 response [pellets floating at 20 h (%)]; (c) contour plots for Y_1 response and (d) Y_2 response.

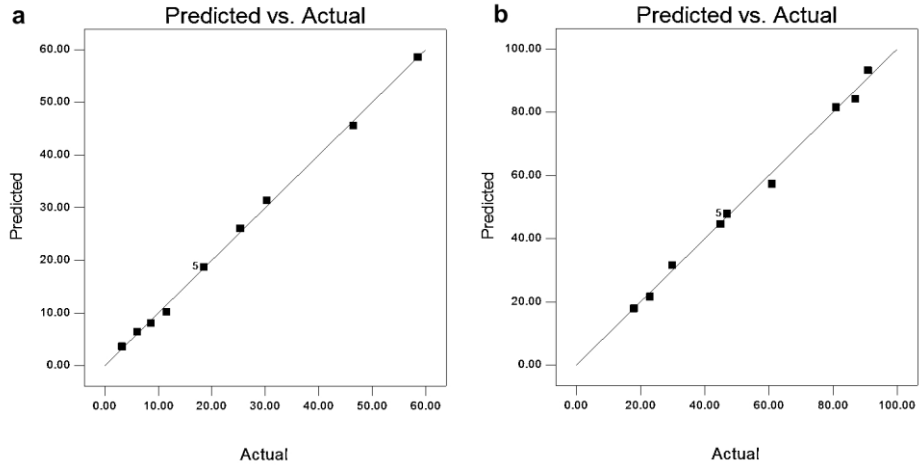


Figure 5.6. Correlation between actual and predicted values for (a) floating lag time (min) and (b) pellets floating at 20 h (%)

Table 5.15. Summary of Experimental Design Data

Parameter		Floating lag time (min)	Pellets floating at 20 h (%)
Least - square second - order polynomial equation		$Y_1 = 18.71 - 19.57A + 7.91B - 5.70AB + 7.27A^2 - 0.60B^2$	$Y_2 = 47.83 + 31.33A + 6.33B - 0.50AB + 5.10A^2 + 3.10B^2$
Predicted R		0.9839	0.9577
Adjusted R		0.9972	0.9906
F - value[a]		844.34	255.15
Prob > F[b]	A	<0.0001	<0.0001
	B	<0.0001	0.0002
	AB	<0.0001	0.6660
	A^2	<0.0001	0.0065
	B^2	0.2735	0.0531

[a]Calculated using Fisher test with ANOVA at 5% level of significance.
[b]Prob > F values < 0.05 indicates that factors in the model have a significant effect on the response.

Table 5.16. Predicted and Observed Responses of Optimum Formulation

Response	Predicted	Observed (Mean ± SD) (n = 3)	Constraints
Y_1 (Floating lag time, min)	8.02	7.54 ± 0.81	Y_1 < 10 min
Y_2 (Pellets floating at 20 h, %)	93.19	92.25 ± 1.50	Y_2 > 85%

Inverse relationship was observed between amount of $NaHCO_3$ and floating lag time. In addition, level of coating of Eudragit® RL30D also influences the floating lag time. Low coating level of Eudragit® RL30D reduces the floating lag time due to immediate hydration of thin layer and faster permeation of the dissolution fluid through coating layer. The time required for pellet to float was found to be minimum in case of formulations coated with effervescent layer (HPMC:$NaHCO_3$ =1:4, weight gain 12%, w/w) and Eudragit® RL30D (weight gain 5%, w/w) compared to other formulations. Further, pellets floating at 20 h (%) were found to be directly related with amount of $NaHCO_3$ as well as with coating level of Eudragit® RL30D. The percentage of pellets floating over dissolution media at 20 h were 81.00 ± 3.61%, 85.00 ± 1.00% and 91.00 ± 2.00% for formulations HPMC/ERL/7, HPMC/ERL/8 and HPMC/ERL/9, respectively (Figure 5.7).

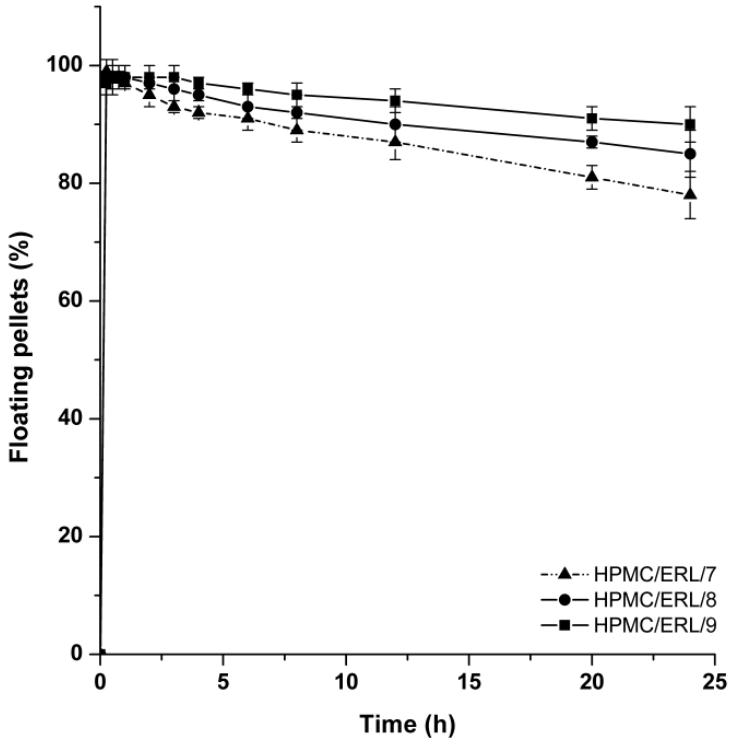


Figure 5.7. Effect of Eudragit® RL30D coating levels on in vitro floating behavior of pellets in 0.1N HCl (Each value represents mean of three independent determinations with standard deviation)

The maximum % of floating pellets (HPMC/ERL/9) was observed due to the higher thickness and impermeability of the Eudragit® RL30D membranes for generated CO_2 gas. Formulations (HPMC/ERL/4, HPMC/ERL/5) exhibit comparatively longer time to float and less than 50% pellets were found to be floating at 20 h. This might be because of the presence of less amount of $NaHCO_3$ in effervescent layer which generate relatively less amount of CO_2. Moreover, 10%, w/w coating level of Eudragit® RL30D was not sufficient to entrap generated CO_2 for longer duration.

The best area for formulation to obtain expected responses was found from contour plots (Figure 5.5c and Figure 5.5d). Therefore the optimum conditions to obtain desired responses (Y_1 < 10 min and Y_2 > 85% floating pellets) corresponded to formulation HPMC/ERL/8 (HPMC:$NaHCO_3$ = 1:4 and Eudragit® RL30D coating level of 15%, w/w) and HPMC/ERL/9 (HPMC:$NaHCO_3$ = 1:4 and Eudragit® RL30D coating level of 20%, w/w). Although both formulations HPMC/ERL/8 and HPMC/ERL/9 showed floating lag time less than 10 min, formulation HPMC/ERL/9 was preferred as it demonstrated maximum percentage of floating pellets at 20 h (91.00 ± 2.00%) (Figure 5.7). Therefore, HPMC/ERL/9 was considered to validate model. Predicted responses were obtained by substituting amounts of A and B of optimized formulations in respective equation for Y_1 and Y_2. A new batch of pellets with predicted levels was manufactured in order to validate the optimization procedure. Selected optimization design was found to be valid since observed results were found to be close to the predicted values and within the range of selected constraints (Table 5.16). Therefore, HPMC:$NaHCO_3$ ratio of 1:4 (12%, w/w coating level) and 20%, w/w of Eudragit® RL30D (HPMC/ERL/9) were considered as optimum composition of effervescent layer and coating level of gas entrapped polymeric layer, respectively for further studies.

5.3.4.2. In Vitro Drug Release

In vitro drug release studies were performed for the coated as well as uncoated formulations (Section 5.3.3). LD and CD exhibit similar drug release profiles in case of all studied formulations because of their similar solubility profiles in dissolution medium. Moreover, LD and CD exhibit almost similar molecular weights (LD = 197.18 g/mol; CD = 226.22 g/mol) which could also be one of the reason for the similar drug release. Therefore,

parameters influencing the drug release from designed formulations are discussed with respect to the release of LD. Further, release profiles of LD and CD were also compared for optimized formulations.

Release profiles of formulation HPMC/ERL/1 to HPMC/ERL/9 are shown in Figure 5.8. Formulation HPMC/ERL/1, HPMC/ERL/4 and HPMC/ERL/7 showed faster drug release when compared to formulation HPMC/ERL/2, HPMC/ERL/5 and HPMC/ERL/8.

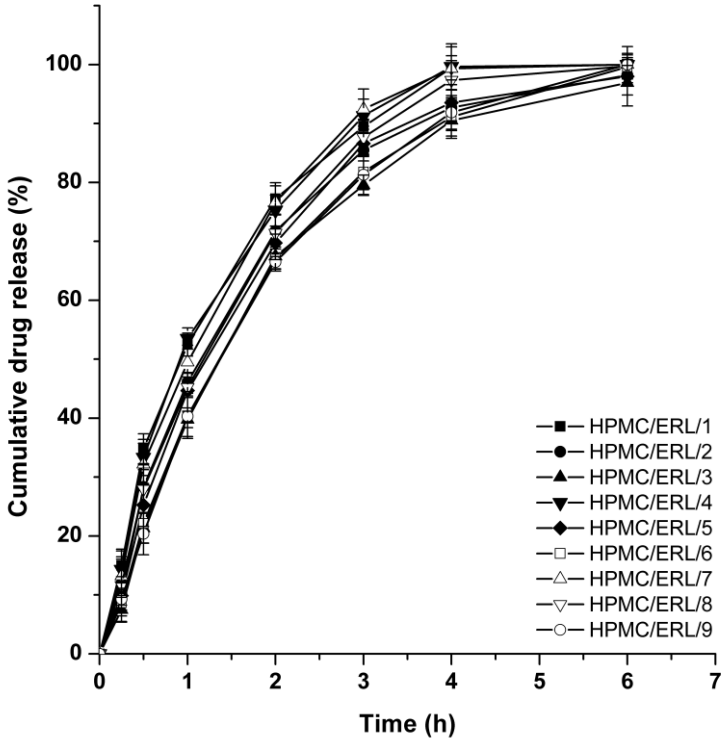


Figure 5.8. Effect of different compositions of HPMC:$NaHCO_3$ (1:4, at fixed weight gain of 12 %,w/w) and different coating levels of Eudragit® RL30D on in vitro release of LD in 0.1N HCl. (Each value represents mean of three independent determinations with standard deviation).

Further, in comparison to formulation coated with 15%, w/w of Eudragit® RL30D (HPMC/ERL/2, HPMC/ERL/5 and HPMC/ERL/8), formulations coated with 20%, w/w of Eudragit® RL30D (HPMC/ERL/3, HPMC/ERL/6 and HPMC/ ERL/9) showed retarded drug release. This could be because of the increase in Eudragit® RL30D coating layer thickness. In addition, swollen Eudragit® RL30D film could also be responsible for the

slower drug release due to increase in diffusion path length. However, statistically significant difference in release profiles of LD was not observed among all formulations coated with HPMC as a protective layer (HPMC/ERL/1 to HPMC/ERL/9).

In addition, observed MDT and $t_{80\%}$ values for HPMC/ERL/1 to HPMC/ERL/9 were in the range of 1.28 to 1.74 h and 1.58 to 1.97 h respectively (Table 5.17a) which further demonstrated similarity in drug release at different coating levels of Eudragit® RL30D.

In order to maintain constant plasma drug concentration of LD, formulation should release drug at controlled rate for longer time. Eudragit® RL30D alone was not able to sustain LD release as formulations HPMC/ERL/1 to HPMC/ERL/9 showed more than 80% of LD release within 2 to 3 h. Therefore, it was decided to replace HPMC layer by EC layer as a protective coating which would also act as additional barrier for LD release along with Eudragit® RL30D. EC is water insoluble polymer mostly used in reservoir type sustained release formulations (Rao et al., 2011; Songsurang et al., 2011).

The effect of different EC and Eudragit® RL30D coating levels on the drug release from pellets was investigated. In EC layer, povidone (PVPK30) was used as pore forming agent in the ratio (EC:PVPK30 = 3.5:1.5). EC coating showed higher impact on the drug release from the pellet compared to HPMC coating. The release profiles of LD from pellets (Formulation EC/ERL/1 to EC/ERL/4) coated with different coating levels of ethyl cellulose and at fixed coating levels of effervescent layer and Eudragit® RL30D are shown in Figure 5.9. Pellets coated with ethyl cellulose as a protective layer showed sustained release of LD. Although pore forming agent was present in the EC coating, drug release was found to be retarded with increase in the coating level of EC layer. Pellets coated with the 6%, w/w of EC layer (EC/ERL/4) showed very slow release (approx. 30% at 24 h) compared to the formulation coated with 3.0%, w/w (EC/ERL/2) and 2%, w/w of EC (EC/ERL/1). The MDT and $t_{80\%}$ values increased from 5.23 to 12.67 h and 5.70 to 69.99 h respectively with increase in EC coating from 2%, w/w to 6%, w/w (Table 5.17a). This could be because of increase in the EC film thickness and diffusion path length (Yang et al., 2014). In addition, approximate zero order release pattern and absence of longer a lag time phase in drug release irrespective of coating level can be attributed to the use of intense hydrophilic PVPK30 as a pore former in coating layers (Sinchaipanid et al., 2004; Yang et al., 2014). Moreover, drug release could be controlled by diffusion mechanism

simultaneously through the water-filled pores or cracks and intact macromolecular networks present in the EC polymer films (Muschert et al., 2009).

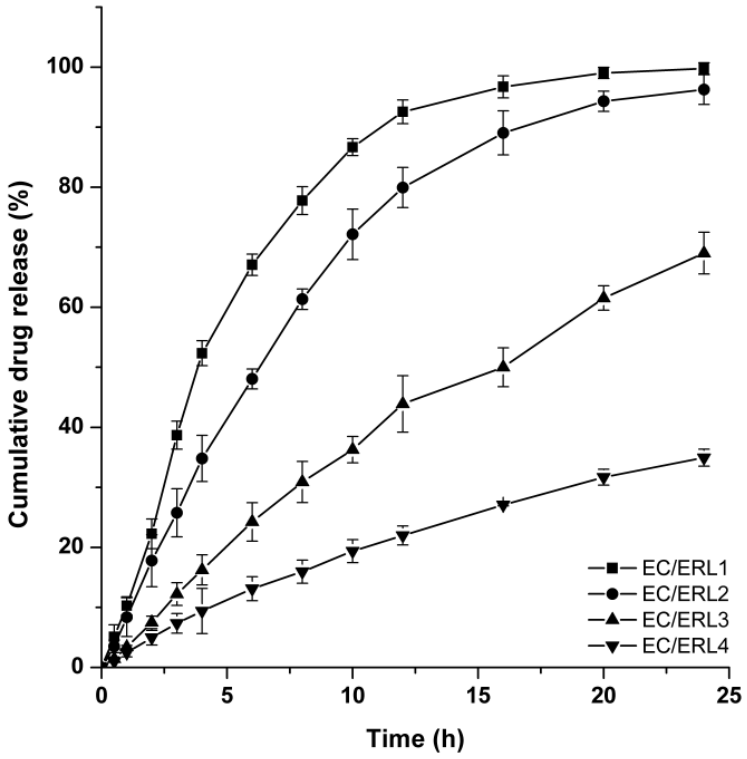


Figure 5.9. Effect of different coating levels of EC on release of LD in 0.1N HCl (Each value represents mean of three independent determinations with standard deviation).

In vitro floating behavior of the pellets (EC/ERL/1 to EC/ERL/4) is shown in Figure 5.10. All formulation (EC/ERL/1 to EC/ERL/4) showed almost similar floating lag time and pellets floating (%) irrespective of different coating levels of EC. This could be because of similar coating level of effervescent layer (HPMC:$NaHCO_3$ = 1:4) and gas entrapped polymeric layer of Eudragit® RL30D. The coating level of EC was considered optimum at 3.0%, w/w, as formulation EC/ERL/2 releases LD over a period of 10 -12 h ($t_{80\%}$ = 10.14 h) at a controlled rate when compared to formulation EC/ERL/1 ($t_{80\%}$ = 5.70 h), EC/ERL/3 ($t_{80\%}$ = 26.24 h) and EC/ERL/4 ($t_{80\%}$ = 69.99 h).

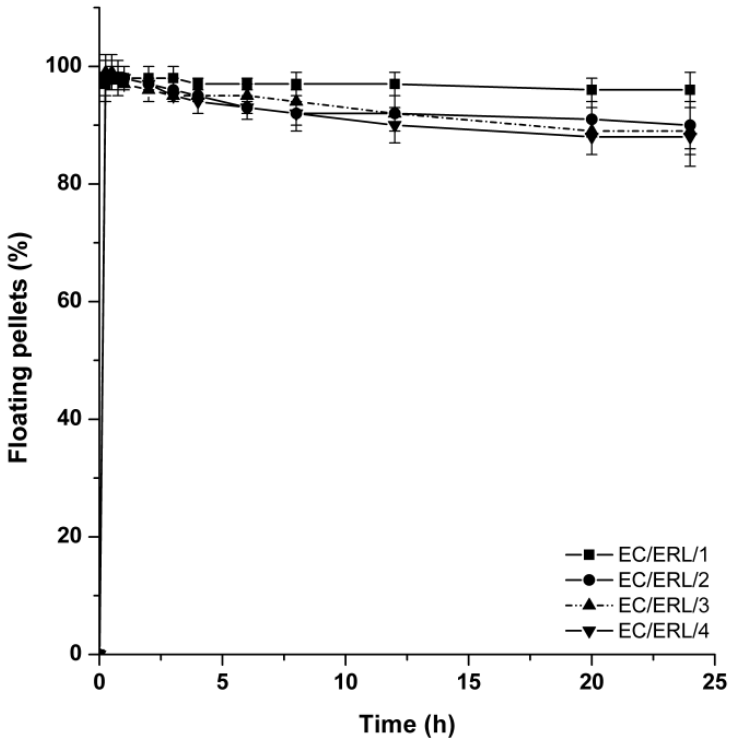


Figure 5.10. In vitro floating behavior of pellets coated at different coating levels of EC in 0.1N HCl (Each value represents mean of three independent determinations with standard deviation).

The effect of the effervescent layer on the release of LD was studied by comparing in vitro release profile of the formulation EC/ERL/2 and EC/ERL/5. The estimated similarity factors (f_2 = 62.33) confirmed that effervescent layer have negligible impact on the release profile of LD (Figure 5.11).

Further, effect of Eudragit® RL30D coating on the drug release was also investigated by comparing release profiles of formulation EC/ERL/2 and EC/ERL/6. Figure 5.12 shows the influence of gas entrapped polymeric membrane and effervescent layer on in vitro release of LD. Both formulations showed sustained release of LD due to presence of EC coating. Further, formulation EC/ERL/6 showed faster LD release compared to formulation EC/ERL/2 which may be attributed to the presence of thick swollen outer membrane formed by Eudragit® RL30D in case of formulation EC/ERL/2.

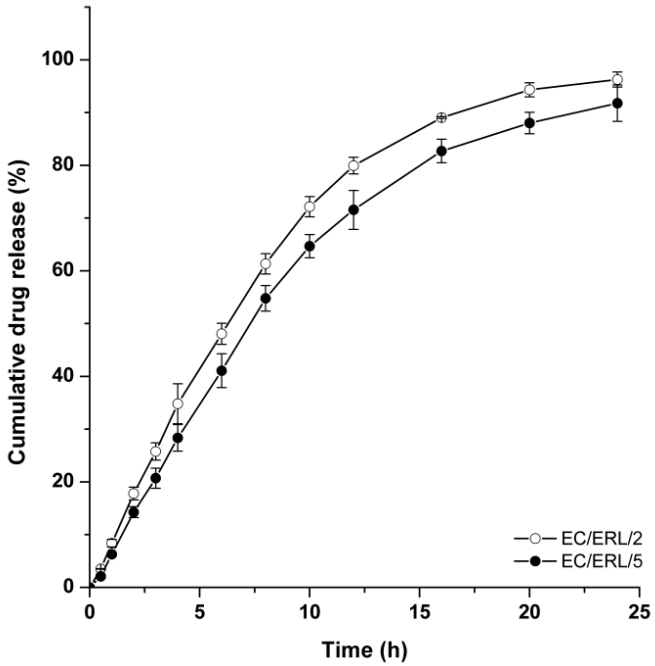


Figure 5.11. Effect of effervescent layer on in vitro release of LD in 0.1N HCl. (Each value represents mean of three independent determinations with standard deviation).

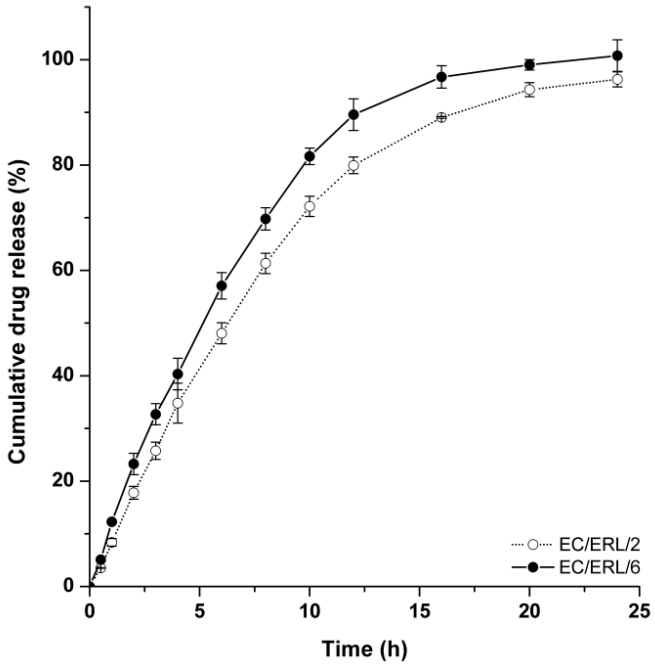


Figure 5.12. Effect of gas entrapment polymeric film and effervescent layer on in vitro release of LD in 0.1N HCl. (Each value represents mean of three independent determinations with standard deviation).

Formulation EC/ERL/2 showed similar LD and CD release profiles (f_2 = 70.38) (Figure 5.13) which suggested that coating levels of EC and gas entrapped polymeric membrane had equal impact on the release of LD and CD. Moreover, formulation EC/ERL/2 showed less floating lag time (7.16 ± 1.65 min) and more than 85% pellets were found to be floating at 20 h (90.00 ± 3.00%) (Figure 5.10). Therefore, this formulation was considered for further evaluation.

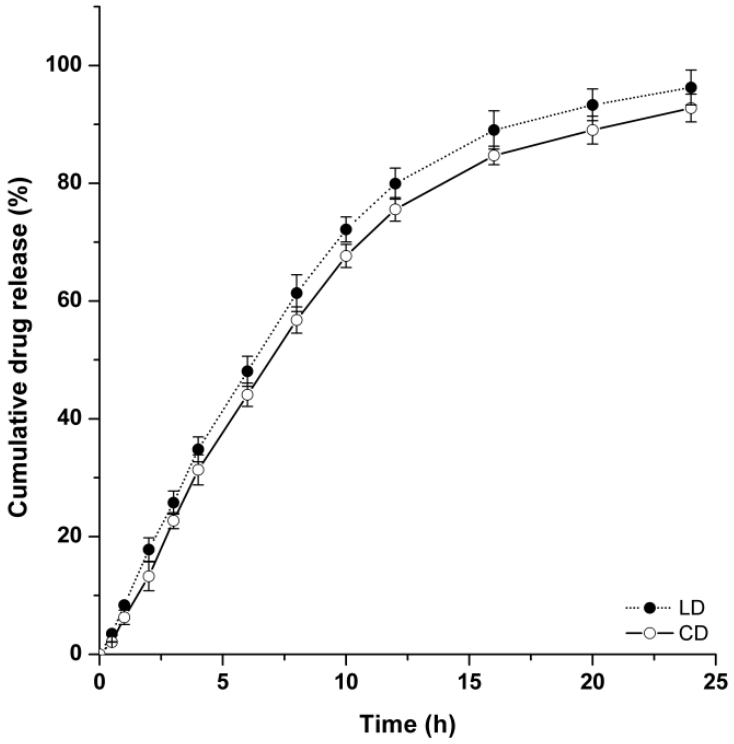


Figure 5.13. In vitro release profiles of LD and CD in 0.1N HCl from formulation EC/ERL/2. (Each value represents mean of three independent determinations with standard deviation).

The effect of different coating levels of Eudragit® RS30D and Eudragit® NE30D polymer (as gas entrapment polymer layer) alone or in presence of pore formers on in vitro release and floating properties was also studied. These studies were carried out at constant coating levels of sustained release layer and effervescent layer at constant composition of HPMC and $NaHCO_3$ (1:4, w/w).

5.3.4.3. Effect of Eudragit® RS30D and Eudragit® NE30D

Eudragit® RS30D was also studied as gas entrapment polymeric membrane at different coating levels (5%, 10% and 15%, w/w). In vitro LD release profiles from pellets coated with Eudragit® RS30D (EC/ERS/1 to EC/ERS/3) are represented in Figure 5.14. LD release retarded with increasing coating level of Eudragit® RS30D. MDT values increased with increase in coating level of polymer. Unlike Eudragit® RL30D, higher MDT values demonstrated that the release of LD was influenced largely by Eudragit® RS30D. The MDT for pellets coated at 5%, 10% and 15%, w/w with Eudragit® RS30D were found to be 9.46, 10.36 and 10.96 h, respectively (Table 5.17a). In addition, time required to release 80% of LD was found to be 15.37, 21.12 and 31.67 h for pellets coated at 5%, 10% and 15%, w/w coating level respectively (Table 5.17a) which further demonstrated retardation of LD release with the increase in coating level of Eudragit® RS30D. Retardation of LD release was attributed to slow permeation of LD through the hydrophobic EC and Eudragit® RS30D film.

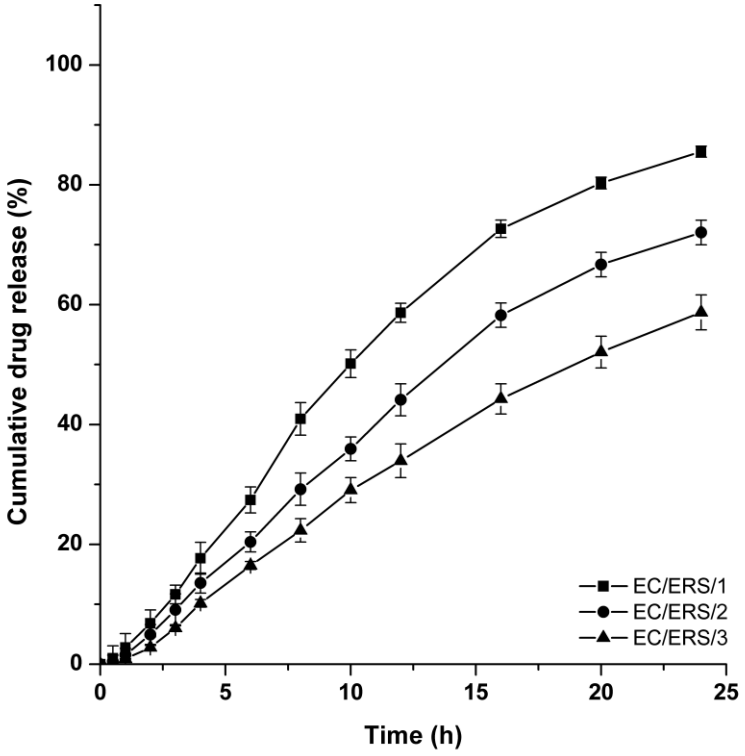


Figure 5.14. Effect of different coating levels of Eudragit® RS30D on in vitro release of LD in 0.1N HCl. (Each value represents mean of three independent determinations with standard deviation).

In vitro floating studies demonstrated that none of formulation manufactured using Eudragit® RS30D layer showed floating. This could be because of slow diffusion of dissolution fluid and slower hydration of Eudragit® RS30D layer which leads to slow generation of CO_2 gas. In addition, dispersion of Eudragit® RS30D contains 19.4 mg of potassium hydroxide (per g of polymer) which could be responsible for the neutralization of dissolution medium (0.1N HCl) during permeation through coated membrane which probably may resulted into generation of insufficient amount of CO_2.

Formulations with Eudragit® NE30D as gas entrapment layer also showed similar results. In vitro release profiles of LD from pellets coated with Eudragit® NE30D (EC/ENE/1 to EC/ENE/3) are shown in Figure 5.15. Retardation of drug release was observed with increasing coating level of Eudragit® NE30D from 5 to 15%, w/w. Slower drug release was observed for formulations coated with Eudragit® NE30D (EC/ENE/3, MDT= 11.25 h, $T_{80\%}$= 49.60 h) when compared to formulations coated at same coating level (15%, w/w) with Eudragit® RS30D (EC/ERS/3, MDT=10.96 h, $T_{80\%}$=31.67 h) (Table 5.17a). This greater retardation of LD release can be attributed to hydrophobic nature and high molecular weight (750,000 g) of Eudragit® NE30D compared to Eudragit® RL30D (32,000 g) and Eudragit® RS30D (32,000 g). Further, floating study demonstrated that pellets coated with Eudragit® NE30D also failed to show floating even at low level of coating (5%, w/w). Eudragit® NE30D is neutral ester polymer comprising of ethyl acrylate and methyl methacrylate (in the ratio of 2:1) without hydrogen bonds or other intermolecular forces. In addition, Eudragit® NE30D exhibit glass transition temperature of approximately 5°C and thus it has high flexibility (static strain > 600%) at room temperature. Hung and coworkers observed that Eudragit® NE30D has higher elongation values in both dry and wet states (Hung et al., 2014). Further, this polymer does not require plasticizer due to low minimum film formation temperature. Therefore, films formed by Eudragit® NE30D are flexible in nature (Krögel and Bodmeier, 1999). Although films are flexible in nature to entrap generated CO_2 gas, hydrophobic character of Eudragit® NE30D film hinders the water permeation. Because of slow water permeation, system would not generate sufficient CO_2 gas immediately to promote pellet floating (Sungthongjeen et al., 2006; Sungthongjeen et al., 2008).

During investigation of Eudragit® RS30D and Eudragit® NE30D as gas entrapment layer, change in the color of pellets was observed within 1 h when pellets (EC/ERS/1 to EC/ERS/3 and EC/ENE/1 to EC/ENE/3) were charged for dissolution study. This could be because of poor permeability of outer gas entrapped coating which hinders the permeation of sufficient amount of dissolution media into the formulations. Therefore, permeation of insufficient quantity of dissolution media might be responsible for generation of basic microenvironmental pH due to incomplete neutralization of the coated $NaHCO_3$ inside the gas entrapped polymeric film. This generation of basic microenvironmental pH during dissolution study could be responsible for the degradation of the LD and CD resulting into bluish black color of the pellets.

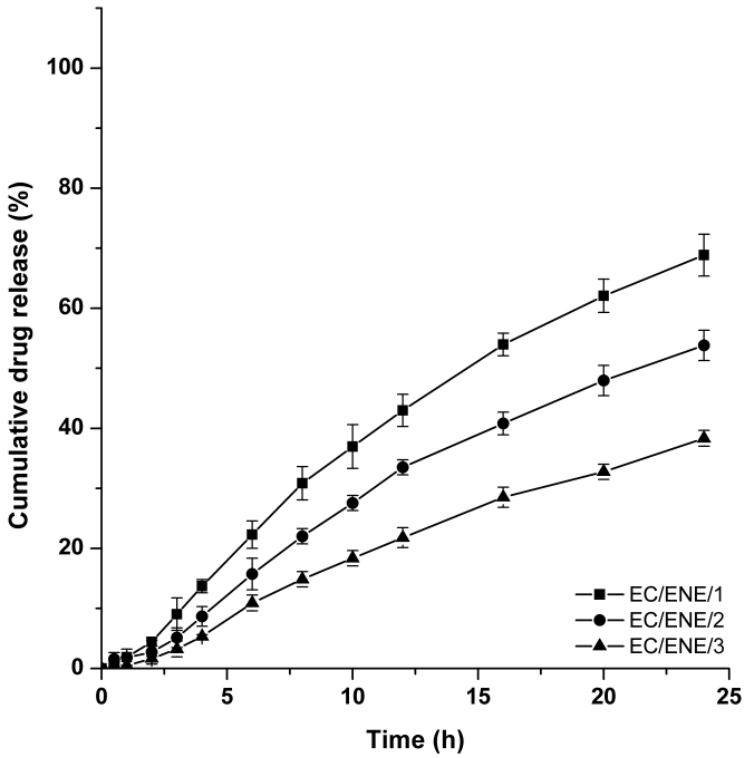


Figure 5.15. Effect of different coating levels of Eudragit® NE30D on in vitro release of LD in 0.1N HCl. (Each value represents mean of three independent determinations with standard deviation).

5.3.4.4. Effect of Pore Formers

Pore forming agents or water permeation enhancer were used in order to increase the water permeability and hydration rate of the Eudragit® RS30D and Eudragit® NE30D. Eudragit® RL30D was used as a pore forming agent in Eudragit® RS30D whereas PEG 6000 was used as pore forming agent in case of Eudragit® NE30D (Table 5.5).

In vitro release profile of LD from pellets coated with Eudragit® RL30D:Eudragit® RS30D layer as gas entrapment film are shown in Figure 5.16a (EC/ERLRS/1 to EC/ERLRS/3), Figure 5.17a (EC/ERLRS/4 to EC/ERLRS/6) and Figure 5.18a (EC/ERLRS/7 to EC/ERLRS/9). The type and composition of Eudragit® polymers influences the drug release to considerable extent. The release of LD was found to be more retarded in case of formulation EC/ERLRS/9 (MDT = 11.79 h, $T_{80\%}$ = 43.19 h) compared to the formulation EC/ERLRS/6 (MDT = 10.89 h, $T_{80\%}$ = 30.96 h) and EC/ERLRS/3 (MDT = 9.45 h, $T_{80\%}$ = 15.82 h) (Table 5.17b). Further, formulation EC/ERLRS/6 showed retarded LD release in comparison to the formulation EC/ERLRS/3. This could be attributed to the decreasing permeability and hydration rate of film resulting from increasing Eudragit® RS30D portion in the outer layer. Lower ratios of Eudragit® RL/RS showed retarded drug release compared to the formulation coated with high ratios of Eudragit® RL/RS (Figure 5.16a and Figure 5.18a). Moreover, retardation in drug release was observed with increasing coating level of the polymer. The $t_{80\%}$ values were found to be increased from 13.34 to 15.82 h with increasing coating level from 10 to 20%, w/w of Eudragit® RL30D/RS30D = 80/20 (Table 5.17b). In vitro floating behavior of pellets coated with Eudragit® RL30D:Eudragit® RS30D layer as gas entrapment film are shown in Figure 5.16b (EC/ERLRS/1 to EC/ERLRS/3), Figure 5.17b (EC/ERLRS/4 to EC/ERLRS/6) and Figure 5.18b (EC/ERLRS/7 to EC/ERLRS/9). The increase in the percentage of Eudragit® RS30D in outer coat increases floating lag time and decreases the % pellets floating (Figure 5.19). The % of pellets floating at 20 h for formulations EC/ERLRS/3, EC/ERLRS/6 and EC/ERLRS/9 were 92 ± 3%, 87 ± 3%, 79 ± 4%, respectively. Increase in floating lag time was observed with increase in proportion of the Eudragit® RS30D which attributed to slow generation of CO_2 gas resulting from slow permeation of dissolution fluid.

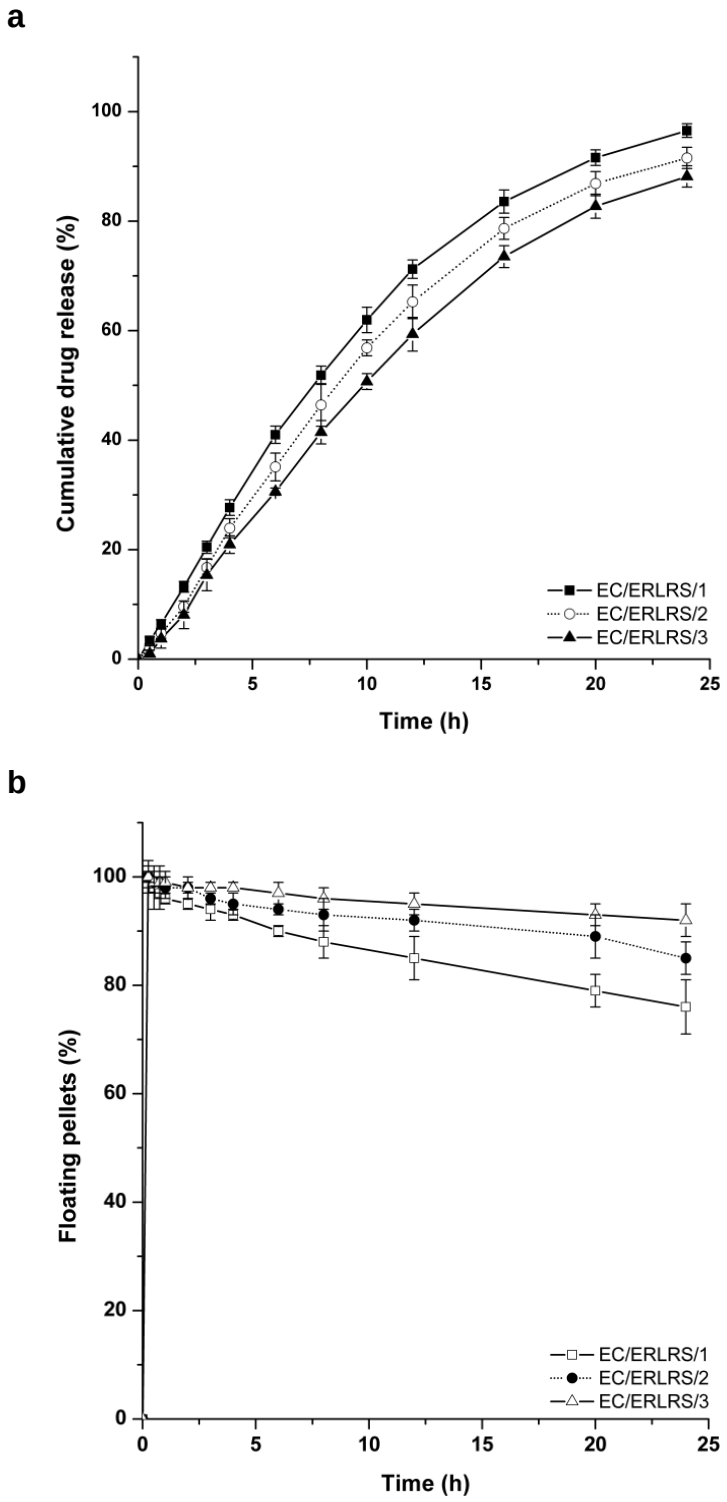


Figure 5.16. Effect of different coating levels of Eudragit® RL30D:Eudragit® RS30D = 80:20 on (a) in vitro release of LD and (b) in vitro floating in 0.1N HCl (Each value represents mean of three independent determinations with standard deviation).

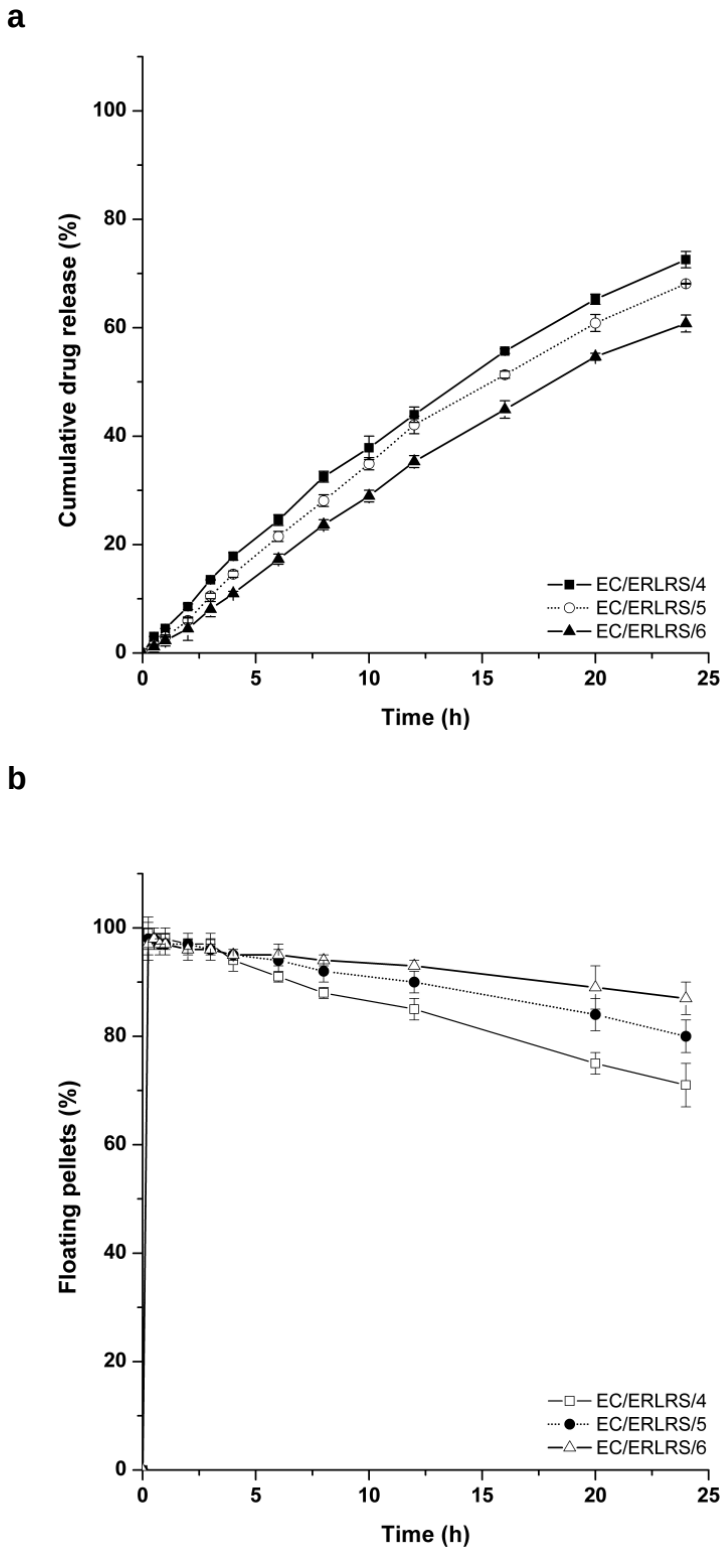


Figure 5.17. Effect of different coating levels of Eudragit® RL30D:Eudragit® RS30D = 60:40 on (a) in vitro release of LD and (b) in vitro floating in 0.1N HCl (Each value represents mean of three independent determinations with standard deviation).

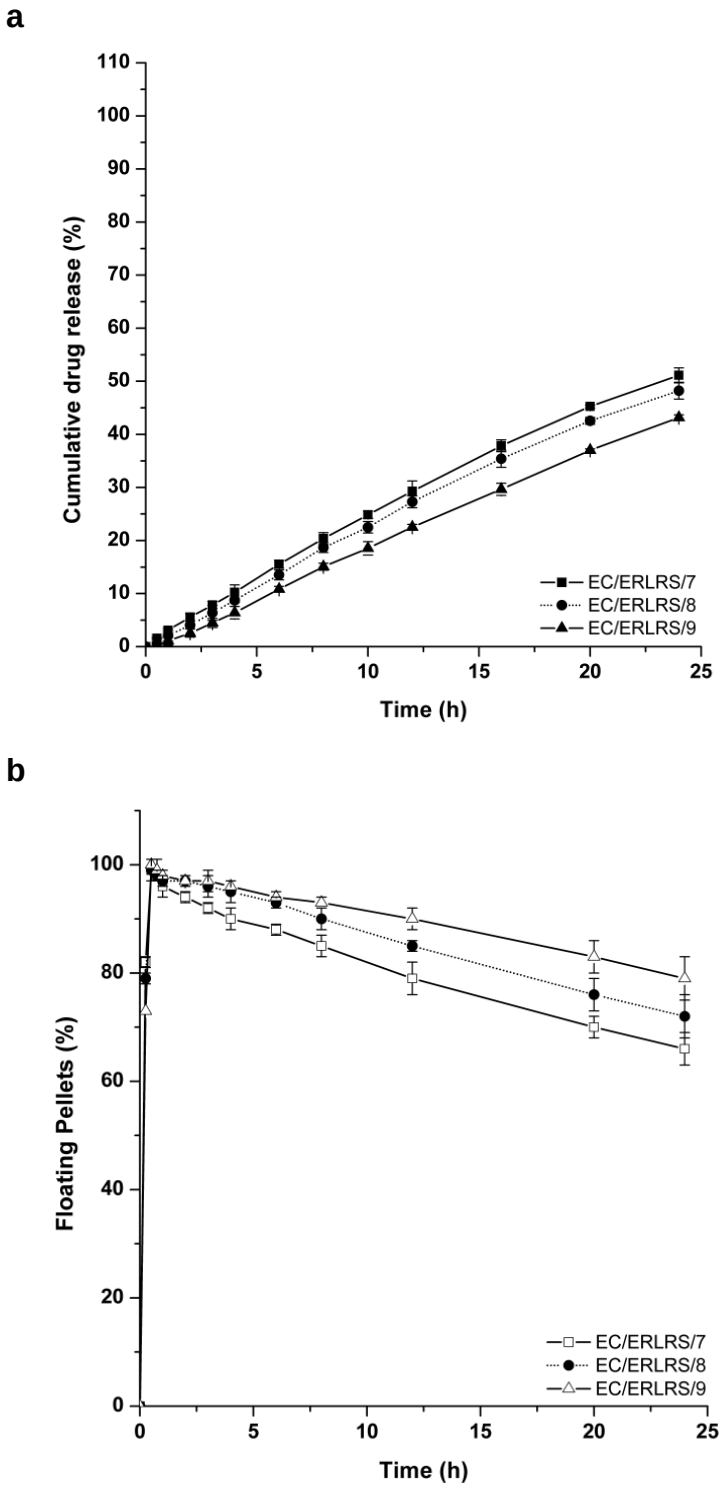


Figure 5.18. Effect of different coating levels of Eudragit® RL30D:Eudragit® RS30D = 40:60 on (a) in vitro release of LD and (b) in vitro floating in 0.1N HCl (Each value represents mean of three independent determinations with standard deviation).

In case of formulations containing Eudragit® RL30D above 50% in outer coat, dissolution fluid penetrated rapidly which leads to immediate formation of CO_2 gas resulting into reduction in floating lag time and high percentage of floating pellets at 20 h even at 15%, w/w coating level (Figure 5.19a and Figure 5.19b).

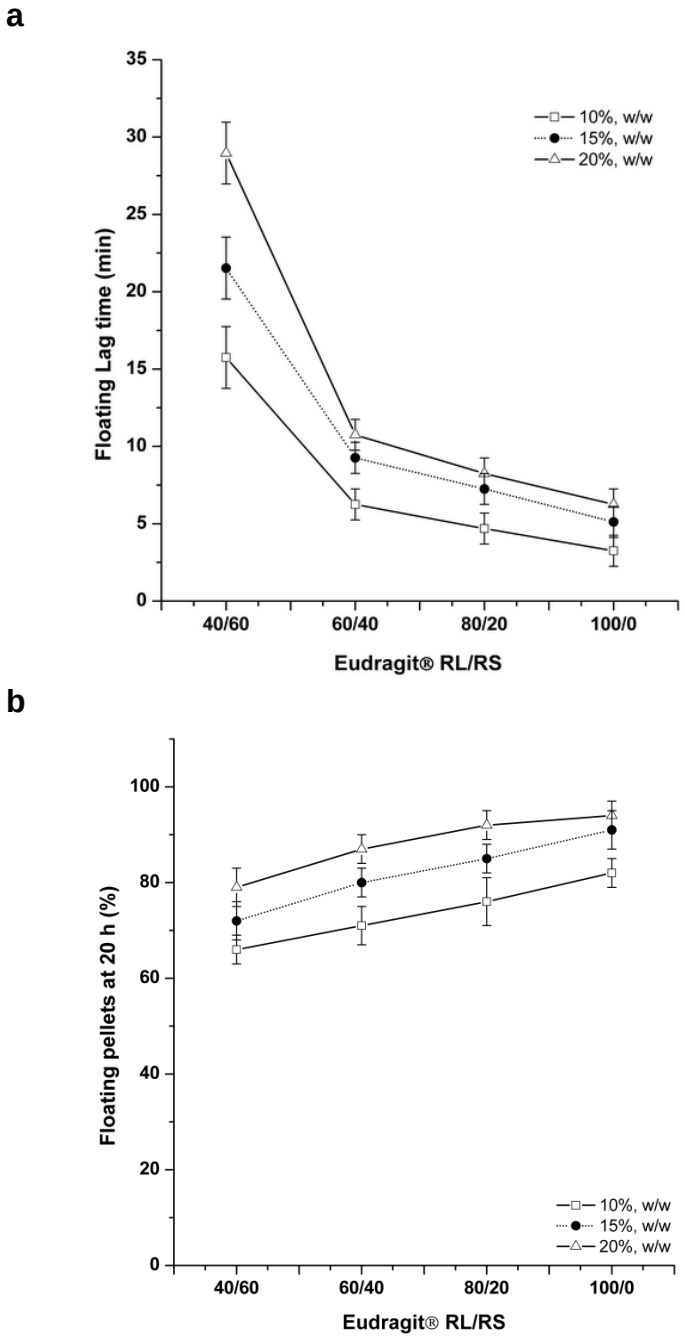


Figure 5.19. Effect of various coating levels and the ratio of Eudragit® RL30D:Eudragit® RS30D on (a) floating lag time (min) and (b) floating pellets at 20 h (%)

Formulation coated at 15%, w/w with Eudragit® RL30D:Eudragit® RS30D = 80:20 (EC/ERLRS/2) showed minimum floating lag time (7.86 ± 1.25 min) and higher % of pellets floating at 20 h (87 ± %). In addition to this, formulation EC/ERLRS/2 showed retardation of drug release over a longer period (MDT = 8.88 h and $t_{80\%}$ =14.38 h) (Table 5.17b). Further, similarity factor for in vitro release profiles of LD and CD from formulation coated with Eudragit® RL30D/RS30D in the ratio of 80/20 at 15%, w/w coating level (EC/ERLRS/2) was found to be more than 50 (f_2 = 80.88) which confirmed similarities in LD and CD release profiles (Figure 5.20). Thus, combination of Eudragit® RL30D:RS30D in the ratio of 80:20 at 15%, w/w coating level was considered as optimum gas entrapment polymeric layer.

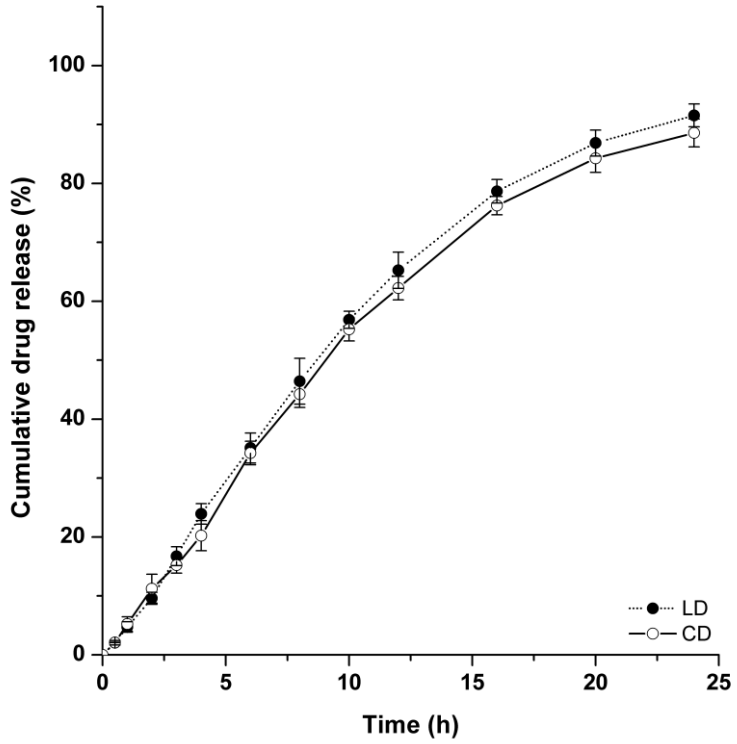


Figure 5.20. In vitro release profiles of LD and CD in 0.1N HCl from formulation EC/ERLRS/2. (Each value represents mean of three independent determinations with standard deviation).

The observed results is due to the physicochemical properties of Eudragit® polymers. The Eudragit® RL30D is a copolymer of ethyl methacrylate, methyl methacrylate and tri methyl ammonioethyl methacrylate chloride in a ratio of 1:2:0.2, whereas Eudragit® RS30D contains same copolymer but in a ratio of 1:2:0.1. The percentage of trimethylammonioethyl methacrylate chloride is more in Eudragit® RL30D which is responsible for the high water absorption, permeability and swelling property of the polymer. The amount of quaternary ammonium groups presents in the Eudragit® RL30D makes polymer hydrophilic and more permeable on the other hand hydrophobicity of Eudragit® RS30D makes it less permeable (Lin et al., 2000). Thus, in comparison to the Eudragit® RS30D, Eudragit® RL30D exhibits faster water permeability and exhibit great swelling property. In addition, pore diameter also play important role in the permeability of dissolution medium. Comparatively, Eudragit® RL30D (pore diameter = 1-5 µm) exhibit large pore size than Eudragit® RS30D (pore diameter = 0.1-0.6 µm) which governs the fast permeability of dissolution medium (Lin et al., 2000).

In vitro drug release profiles of LD from the pellets coated using different compositions of Eudragit® NE30D:PEG 6000 (HPMC/ENEPEG/1 to HPMC/ENEPEG/3) are shown in the Figure 5.21a. Drug release increased with increase in the percentage of PEG 6000 in Eudragit® NE30D layer. MDT decreased from 9.89 to 8.98 h with increasing PEG6000 concentration in outer coating layer from 10 to 30%, w/w respectively. In addition, time required to release 80% of drug was also found to be reduced from 26.07 to 15.91 h which demonstrated increase in drug release with increase in pore forming agent (Table 5.17b). Due to hydrophilic nature, PEG 6000 dissolves immediately creating pore in the Eudragit® NE30D film resulting in increase in permeability of film and increase in drug release rate. Further, increase in coating level showed retardation of the drug release which might be because of increase in diffusion path length resulting from increased thickness and swelling of coating layer. Further, reduction in floating lag time and increment in % of floating pellets were observed with increase in percentage of the PEG 6000 in outer coat of Eudragit® NE30D (Figure 5.21b). However, formulations coated with Eudragit® NE30D:PEG 6000 showed comparatively longer floating lag time and low % of floating pellets than the formulations coated with Eudragit® RL30D which may attributed to the rate of hydration, swelling and permeability of the hydrated film.

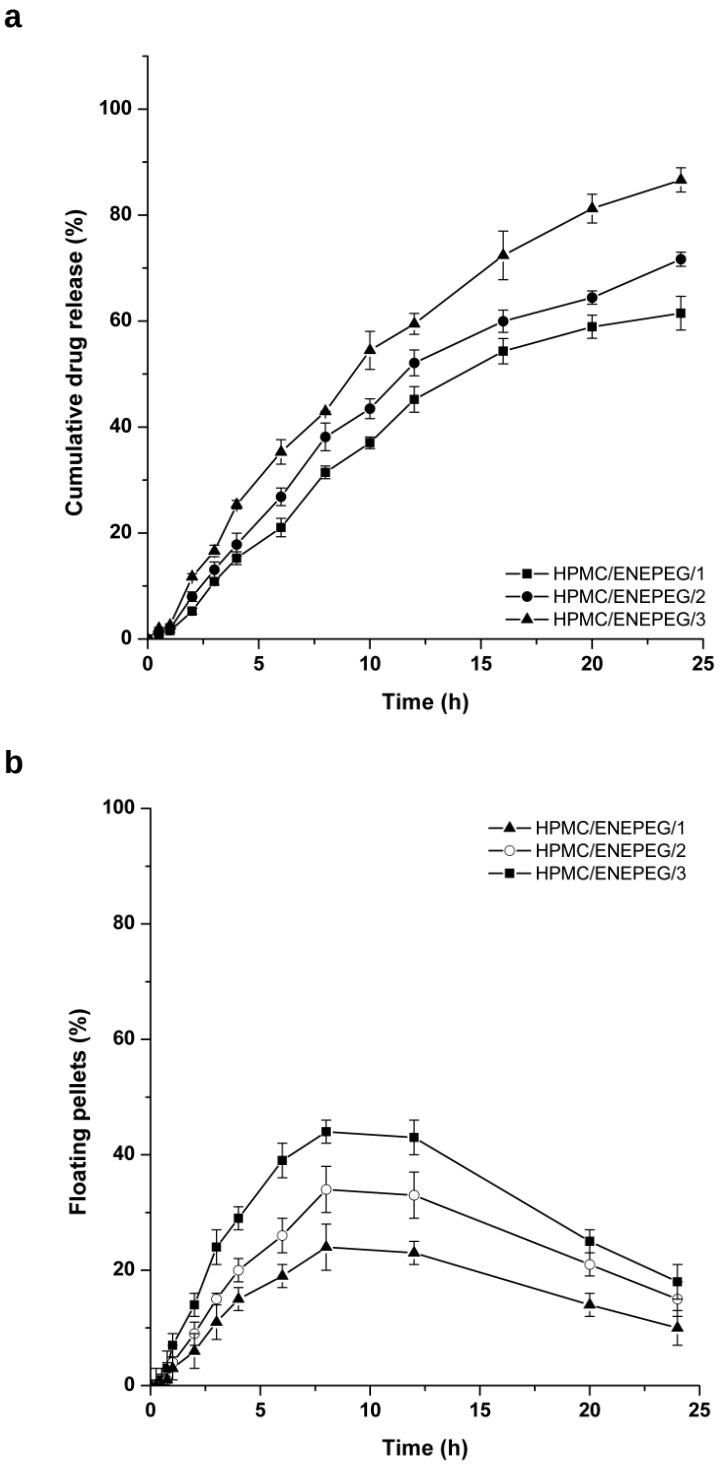


Figure 5.21. Effect of Eudragit® NE30D layer (containing different amount of PEG 6000) on (a) in vitro release of LD and (b) in vitro floating behavior of pellets in 0.1N HCl (Each value represents mean of three independent determinations with standard deviation).

Film formed by Eudragit® NE30D:PEG 6000 probably could have hydrated and swelled relatively at slower rate than Eudragit® RL30D therefore, it would failed to entrap generated CO_2 gas. In addition, even at lower coating level, formulation HPMC/ENEPEG/3 showed longer floating lag time. Moreover, it has failed to float for longer duration (24 ± 2% floating pellets at 20 h) (Figure 5.21b) which might be because of lower thickness and high permeability (due to PEG 6000) of coating layer.

Although, retardation in LD release was observed in case of formulations coated using Eudragit® RS30D and Eudragit® NE30D alone, they had failed to show expected floating behavior. Moreover, use of pore forming agent did not showed considerable improvement in the floating and in vitro release behavior except formulation coated with Eudragit® RL30D:Eudragit® RS30D = 80:20 at 15% coating level (EC/ERLRS/2). Therefore, formulation EC/ERLRS/2 was considered for further investigation of stability and batch reproducibility study.

5.3.5. Systems Coated using Tartaric Acid and Sodium Bicarbonate as Gas Generating Layers

Eudragit® RL100 film exhibits high water absorption, permeability, flexibility and swelling property compared to other cellulosic polymers (Krögel and Bodmeier, 1999; Lin et al., 2000). The effect of TA and $NaHCO_3$ on floating properties of pellets was investigated by using Eudragit® RL100 as gas entrapped polymeric film. In this type of formulations, TA and $NaHCO_3$ were suspended individually in HPC solution prepared in isopropyl alcohol and then individual coating of TA and $NaHCO_3$ layer were carried out. Further, coating of gas entrapped polymeric layer (Eudragit® RL100) was carried out using organic solvent in order to avoid reaction between TA and $NaHCO_3$ layered on the surface of pellet during coating process.

5.3.5.1. Coating Levels of Effervescent Layers and Gas Entrapped Polymeric Film

During formulation development, formulations were prepared by successive coating of core pellet with TA layer followed by application of $NaHCO_3$ layer and then Eudragit® RL100 layer. Drug degradation problem was rectified by avoiding direct contact of drug loaded pellets with $NaHCO_3$ layer by intermediate application of TA layer on surface of

core pellets (Figure 5.3). In addition, $NaHCO_3$ layer was applied on pellets surface by suspending $NaHCO_3$ in binder solution prepared in organic solvent. For initial formulations (ERL100/1 to ERL100/9), core pellets were coated with three successive layers viz. TA, $NaHCO_3$ and Eudragit® RL100 layer in order to optimize coating levels of effervescent layers and gas entrap polymeric layer.

It was postulated that, permeation of dissolution medium through outermost gas entrapped polymeric layer would results in generation of CO_2 due to reaction between TA and $NaHCO_3$. The evolved gas would get entrapped around the core pellets due to the flexible swollen polymeric membrane. This would results in reduction of density (increased buoyancy) of the pellets and hence would aid in floatation. The TA and $NaHCO_3$ were coated on the surface of core pellets using low viscosity grade HPC. The ratio of 1:4 (w/w) for both HPC:TA and HPC:$NaHCO_3$ was kept constant during evaluation of impact of effervescent layers coating levels on the floating behavior of the pellets.

The effect of effervescent layers (coating levels of $NaHCO_3$ and TA layer in the ratio of 8:14, 10:10 and 14:8) and coating level of gas entrapped polymeric membrane (Eudragit® RL100) (5, 10, 15% w/w) on the floating ability and the drug release from designed pellets was investigated. During formulation development, initial task was to optimize the coating levels of gas generating layers and gas entrapped polymeric membrane so that pellets should start floating within short time (10 min) to avoid passage into the small intestine along with food (Iannuccelli et al., 1998). Further, floating of pellets should sustain for longer time (maximum numbers of pellets should show floating) in stomach so that complete amount of drug would release from formulation above absorption site and thus increasing bioavailability. Therefore, % of pellets floating at 20 h was used for comparison of formulation in case of in vitro floating study data.

The coating levels of TA, $NaHCO_3$ and Eudragit® RL100 plays a key role in the floating of the pellets. Inverse relationship was observed between coating level of $NaHCO_3$ layer and floating lag time. Time required for pellet to float decreases with the increase in coating level of $NaHCO_3$ layer. Further, coating level of Eudragit® RL100 layer also influenced the floating lag time. The time required for pellet to float was found to be minimum in case of formulations coated with effervescent layers (8%, w/w of TA and 14%, w/w of $NaHCO_3$) and Eudragit® RL100 (coating level 5%, w/w) (ERL100/3) compared to other formulations.

Low coating level of Eudragit® RL100 reduces the floating lag time due to immediate penetration of the dissolution fluid through thin coating layer and instant gas formation. The effect of coating levels of TA and $NaHCO_3$ layers and Eudragit® RL100 on floating lag time is represented in Figure 5.22. Not only floating lag time but % of pellets floating at the end of 20 h also plays important role in formulation development.

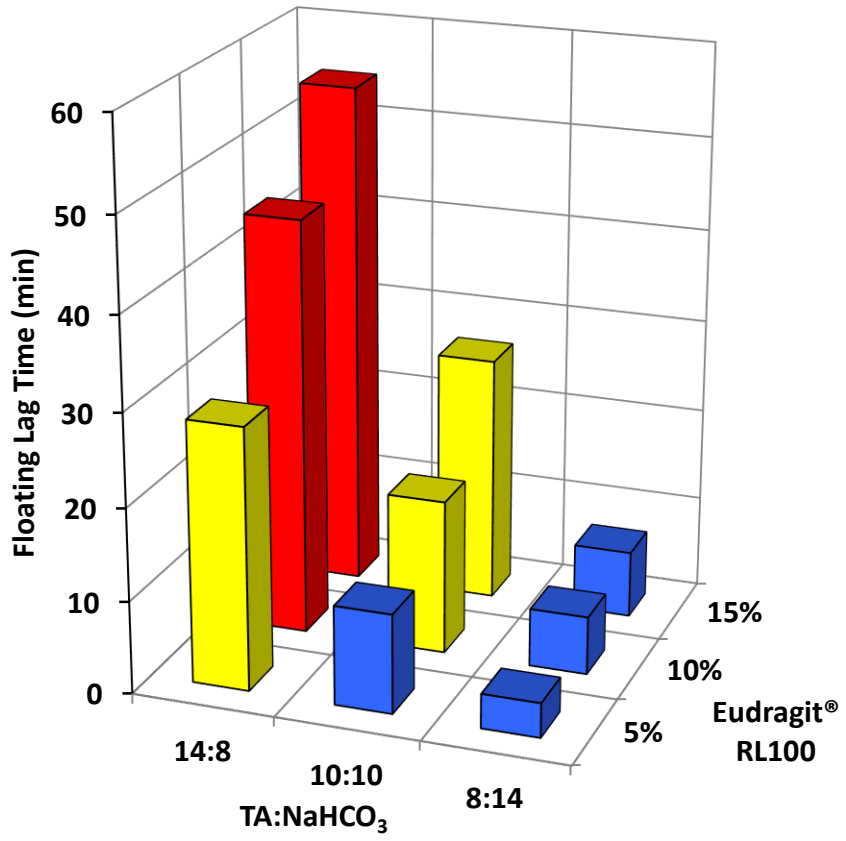


Figure 5.22. The effect of coating levels of effervescent layers (TA and $NaHCO_3$) and gas entrapped polymeric layer (Eudragit® RL100) on floating lag time (min) of designed pellets.

In this study, formulation showing more than 85% floating pellets at the end of 20 h was considered as optimum formulation. The % of floating pellets was found to be directly related to coating levels of gas generating agents (TA and $NaHCO_3$) as well as with the coating level of gas entrapped polymeric film. The influence of coating levels of

effervescent layer and gas entrapped polymeric film on % of floating pellets at 20 h is shown in Figure 5.23.

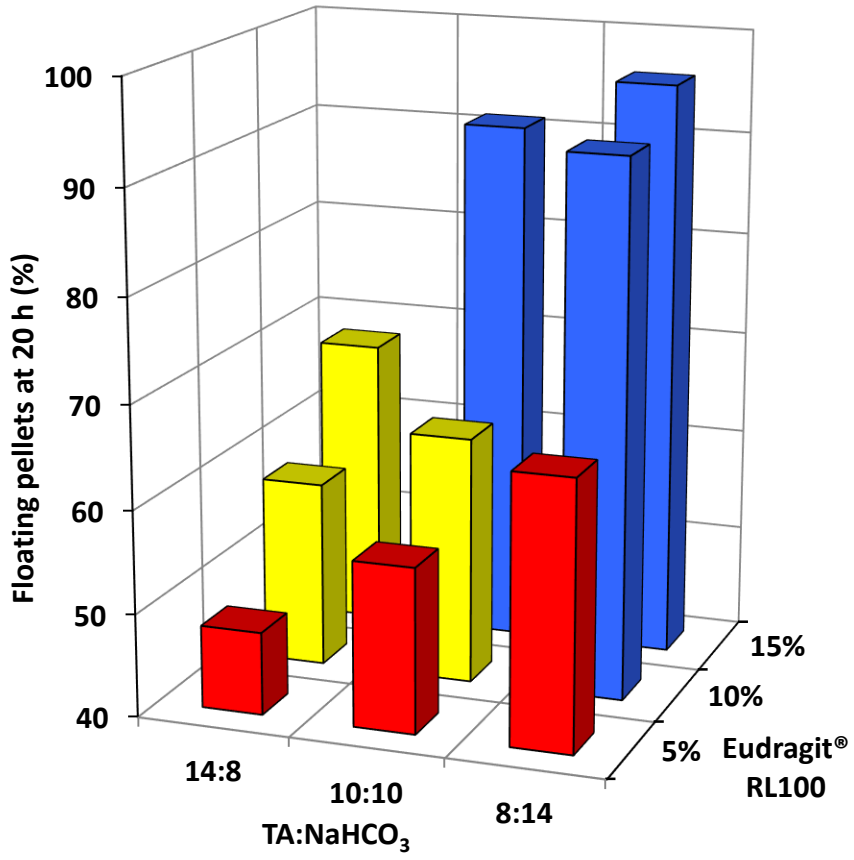


Figure 5.23. The effect of coating levels of effervescent layers (TA and $NaHCO_3$) and gas entrapped polymeric layer (Eudragit® RL100) on floating pellets (%) at 20 h.

Formulations coated with 15%, w/w of Eudragit® RL100 (ERL100/9) showed highest % of floating pellets at 20 h due to increased coating layer thickness and impermeability of generated CO_2 gas. Thus CO_2 gas retained inside polymeric film for longer duration resulting in longer floatation of pellets. However, formulation ERL100/9 showed longer but acceptable time to float compared to formulations coated at low coating level of Eudragit® RL100 which could be because of decrease in rate of dissolution fluid permeation with increase in outer coating layer thickness.

Formulation ERL100/4 and ERL100/5 had taken comparatively longer time to float as well as % of floating pellets were 58 ± 2 and 64 ± 3% respectively. This could be because of the different coating levels of TA and $NaHCO_3$ layers which in presence of dissolution medium generate relatively less amount of CO_2 gas. In addition, coating levels of Eudragit® RL100 (10%, w/w) may not be sufficient to entrap generated CO_2 for longer duration. Further, formulation ERL100/6 and ERL100/9 showed acceptable floating lag time (< 10 min). The percentage of pellets floating over dissolution medium for formulations ERL100/6, ERL100/8 and ERL100/9 were found to be 93 ± 2%, 91 ± 1% and 96 ± 2% respectively at 20 h (Figure 5.24). ERL100/9 showed comparatively higher % of floating pellets at 20 h than formulation ERL100/6 which could be because of 10% w/w coating level of Eudragit® RL100 (ERL100/6) which may not be sufficient to entrap generated CO_2 for longer duration.

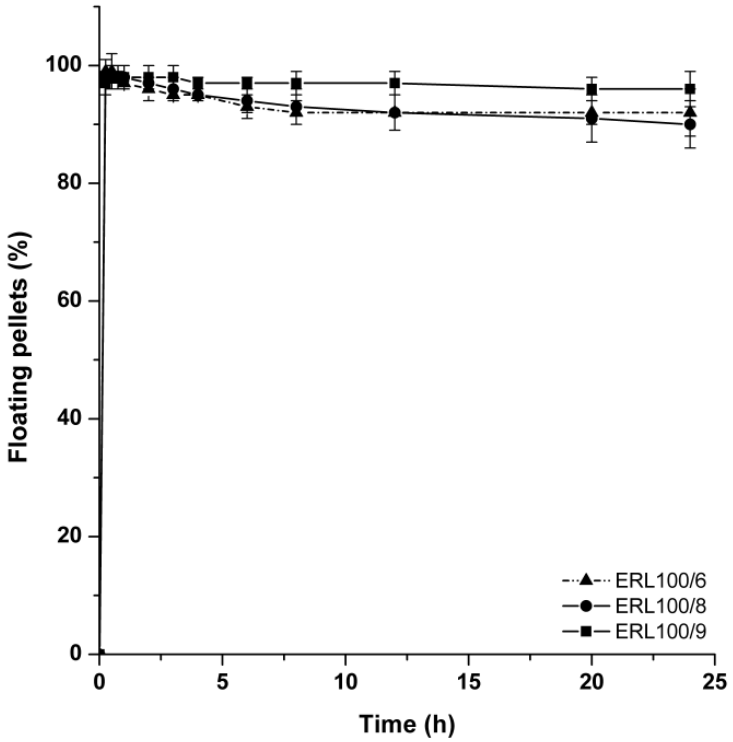


Figure 5.24. Effect of different coating levels of Eudragit® RL100 on floating behavior of pellets. (Each value represents mean of three independent determinations with standard deviation).

Formulation ERL100/9 showed acceptable floating lag time (< 10 min) and higher % of floating pellets at the end of 20 h. Therefore, optimum coating levels of effervescent layers and gas entrapped polymeric film was considered as 8%, w/w of TA layer, 14%, w/w of $NaHCO_3$ layer and 15%, w/w of Eudragit® RL100 layer.

5.3.5.2. In Vitro Drug Release

The effect of coating levels of effervescent layers and Eudragit® RL100 on LD release was evaluated. In vitro LD release profile of formulations ERL100/1 to ERL100/9 are represented in Figure 5.25.

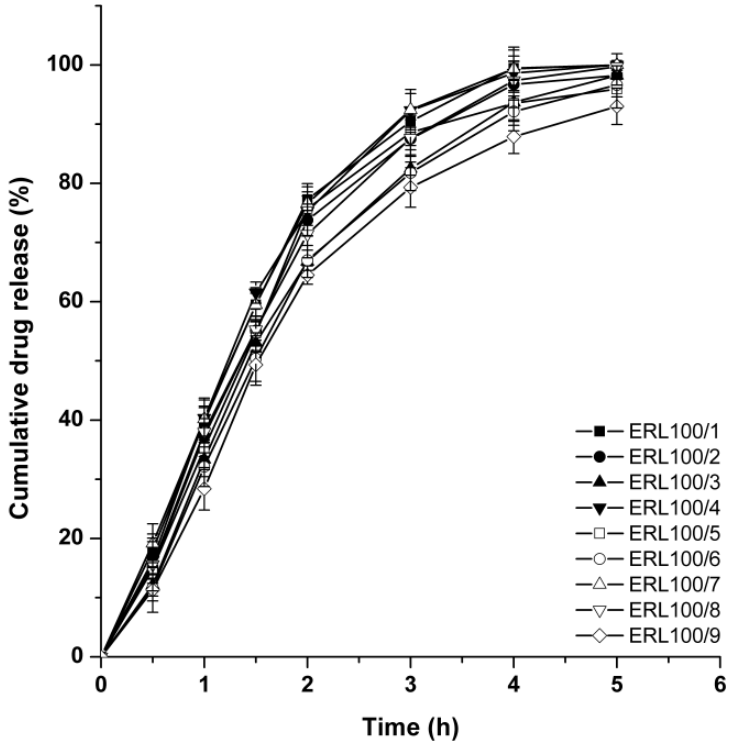


Figure 5.25. In vitro release profile of LD from pellets coated at different coating levels of TA, $NaHCO_3$ and Eudragit® RL100 layer. (Each value represents mean of three independent determinations with standard deviation).

Slow drug release was observed for initial few minutes in case of formulations coated with 10%, w/w and 15%, w/w of Eudragit® RL100 which could be due to slow permeation of dissolution fluid through larger thickness of membrane. However, formulations ERL100/1

to ERL100/9 showed MDT in the range of 1.41 to 1.70 h whereas $t_{80\%}$ in the range of 1.87 to 2.15 h (Table 5.17c). This may be because of high permeability of Eudragit® RL100. The observed MDT and $t_{80\%}$ values demonstrated insignificant influence of Eudragit® RL100 on LD release. Further, binder (HPC) used in effervescent agents coating also did not influence LD release profile. This may be due to hydrophilic nature of HPC. During dissolution HPC dissolves immediately along with $NaHCO_3$ and TA in the permeated dissolution fluid and generate CO_2 gas resulting in reduction of density and floating of pellets. The observed results are in good agreement with the results obtained for formulations HPMC/ERL/1 to HPMC/ERL/9 confirming Eudragit® RL100 alone was not sufficient to retard drug release. In addition, observed results also confirmed that the organic coating of Eudragit® RL100 did not retard drug release. Therefore, in order to control release of the drug the core was coated using sustained release polymeric layer before coating of effervescent and Eudragit®RL100 layers. EC and Kollicoat® SR30D were investigated as sustained release polymer layer.

The Drug release from formulations coated with Kollicoat® SR30D and EC as sustained release layer was evaluated at different coating levels. LD and CD showed similar drug release profiles from all studied formulations due to similar molecular weight and solubility profile. Therefore, in vitro release results of all formulations is discussed by comparing release profiles of LD. Moreover, release profiles of LD and CD for optimized batch were also compared by calculation of similarity factor (f_2). LD release in 0.1 N HCl from pellets coated with 2%, 3% and 4.5%, w/w of EC (EC/ERL100/1 to EC/ERL/3) and 4%, 6% and 10%, w/w of Kollicoat® SR30D (KSR/ERL100/1 to KSR/ERL100/3) are shown in Figure 5.26. Increasing coating levels of both EC and Kollicoat® SR30D retarded release of LD. MDT values were found to be in the range of 4.52 to 9.44 h for formulations coated with EC whereas 6.01 to 10.15 for formulations coated with Kollicoat® SR30D (Table 5.17c). In addition, time required to release 80% of drug was found to be in the range of 5.66 to 19.27 h and 8.08 to 26.97 h for EC and Kollicoat® SR30D coated formulations, respectively (Table 5.17c). This could be because of increased coating layer thickness and decrease in the permeability of LD. Further, EC coated pellets showed greater retardation of drug release when compared to Kollicoat® SR30D. Pellets coated with 3%, w/w of EC (EC/ERL100/2, MDT = 6.61 h) showed sustained release of LD with 80% drug release

within 9.55 h. In contrast, 4% coating level of Kollicoat® SR30D (KSR/ERL100/1, MDT = 6.01 h) was required to achieve 80% of LD release over period of 8 - 9 h, ($t_{80\%}$ = 8.08 h) (Table 5.17c).

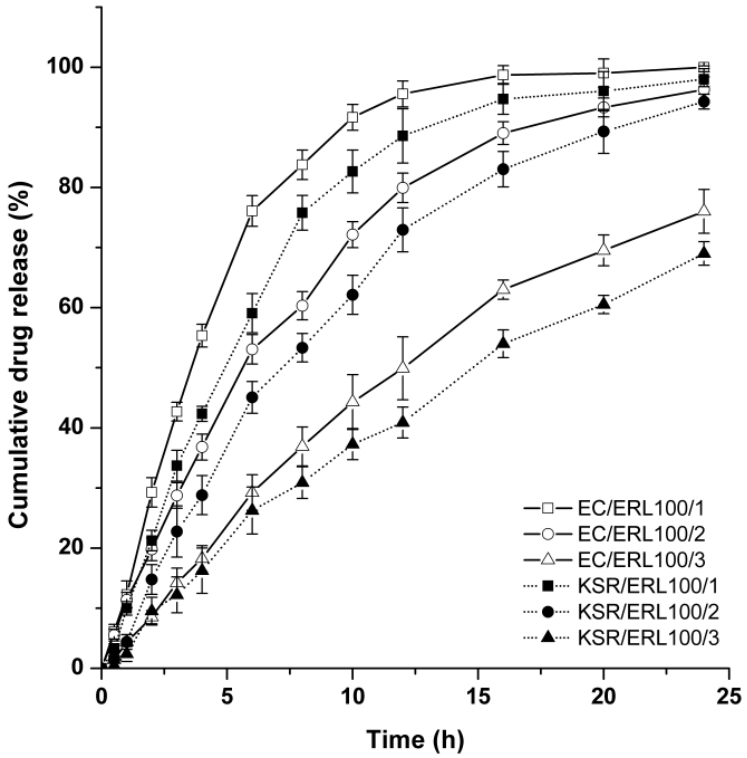


Figure 5.26. Effect of different coating levels of EC and Kollicoat® SR30D on in vitro release of LD in 0.1N HCl (Each value represents mean of three independent determinations with standard deviation).

EC is relatively less permeable than Kollicoat®SR30D due to higher hydrophobicity of it. Thus, faster release from Kollicoat® SR30D coated pellets can be attributed to the higher permeability (rate and extent) of the coating layer. In vitro floating behavior of formulations coated with EC and Kollicoat® SR30D is represented in Figure 5.27. Formulation coated with different coating level of EC and Kollicoat® SR30D showed similar floating behavior confirming insignificant impact of the sustained release layer on floating properties. This could be because of similar coating level of effervescent layers and gas entrapped polymeric layer.

Although, formulation EC/ERL100/2 and KSR/ERL100/2 showed similar release profiles, formulation coated with 3%, w/w of EC (EC/ERL100/2) was considered as the optimum formulation because increased coating level increases pellet size. Since, Kollicoat® SR30D does not play any role in floating of the formulation therefore high coating level merely increases the weight of pellet without providing additional benefits over EC coated formulation. In addition, density of the pellet is considered as important factor in floating of the pellets. Further, EC/ERL100/2 showed retarded drug release ($t_{80\%}$ = 9.55 h) (Table 5.17c), less floating lag time (< 10 min) and high % of floating pellets at 20 h (92 ± 2%) (Figure 5.27).

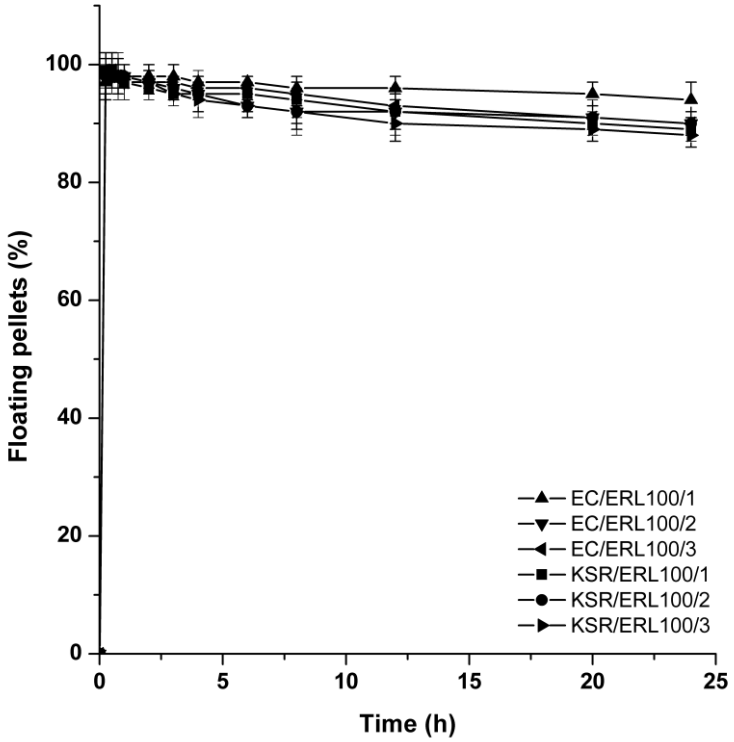


Figure 5.27. Effect of different coating levels of EC and Kollicoat® SR30D on in vitro floating behavior of pellets in 0.1N HCl. (Each value represents mean of three independent determinations with standard deviation).

Moreover, similarity factor higher than 50 (f_2 = 71.17) demonstrated the similarity of LD and CD release profiles from formulation EC/ERL100/2 (Figure 5.28). Therefore, it was expected that formulation EC/ERL100/2 would float immediately in vivo and remain buoyant for prolonged period, thereby formulation would release complete dose of drug at

controlled rate above absorption site. Thus, among all the formulations, EC/ERL100/2 was considered for further evaluation.

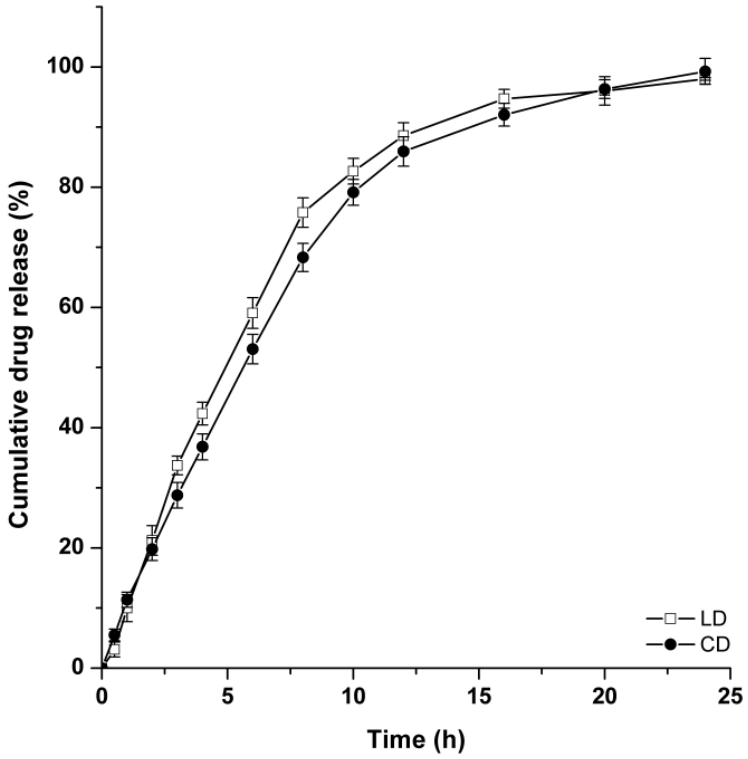


Figure 5.28. In vitro release profiles of LD and CD from formulation EC/ERL100/2 in 0.1N HCl. (Each value represents mean of three independent determinations with standard deviation).

5.3.6. Effect of Dissolution Conditions

Designed dosage form for controlled release must ideally release drug irrespective of the release conditions e.g. dissolution media pH and mechanical shear force. This would minimize the variability and bioavailability during in vivo pharmacokinetic studies (Dashevsky et al., 2004). Therefore, effect of dissolution conditions like agitation rate, dissolution media pH on in vitro release profile of LD and CD were investigated for formulation EC/ERL/2 (Table 5.4) and EC/ERL100/2 (Table 5.6).

5.3.6.1. Effect of Dissolution Media pH on In Vitro Drug Release

LD and CD exhibit pH dependent solubility. Therefore, influence of the pH of dissolution fluid on the drug release was also evaluated. The dissolution test for formulations was carried in simulated gastric media (pH=1.2) and phosphate buffer pH 3.0. The rationale behind the selection of dissolution media pH was to simulate gastric pH in fasted (1.5) and fed condition (3). In vitro release studies for EC/ERL/2 and EC/ERL100/2 was performed in dissolution media having pH 1.2 and pH 3.0 individually keeping all other dissolution parameters constant.

For, formulation EC/ERL100/2, release profile of LD [$f_{2(1.2/3.0)}$= 75.36] was found to be similar [$MDT_{(pH= 1.2)}$=6.61 h, $MDT_{(pH=3.2)}$=6.79 h] in dissolution media with different pH Figure 5.29a. In addition, EC/ERL100/2 showed similar LD and CD profile at different pH conditions [pH=1.2: $f_{2\ (LD/CD)}$ = 69.23 and pH=3.0: $f_{2(LD/CD)}$ = 68.26] (Figure 5.29b and Figure 5.29c). The observed dissimilarity factor for LD release profiles and CD release profiles from EC/ERL100/2 in different media further confirmed the LD and CD release were independent of dissolution media pH. In case of formulation EC/ERL/2, LD release profiles were differed significantly at different pH of dissolution media ($f_{2\ (1.2/3)}$ = 27.03) (Figure 5.29d). Retarded LD release for formulation EC/ERL/2 was observed in phosphate buffer pH 3.0 and this probably would be attributed to the generation of basic microenvironmental pH inside gas entrapped polymeric layer. Amount of phosphate buffer permeated through the gas entrapped polymeric layer could not be sufficient to neutralize the sodium bicarbonate completely. In addition, percentage of pellets floating over dissolution medium for formulation EC/ERL/2 were found to be 89% and 0% in pH 1.2 and 3.0 respectively (Figure 5.31a). These, floating study results further confirmed that the formulation EC/ERL/2 failed to produce sufficient CO_2 gas in order to sustain floating for longer duration in phosphate buffer media (pH 3.0). In contrast, formulation EC/ERL100/2 showed acceptable % floating pellets in both dissolution media (pH 1.2 and pH 3.0) (Figure 5.31a) probably due to presence of TA which neutralize $NaHCO_3$ and generate sufficient CO_2 gas regardless of dissolution media pH. Although, LD and CD exhibit different solubilities at pH 1.0 and pH 3.0, both drug showed similar drug release profiles in case of EC/ERL100/2.

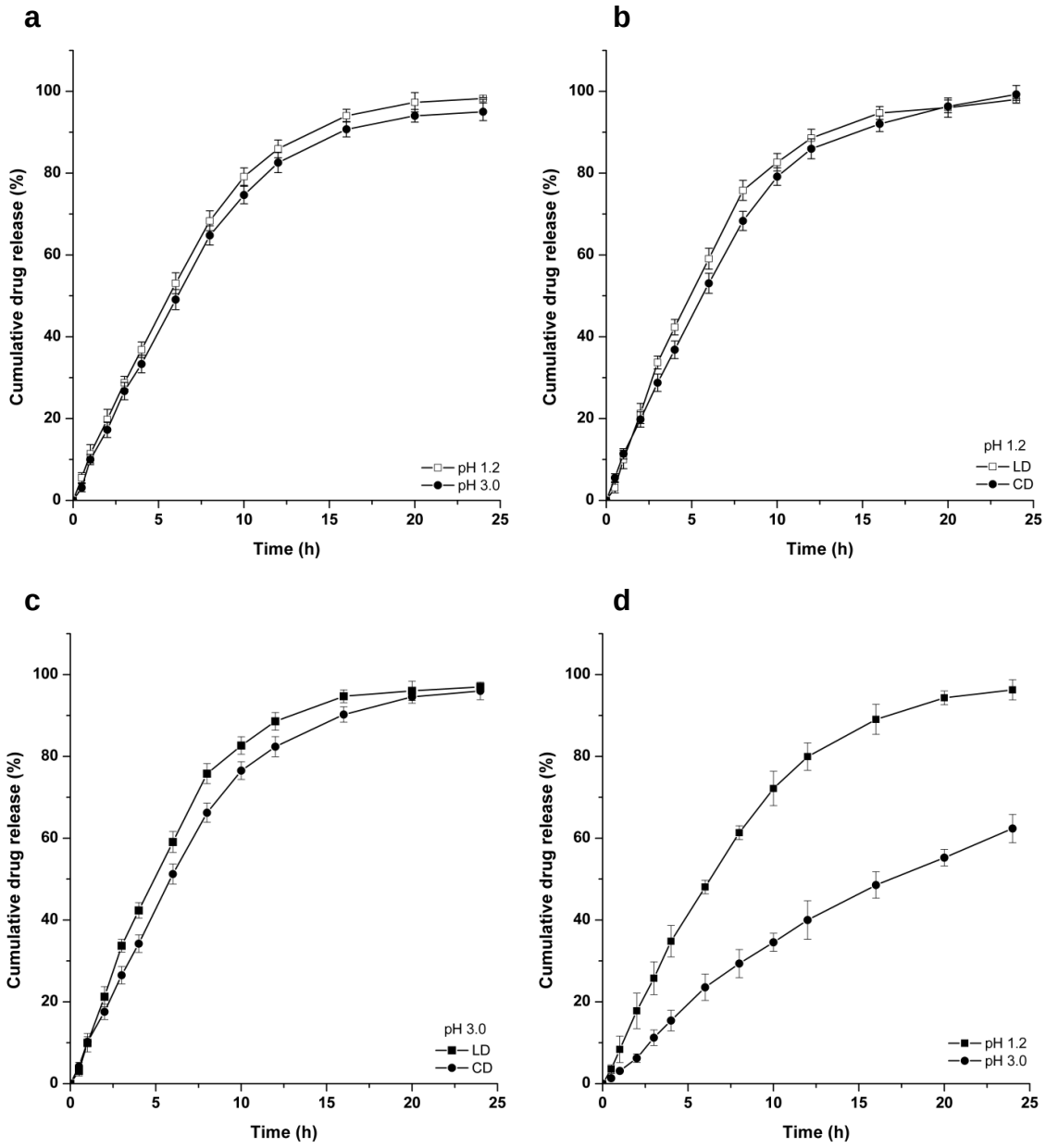


Figure 5.29. Effect of dissolution media pH on in vitro release of (a) LD from formulation EC/ERL100/2 at pH 1.2 and 3; (b) LD and CD from formulation EC/ERL100/2 at pH 1.2; (c) LD and CD from formulation EC/ERL100/2 at pH 3.0 and (d) LD from formulation EC/ERL/2 at pH 1.2 and 3 (Each value represents mean of three independent determinations with standard deviation).

The observed results demonstrated that instead of pH dependent solubility, the release rate of LD and CD from the formulation seems to be depends more on the diffusion of drug through barrier membrane (EC and Eudragit® RL100 layers). Therefore, it was expected that formulation (EC/ERL100/2) would maintain controlled release of LD and CD irrespective of pH environment. Thus, formulation EC/ERL100/2 was considered for evaluation of the effect of agitation rate, type of plasticizer and spheronization aid on in vitro release of LD and CD.

5.3.6.2. Effect of Agitation on In Vitro Drug Release

The robustness of the drug delivery system was also evaluated by estimating release profile at different agitation rates using dissolution testing apparatus (USP type 2). The drug release from the formulation involves various mass transport processes like diffusion of dissolution fluid inside the membrane, drug dissolution and diffusion of drug though the hydrated polymeric membrane. The agitation speed during dissolution studies influences these mass transport processes and thus it affects release profile. Therefore, effect of agitation speed on release rate of the optimized formulation was evaluated at 0.1N HCl. According to Noyes-Whitney equation, release rate of drug depend on the thickness of the stagnant layer (Fukunaka et al., 2006). Increase in agitation rate reduces the thickness of stagnant layer thereby increasing release rate of the drug. The drug release rate from the formulation EC/ERL100/2 did not influence by the agitation rate (Figure 5.30a). In addition, floating behavior of pellets also did not influence by agitation rate. Significant variation in percentage floating pellets at 20 h was not observed indicating flexibility and impermeability (for CO_2 gas) of selected gas entrapment polymer film at high agitation speed (Figure 5.31b). Moreover, dissolution profiles were also found to be similar [$f_{2(\text{LD, }50/100)} = 74.96$; $f_{2(\text{CD, }50/100)} = 76.39$] (Figure 5.30a). Similarity in the LD release profile from EC/ERL100/2 at 50 and 100 rpm agitation rates indicated the robustness of the designed formulation. Therefore, from in vitro release and floating studies, it can be expected that formulation EC/ERL100/2 would provide constant supply of the LD and CD in vivo.

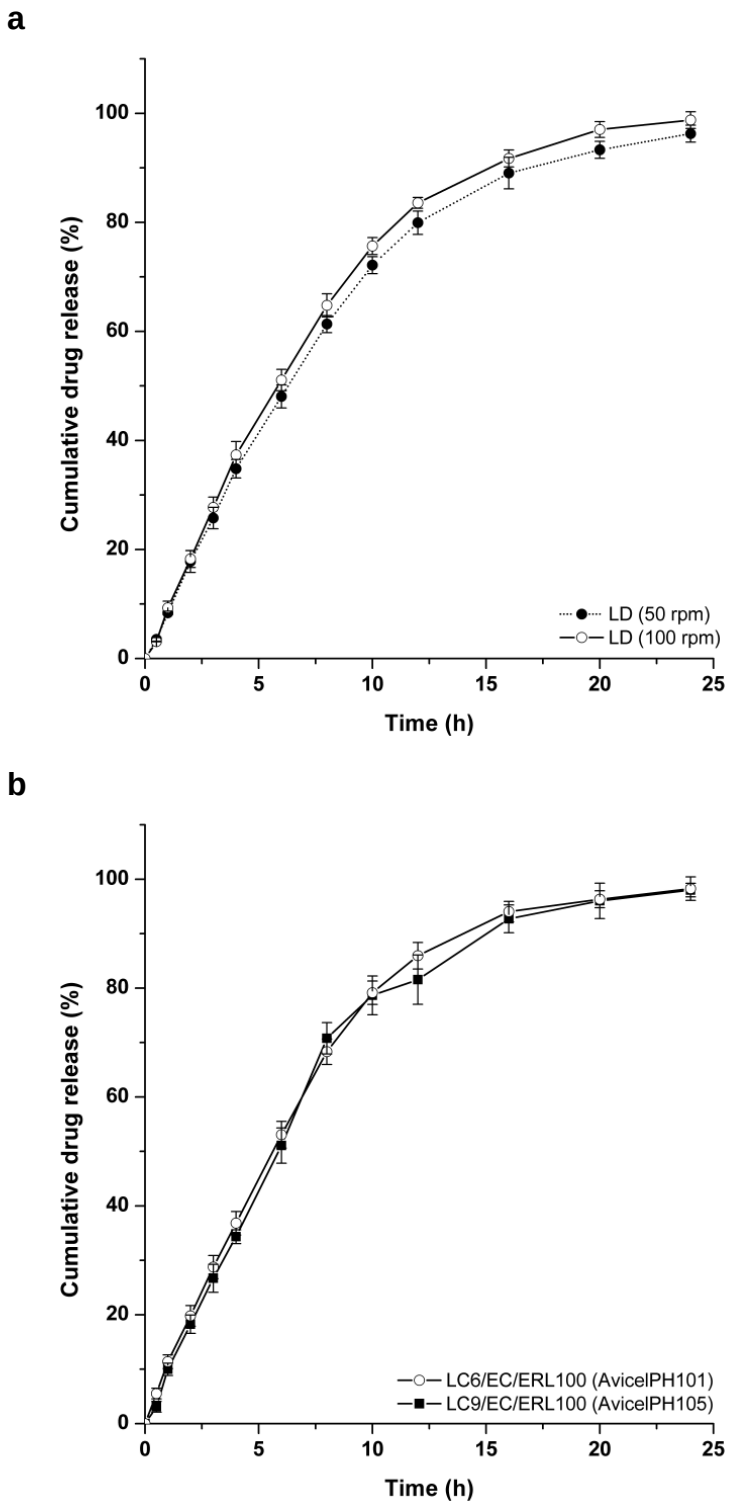


Figure 5.30. Effect of (a) agitation rate and (b) spheronization aid on in vitro release of LD in 0.1N HCl (Each value represents mean of three independent determinations with standard deviation).

5.3.7. Effect of Spheronization Aid Used for the Manufacture of Drug Loaded Core Pellets

Effect of spheronization aid on drug release from the designed pellet was evaluated by manufacturing pellet core using different grades of MCC viz. Avicel® PH101 and Avicel PH105. All other manufacturing parameters including coating levels were kept constant. In vitro release of LD from the pellets manufactured using Avicel® PH101 (LC6/EC/ERL100) and Avicel® PH105 (LC9/EC/ERL100) are shown in Figure 5.30b. Slightly slower LD release was observed from pellets manufactured using Avicel PH105 when compared to the pellets manufactured with Avicel®PH101. Slower drug release might be attributed to the smaller particle size of Avicel® PH105 which results into lower porosity when compared to the Avicel® PH101. Estimated similarity factor f_2 value (82.91) confirmed insignificant difference in the LD release profile which could be because of the high drug loading in the pellet core. In addition, floating properties of the pellets manufactured using Avicel® PH101 and Avicel® PH105 were found to be similar indicating negligible impact of the spheronization aid on the floating lag time and % pellets floating at 20 h (Figure 5.31a). Further, release rate of the LD and CD were found to be unchanged as confirmed by estimating similarity values for formulations manufactured using Avicel® PH101 [$f_{2(LD/CD)}$ = 75.23] and Avicel® PH105 [$f_{2(LD/CD)}$ = 78.35].

5.3.8. Effect of Plasticizer

Plasticizers are low molecular weight non-volatile additives when incorporated into polymeric material improves the workability and flexibility of the polymer by increasing intermolecular separation of the polymer molecules. Addition of plasticizers in polymeric material results in reduction in the elastic modulus, tensile strength, polymer melt viscosity and glass transition temperature (Tg). In addition, polymer toughness and flexibility and reduction in thermal processing temperatures (Tg) can be achieved with addition of plasticizers (Zhu et al., 2002). Plasticizer improves polymer particle coalescence by reducing Tg resulting in to continuous polymeric structure which either increase or decrease permeability of the film depending on physicochemical properties of the plasticizer. Hydrophilic plasticizer renders polymer film more permeable by formation of pores in presence of dissolution media than the water insoluble plasticizer.

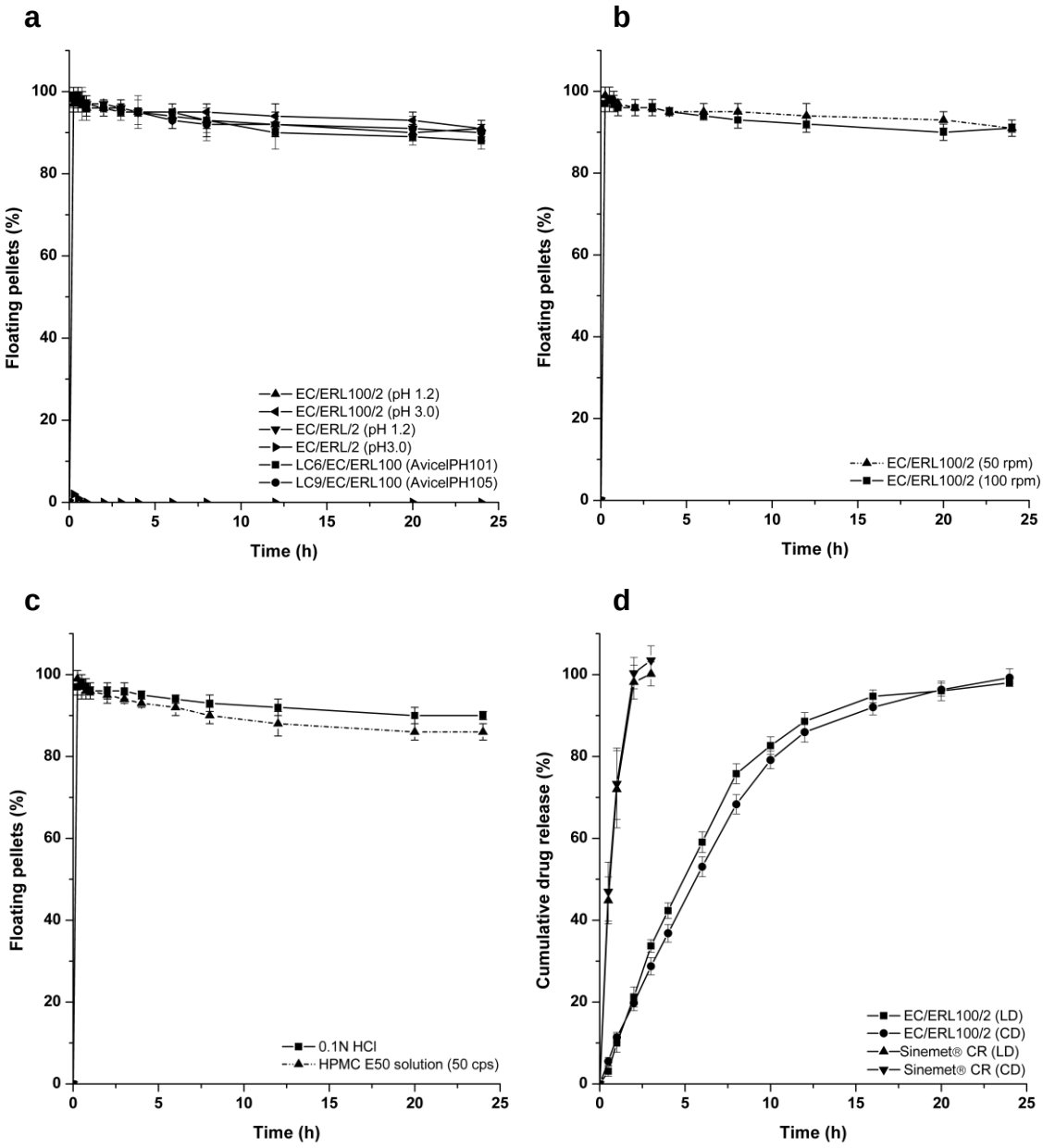


Figure 5.31. In vitro floating behavior of (a) EC/ERL/2 and EC/ERL100/2 at different pH conditions and effect of spheronization aid on floating behavior of pellets; (b) EC/ERL100/2 at different agitation speed; (c) EC/ERL100/2 at different viscosities and (d) in vitro release profiles of Sinemet® CR (LD/CD = 200/50) and EC/ERL100/2 in 0.1N HCl. (Each value represents mean of three independent determinations with standard deviation).

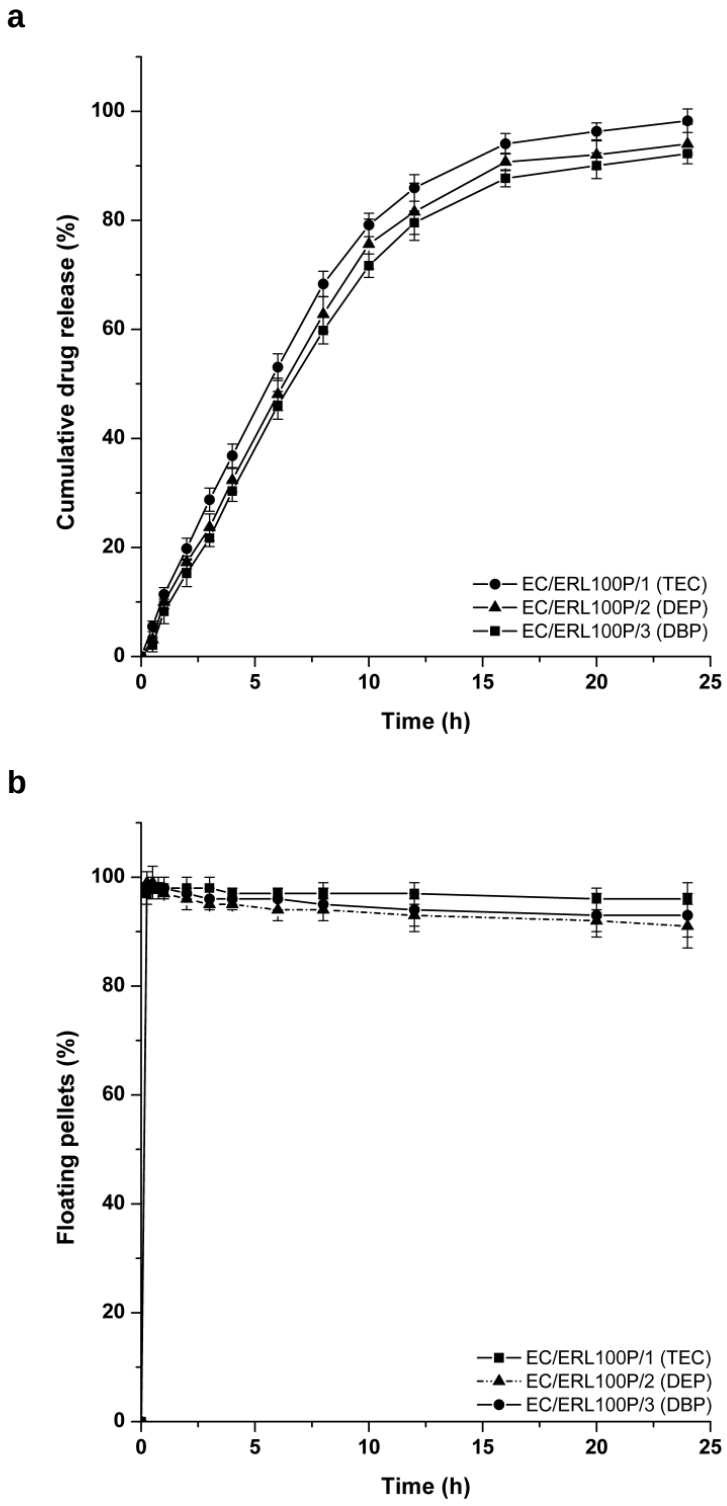


Figure 5.32. Effect of plasticizer on (a) in vitro release of LD and (b) in vitro floating behavior in 0.1N HCl. (Each value represents mean of three independent determinations with standard deviation).

Therefore, effect of plasticizer type (TEC, DEP and DBP) on in vitro release was investigated. The influence of plasticizer type in Eudragit® RL100 film was studied by comparing dissolution profiles of LD from formulations EC/ERL100P/1 to EC/ERL100P/3 (Figure 5.32) (pellets coated with 3%,w/w of EC, 8%,w/w of TA, 14%,w/w of $NaHCO_3$ and 15%,w/w of Eudragit® RL100). DEP exhibit comparatively higher solubility than DBP (Frohoff-Hülsmann et al., 1999) resulting into faster drug release from EC/ERL100P/2 compared to formulation EC/ERL100P/3 (Figure 5.32a). Formulation EC/ERL100P/3 showed retarded drug release compared to formulation EC/ERL100P/1 and EC/ERL100P/2 which attributed to the higher solubilities of TEC and DEP compared to DBP (Figure 5.32a). (Frohoff-Hülsmann et al., 1999). However, significant difference had not been observed, when dissolution profiles of EC/ERL100P/2 and EC/ERL100P/3 were compared (f_2 = 79.59). In addition, similarity in dissolution profile between EC/ERL100P/3 and EC/ERL100P/1 indicated TEC also had insignificant influence on the drug release (f_2 = 71.56). Moreover, influence of plasticizer type in Eudragit® RL100 film on floating lag time and % floating pellets was also evaluated. Similar floating behavior was observed for all formulations indicating negligible influence of type of plasticizer on floating nature of designed pellets (Figure 5.32b).

Gastric juice is viscous in nature. Thus, the effect of viscosity on floating behavior of EC/ERL100/2 was also studied. EC/ERL100/2 showed similar floating behavior in 0.1N HCl and in 50cps viscous solution (HPMC E50, 50cps) (Figure 5.31c). The observed results confirms that the levels of effervescent agent and gas entrapment polymer layer was optimum to show desired floating behavior in viscous solution. However in vivo gastroretention studies in humans needed in order to confirm the observed results. Further, in vitro release profiles of LD and CD from EC/ERL100/2 were compared with the in vitro release profiles of LD and CD from Sinemet® CR (Figure 5.31d). Sinemet® CR controls release of both LD and CD up to 4 h whereas EC/ERL100/2 demonstrated controlled release of both LD and CD for 8-10 h. Thus, controlled release of LD and CD for longer period may reduce the dosing frequency and thereby more stable plasma concentration can be expected from designed formulation compared to the Sinemet® CR.

5.3.9. Release Kinetics

Results of release data was fitted to the various kinetic models and observed results are represented in Table 5.17a, Table 5.17b, Table 5.17c, Table 5.18 a, Table 5.18b and Table 5.18c. The observed lower AIC and regression coefficient values indicated that data was best fitted to the first order release kinetics and Korsmeyer peppas model. LD was released by first order release kinetics from almost all formulations (Table 5.17a, 5.17b and 5.17c). Further, n values for all formulations was observed in the range of 0.75 to 1.27 which demonstrated that LD was released by polymer relaxation and erosion process (Ritger and Peppas, 1987a).

Although overall release kinetic for formulations was found to be first order, optimized formulation EC/ERL100/2 showed constant drug release rate for initial 12 h for both LD and CD. A good regression coefficient for straight line fitted to the observed drug release data of LD (R= 0.9925) and CD (R= 0.9940) indicated zero order release for initial 12 h (Figure 5.33a). To understand the possible mechanism behind this type of release of drug from the designed formulation, some assumptions were made. It can be assumed that the drugs were uniformly distributed in spherical pellet, diffused dissolution medium build up the osmotic pressure inside coating layer and coating film has uniform thickness. In addition, it was also assumed that, coating film has pores of appropriate size there by drug diffusion transport and generation of hydrostatic pressure would be minimum. Possible release process of drug from the designed pellet is shown in Figure 5.33b.

The release of drug at constant rate for initial 12 h could be because of initial concentration of drug present in the pellets exceeds solubility of drug in permeated dissolution medium inside the coated layers. Therefore for initial 12 h, released drug molecules were replenished by partial dissolution of the drug present in undissolved form at core of the pellets resulting in maintenance of constant drug concentration in the form of saturated solution at inner surface of the coated membrane and thus, constant release rate was maintained. It has already been reported that, if drug release rate controlling membrane exhibit uniform thickness and constant permeability for the drug (due to formation of pores); in presence of perfect sink condition provided throughout release study such system follows constant (zero order) drug release rate till the end of saturation solution formation

irrespective of the geometry of the formulation (equation 5.15) (Baker, 1987; Siepmann and Siepmann, 2008).

$$\frac{dMt}{dt} = \frac{AJlim}{I} = \frac{ADKC_s}{I} \quad (5.15)$$

Where, Mt: amount of drug release at time t; dMt/dt: steady state release rate at time t; A: total surface area of the device; Jlim: membrane-limiting flux, I: thickness of the membrane, D: the diffusion coefficient of the drug within the membrane, K: the partition coefficient of the drug between the membrane and the reservoir, and C_s: the solubility of drug in the reservoir.

Therefore, it can be assumed that release of the drug is driven by generated osmotic pressure. Further, saturated solution formation process ends due to complete dissolution of the undissolved drug, thus release rates thereafter would be decided by the concentration of the drug present in the reservoir. Further, drug release driven by osmotic pressure is directly related to the solubility of the drug within core (Verma et al., 2002). The fraction of drug released (theoretical value) for EC/ERL100/2 can be calculated according to following equation 5.16 (McClelland et al., 1991; Zentner et al., 1991; Verma et al., 2002):

$$F = 1 - \frac{S}{\rho} \quad (5.16)$$

Where, F is the fraction of drug released by zero order kinetics, S is the saturation solubility (g/cm^3) of the drug in 0.1N HCl (at 37°C) and ρ is the density (g/cm^3) of solid drug. As discussed earlier in this case critical time point observed was 12 h. The observed cumulative LD and CD release at critical time point were found to be 75.23% and 70.25% whereas theoretical drug release values for LD and CD were 90.52% and 86.23%, respectively. Observed drug release values were found to be less than the theoretical values in case of both LD and CD which could be because of the ignoring the volume and thickness of ethyl cellulose and Eudragit® RL100 membrane swelling. Therefore, in order to confirm the validity of the assumptions, values of membrane volume and thickness has to be considered while estimating theoretical values.

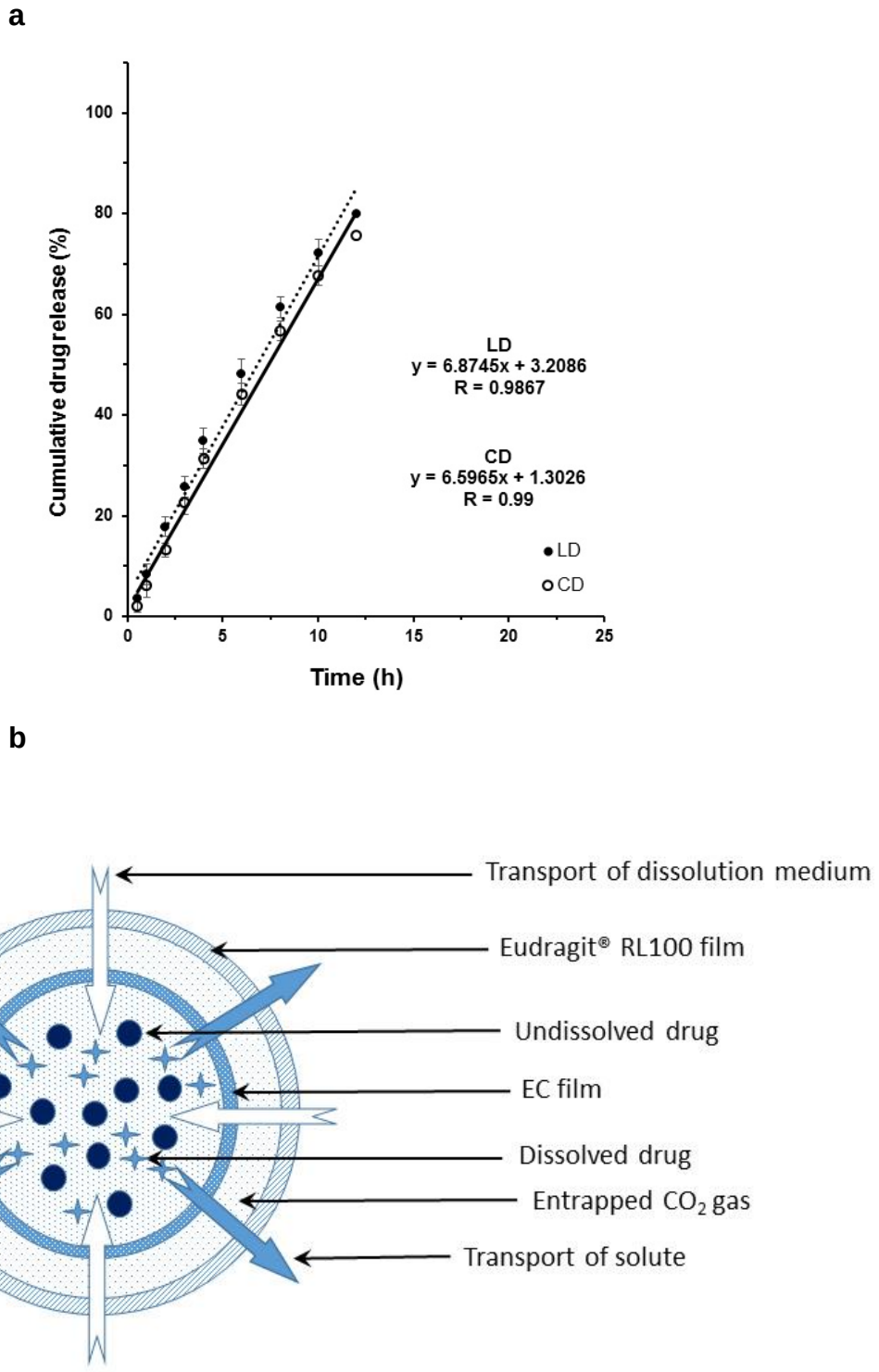


Figure 5.33. (a) Release of LD and CD in 0.1N HCl and (b) illustration of assumed release mechanism from the designed pellet.

Table 5.17a. Data of Release Kinetic Study (Zero Order, First Order) of Designed Formulations Coated with Single Gas Generating Layer (Sodium Bicarbonate Layer Alone) and Gas Entrapment Layer

Formulation code	MDT[a] (h)	$t_{80\%}$[b] (h)	Zero order model			First order model		
			R[c]	k_0 (mg% h^{-1})[d]	AIC[e]	R[c]	k_1 (h^{-1})[f]	AIC[e]
HPMC/ERL/1	1.28	1.67	0.3465	22.53	60.69	0.9947	0.77	26.94
HPMC/ERL/2	1.50	1.79	0.5414	21.46	58.49	0.9967	0.63	23.92
HPMC/ERL/3	1.67	1.93	0.6900	20.65	56.12	0.9943	0.53	28.15
HPMC/ERL/4	1.28	1.58	0.3734	22.57	60.61	0.9938	0.76	28.31
HPMC/ERL/5	1.51	1.81	0.5915	21.43	58.02	0.9955	0.61	26.47
HPMC/ERL/6	1.73	1.97	0.7005	21.04	56.02	0.9945	0.55	28.09
HPMC/ERL/7	1.29	1.76	0.4292	22.57	60.33	0.9923	0.74	30.20
HPMC/ERL/8	1.47	1.91	0.5651	21.95	58.53	0.9933	0.65	29.28
HPMC/ERL/9	1.74	1.95	0.7162	21.07	55.75	0.9932	0.54	29.60
EC/ERL/1	5.23	5.70	0.5213	5.79	107.67	0.9882	0.18	63.20
EC/ERL/2	7.12	10.14	0.7837	5.17	96.97	0.9917	0.12	57.82
EC/ERL/3	10.29	26.24	0.9669	3.17	64.91	0.9970	0.05	36.26
EC/ERL/4	12.67	69.99	0.9496	1.65	53.34	0.9828	0.02	40.43
EC/ERL/5	7.81	11.65	0.8566	4.79	91.16	0.9921	0.10	63.02
EC/ERL/6	6.15	8.84	0.7375	5.64	111.21	0.9878	0.15	63.51
EC/ERS/1	9.46	15.37	0.9558	4.13	75.83	0.9734	0.07	69.76
EC/ERS/2	10.36	21.12	0.9827	3.32	60.02	0.9783	0.05	62.68
EC/ERS/3	10.96	31.67	0.9886	2.61	49.62	0.9856	0.03	52.39
EC/ENE/1	10.25	22.87	0.9742	3.19	63.20	0.9896	0.05	52.32
EC/ENE/2	10.68	35.02	0.9813	2.43	53.45	0.9877	0.03	48.40
EC/ENE/3	11.25	49.60	0.9873	1.68	40.51	0.9889	0.02	38.89

[a]Mean dissolution time
[b]Time required to release 80% of drug
[c]Regression coefficient
[d,f]Drug release rate coefficient for zero order (k_0) and first order (k_1)
[e]Akaike information criterion

Table 5.17b. Data of Release Kinetic Study (Zero Order, First Order) of Designed Formulations Coated with Single Gas Generating Layer (Sodium Bicarbonate Layer Alone) and Gas Entrapment Layer Containing Pore Former

Formulation code	MDT[a] (h)	$t_{80\%}$[b] (h)	Zero order model			First order model		
			R^c	k_0 (mg% h^{-1})[d]	AIC[e]	R^c	k_1 (h^{-1})[f]	AIC[e]
EC/ERLRS/1	8.47	13.34	0.8999	4.87	87.49	0.9847	0.10	64.92
EC/ERLRS/2	8.88	14.38	0.9303	4.54	82.37	0.9822	0.08	66.01
EC/ERLRS/3	9.45	15.82	0.9560	4.25	75.82	0.9807	0.07	65.93
EC/ERLRS/4	10.16	24.30	0.9691	3.35	65.13	0.9970	0.05	37.19
EC/ERLRS/5	10.47	24.82	0.9814	3.10	58.23	0.9941	0.04	44.44
EC/ERLRS/6	10.89	30.96	0.9919	2.71	45.70	0.9903	0.04	47.89
EC/ERLRS/7	10.78	39.01	0.9889	2.29	44.56	0.9989	0.03	17.18
EC/ERLRS/8	11.04	40.16	0.9938	2.13	36.75	0.9961	0.03	31.02
EC/ERLRS/9	11.79	43.19	0.9977	1.83	22.09	0.9894	0.02	40.39
HPMC/ENEPEG/1	9.89	26.07	0.9357	3.06	72.68	0.9863	0.04	54.11
HPMC/ENEPEG/2	9.15	20.59	0.9235	3.50	77.38	0.9911	0.06	51.55
HPMC/ENEPEG/3	8.98	15.91	0.9237	4.27	81.58	0.9922	0.08	54.18

[a]Mean dissolution time
[b]Time required to release 80% of drug
[c]Regression coefficient
[d,f]Drug release rate coefficient for zero order (k_0) and first order (k_1)
[e]Akaike information criterion

Table 5.17c. Data of Release Kinetic Study (Zero Order, First Order) of Designed Formulations Coated with Two Gas Generating Layers (Tartaric Acid and Sodium Bicarbonate) and Gas Entrapment Layer

Formulation code	MDT[a] (h)	$t_{80\%}$[b] (h)	Zero order model			First order model		
			R^2[c]	k_0 (mg% h^{-1})[d]	AIC[e]	R^2[c]	k_1 (h^{-1})[f]	AIC[e]
ERL100/1	1.43	1.97	0.6089	25.42	56.28	0.9736	0.64	46.74
ERL100/2	1.51	2.08	0.6830	24.62	54.89	0.9735	0.58	46.54
ERL100/3	1.69	2.06	0.7851	23.76	52.29	0.9734	0.52	46.34
ERL100/4	1.44	1.87	0.6080	25.44	56.43	0.9721	0.64	47.26
ERL100/5	1.46	2.08	0.6445	24.27	55.59	0.9697	0.57	47.47
ERL100/6	1.69	2.15	0.7912	23.38	52.03	0.9731	0.50	46.28
ERL100/7	1.41	2.00	0.5955	25.51	56.43	0.9745	0.64	46.45
ERL100/8	1.56	2.09	0.7106	24.74	54.28	0.9748	0.58	46.16
ERL100/9	1.70	2.15	0.7938	22.46	51.53	0.9751	0.46	45.11
EC/ERL100/1	4.52	5.66	0.3624	5.96	110.94	0.9882	0.21	63.08
EC/ERL100/2	6.61	9.55	0.7200	5.45	100.71	0.9879	0.14	62.97
EC/ERL100/3	9.44	19.27	0.9459	3.66	74.04	0.9964	0.06	41.35
KSR/ERL100/1	6.01	8.08	0.6457	5.63	104.16	0.9877	0.16	63.79
KSR/ERL100/2	8.06	11.64	0.8652	4.85	90.77	0.9902	0.10	59.34
KSR/ERL100/3	10.15	26.97	0.9614	3.19	66.89	0.9963	0.05	38.91
EC/ERL100P/1	6.51	9.55	0.7300	5.45	109.23	0.9879	0.14	68.94
EC/ERL100P/2	6.71	10.25	0.7635	5.23	106.41	0.9965	0.12	71.16
EC/ERL100P/3	6.56	10.59	0.7123	5.24	104.33	0.9856	0.15	70.79
LC6/EC/ERL100	6.23	9.55	0.7356	5.45	109.23	0.9758	0.14	68.94
LC9/EC/ERL100	6.21	9.60	0.7545	5.40	108.19	0.9856	0.13	73.06

[a]Mean dissolution time
[b]Time required to release 80% of drug
[c]Regression coefficient
[d,f]Drug release rate coefficient for zero order (k_0) and first order (k_1)
[e]Akaike information criterion

Table 5.18a. Data of Release Mechanism (Korsmeyer-Peppas, Higuchi) of Designed Formulations Coated with Single Gas Generating Layer (Sodium Bicarbonate Layer Alone) and Gas Entrapment Layer

Formulation code	Korsmeyer-Peppas model				Higuchi model		
	R^a	k_{kp} (h^{-n})[b]	n[c]	AIC[d]	R^a	k_H ($h^{-0.5}$)[e]	AIC[d]
HPMC/ERL/1	0.9768	53.54	0.78	16.76	0.9355	48.34	20.48
HPMC/ERL/2	0.9735	47.00	0.91	16.35	0.8926	40.70	21.58
HPMC/ERL/3	0.9879	40.22	1.05	12.00	0.8574	33.48	21.49
HPMC/ERL/4	0.9847	54.35	0.84	15.23	0.9230	48.13	21.31
HPMC/ERL/5	0.9887	45.20	0.96	12.61	0.8863	38.56	21.45
HPMC/ERL/6	0.9911	40.79	0.99	10.81	0.8779	34.49	20.92
HPMC/ERL/7	0.9756	50.48	0.82	16.52	0.9242	45.05	20.69
HPMC/ERL/8	0.9832	45.95	0.86	14.26	0.9163	40.46	20.32
HPMC/ERL/9	0.9992	40.46	1.02	1.14	0.8753	33.95	20.90
EC/ERL/1	0.9976	10.31	1.18	9.20	0.7576	20.675	31.65
EC/ERL/2	0.9957	9.14	0.94	13.45	0.8340	16.244	34.67
EC/ERL/3	0.9890	5.15	0.84	36.57	0.8791	10.919	59.72
EC/ERL/4	0.9965	3.37	0.75	22.13	0.9258	6.365	57.98
EC/ERL/5	0.9982	6.91	0.99	12.06	0.8166	15.348	43.86
EC/ERL/6	0.9967	12.45	0.85	13.48	0.8705	19.753	34.75
EC/ERS/1	0.9953	3.67	1.13	28.41	0.7822	13.19	62.10
EC/ERS/2	0.9974	2.95	1.08	24.80	0.7942	10.94	67.68
EC/ERS/3	0.9891	3.06	0.94	49.87	0.8380	9.83	81.44
EC/ENE/1	0.9893	3.63	0.99	38.03	0.8197	10.81	65.43
EC/ENE/2	0.9840	3.12	0.91	52.48	0.8457	9.19	78.79
EC/ENE/3	0.9868	1.87	0.96	41.85	0.8287	6.29	71.73

[a]Regression coefficient
[b,e]Drug release rate coefficient for Korsmeyer-Peppas model (k_{kp}) and Higuchi model (k_H)
[c]Drug release exponent indicative of release mechanism
[d]Akaike information criterion

Table 5.18b. Data of Release Mechanism (Korsmeyer-Peppas, Higuchi) of Designed Formulations Coated with Single Gas Generating Layer (Sodium Bicarbonate Layer Alone) and Gas Entrapment Layer Containing Pore Former

Formulation code	Korsmeyer-Peppas model				Higuchi model		
	R^a	k_{kp} (h^{-n})[b]	n^c	AIC[d]	R^a	k_H ($h^{-0.5}$)[e]	AIC[d]
EC/ERLRS/1	0.9971	7.87	0.90	26.78	0.8667	17.38	60.29
EC/ERLRS/2	0.9962	5.91	0.98	27.99	0.8350	15.48	61.08
EC/ERLRS/3	0.9964	4.96	1.01	25.78	0.8246	13.82	59.93
EC/ERLRS/4	0.9984	5.39	0.85	18.06	0.8861	11.59	59.90
EC/ERLRS/5	0.9957	3.95	0.94	27.19	0.8464	10.46	62.23
EC/ERLRS/6	0.9948	3.41	0.92	41.17	0.8539	10.24	80.43
EC/ERLRS/7	0.9982	3.30	0.87	23.38	0.8773	8.69	73.36
EC/ERLRS/8	0.9966	2.66	0.92	30.37	0.8548	8.04	74.51
EC/ERLRS/9	0.9976	1.76	1.01	23.63	0.8207	6.83	74.33
HPMC/ENEPEG/1	0.9786	4.80	0.86	52.95	0.8596	11.56	72.80
HPMC/ENEPEG/2	0.9833	5.39	0.89	44.93	0.8524	12.86	65.93
HPMC/ENEPEG/3	0.9877	6.60	0.90	37.15	0.8558	14.76	58.49

[a]Regression coefficient
[b,e]Drug release rate coefficient for Korsmeyer-Peppas model (k_{kp}) and Higuchi model (k_H)
[c]Drug release exponent indicative of release mechanism
[d]Akaike information criterion

Table 5.18c. Data of Release Mechanism (Korsmeyer-Peppas, Higuchi) of Designed Formulations Coated with Two Gas Generating Layers (Tartaric Acid and Sodium Bicarbonate) and Gas Entrapment Layer

Formulation code	Korsmeyer-Peppas model				Higuchi model		
	R^a	k_{kp} (h^{-n})[b]	n[c]	AIC[d]	R^a	k_H ($h^{-0.5}$)[e]	AIC[d]
ERL100/1	0.9990	38.94	1.06	5.25	0.8973	41.80	23.31
ERL100/2	0.9954	35.61	1.11	10.74	0.8809	38.52	23.40
ERL100/3	0.9970	32.05	1.27	8.73	0.8393	35.66	24.32
ERL100/4	0.9974	38.89	1.15	9.33	0.8704	42.40	24.59
ERL100/5	0.9990	34.48	1.15	4.43	0.8731	37.58	23.51
ERL100/6	0.9961	30.69	1.25	9.46	0.8434	34.02	23.81
ERL100/7	0.9996	39.60	1.01	1.77	0.9103	42.20	22.74
ERL100/8	0.9948	36.11	1.08	11.26	0.8874	38.88	23.19
ERL100/9	1.0000	28.52	1.35	-9.64	0.8241	32.27	23.99
EC/ERL100/1	0.9952	13.74	1.01	13.02	0.8076	23.106	30.91
EC/ERL100/2	0.9996	10.83	0.88	0.14	0.8594	17.984	34.22
EC/ERL100/3	0.9937	5.23	0.92	37.70	0.8531	12.206	55.26
KSR/ERL100/1	0.9904	11.16	0.94	20.96	0.8269	20.001	37.63
KSR/ERL100/2	0.9842	7.45	0.96	27.86	0.8157	15.701	44.36
KSR/ERL100/3	0.9898	5.55	0.81	43.90	0.8740	11.155	60.79
EC/ERL100P/1	0.9996	10.83	0.88	-0.50	0.8594	17.984	39.59
EC/ERL100P/2	0.9956	10.85	0.87	15.13	0.8542	17.89	39.96
EC/ERL100P/3	0.9986	10.96	0.85	17.21	0.8456	18.25	51.40
LC6/EC/ERL100	0.9996	10.23	0.86	13.14	0.8652	17.85	40.89
LC9/EC/ERL100	0.9997	10.56	0.84	-0.50	0.8594	17.98	39.59

[a]Regression coefficient
[b,e]Drug release rate coefficient for Korsmeyer-Peppas model (k_{kp}) and Higuchi model (k_H)
[c]Drug release exponent indicative of release mechanism
[d]Akaike information criterion

5.3.10. Microscopic Studies

Scanning electron microscopy was used to examine surface morphology and cross section of the designed pellets (Figure 5.34). LD and CD loaded core pellet exhibits rough surface (Figure 5.34a) whereas comparatively smooth surface was observed in case of pellets coated with EC layer (Figure 5.34b). The rough surface was also observed in case of effervescent layer (Figure 5.34c). This may be because of the crystalline nature of the $NaHCO_3$. In comparison to the surface of EC coated pellet, pellet coated with Eudragit® RL30D exhibits more smoother surface this could be because of the fine particle size of the Eudragit® RL30D (Figure 5.34d). The protective layer, effervescent layer and outer layer (EC layer, HPMC:$NaHCO_3$ layer and Eudragit® RL30D layer respectively) were observed in SEM image (Figure 5.34e). During dissolution outermost polymeric membrane swells and entraps generated CO_2 gas into the swollen membrane. The floating pellet were taken out carefully and dried in oven. Before taking SEM photographs hole was made in outer membrane. The dried pellet and gap between swollen outer membrane and EC coated pellet are seen in SEM photograph (Figure 5.34f).

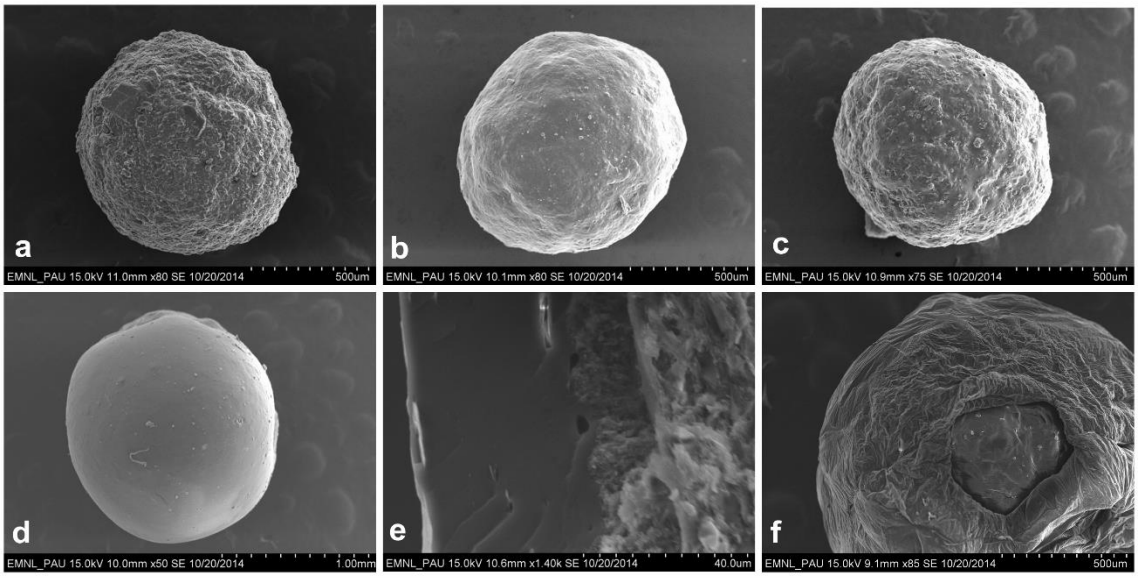


Figure 5.34. Scanning electron microscopy photomicrographs of (a) drug loaded core pellets (LC6); (b) ethyl cellulose coated pellet; (c) pellet coated with effervescent layer; (d) pellet coated with Eudragit® RL30D; (e) cross-section hemisphere of the optimized formulation (EC/ERL/2) and (f) surface of the dried pellet (EC/ERL/2) after exposure to 0.1N HCl.

Figure 5.35a and Figure 5.35b shows the morphology of core pellets (LC6) and the pellet coated with TA and NaHCO3 layer respectively. The outer Eudragit® RL100 layer, inner effervescent layer were observed in SEM image (EC/ERL100/2) (Figure 5.35c)

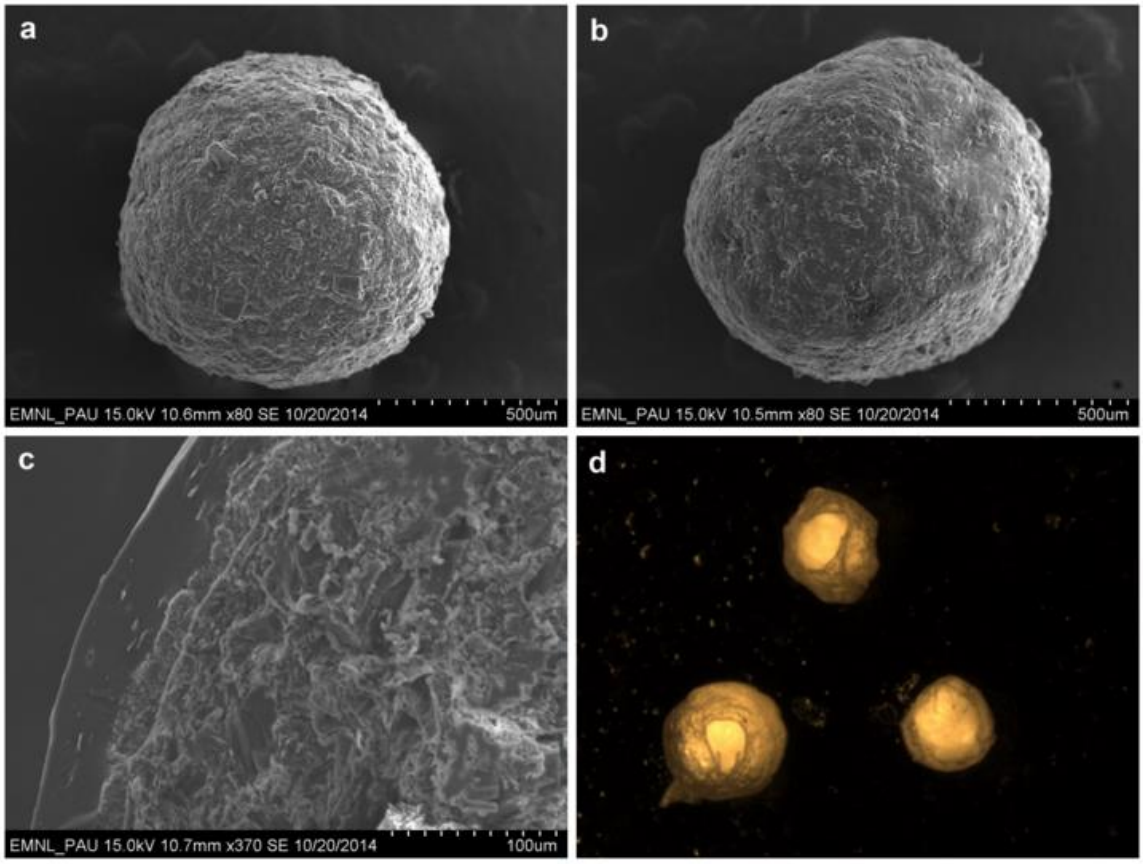


Figure 5.35. SEM images of (a) uncoated core pellet (LC6); (b) TA and $NaHCO_3$ coated pellet; (c) morphology of cross-section of optimized formulation and (d) stereo microscopic image of the pellets after exposure to the 0.1N HCl. (Broken hole was made in outer transparent Eudragit® RL100 membrane to visualizes the gap between outer membrane and the internal core of pellet)

Pellets (EC/ERL100/2) were exposed to dissolution medium and floating pellets taken out carefully and dried. The outer transparent gas entrapment polymeric membrane was clearly visible in photographs taken using stereo zoom microscope (Figure 5.35d). Internal core of the pellet coated with sustained release polymer was found to be intact. Further, when pellets were exposed to dissolution medium, gap generated between outer polymeric

membrane and internal intact core of the pellet demonstrated that effervescent layers had undergone dissolution and thereby generating CO_2. This gap was clearly seen in the photograph taken using stereo zoom microscope (Figure 5.35d).

Microscopic examination of floating process of designed pellets was also done. Formation and entrapment of CO_2 gas in outer gas entrapped polymeric membrane was monitored with the help of microscope. Photographs taken at different time interval after exposure of pellets to dissolution medium for different formulations (EC/ERL/2, EC/ERLRS/2 and EC/ERL100/2) are represented in Figure 5.36, Figure 5.37 and Figure 5.38. Transparent thin border around the pellets indicating hydration and permeation of dissolution medium was observed in the photograph taken immediately after exposure of pellets to dissolution medium (Figure 5.36a, Figure 5.37a and Figure 5.38a). Polymer swelling and CO_2 generation can be seen in microphotograph as thickness of transparent layer around solid core pellet increased. A thick broader film around the pellet core was observed which indicated completion of hydration process, CO_2 gas formation and entrapment of gas in outer polymer membrane.

5.3.11. Batch Reproducibility

Batch to batch variability and reproducibility of the manufacturing process was studied based on evaluation of the physical properties, floating behavior and release characteristic in triplicate from three batches of each of the designed formulations. Low values of standard deviation for assay, crushing strength and angle of repose for three independently prepared batches, indicated that the manufacturing process employed was reliable and reproducible (Table 5.12 to 5.14). Insignificant difference was observed in the in vitro release profile and floating behavior as indicated by low SD values, confirming excellent batch to batch reproducibility (Table 5.17 to 5.18).

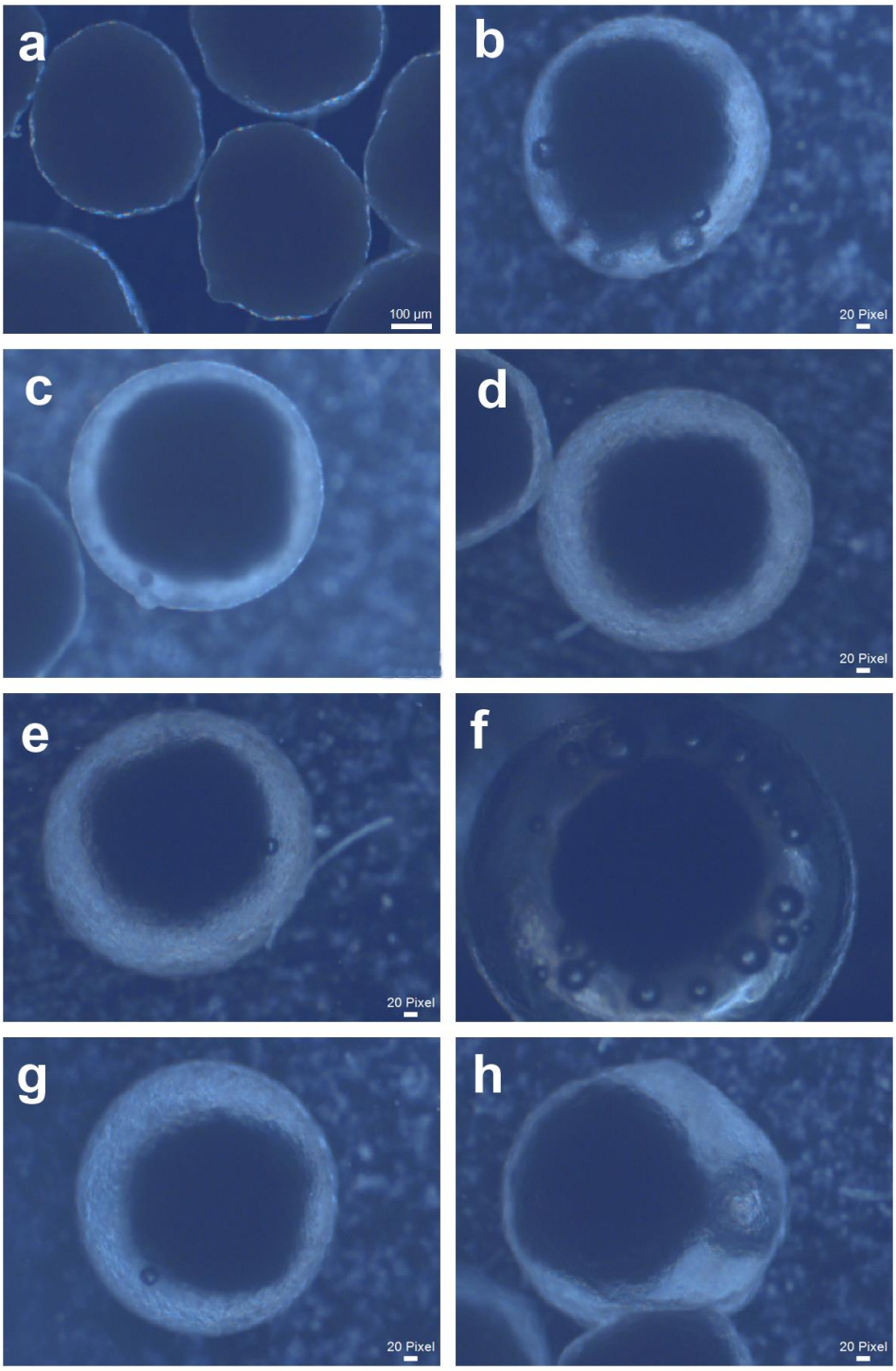


Figure 5.36. Photomicrographs of floating pellets of formulation EC/ERL/2 in 0.1N HCl: (a) 0 min; (b) 5 min; (c) 10 min; (d) 30 min; (e) 2 h; (f) 4 h; (g) 8 h and (h) 20 h.

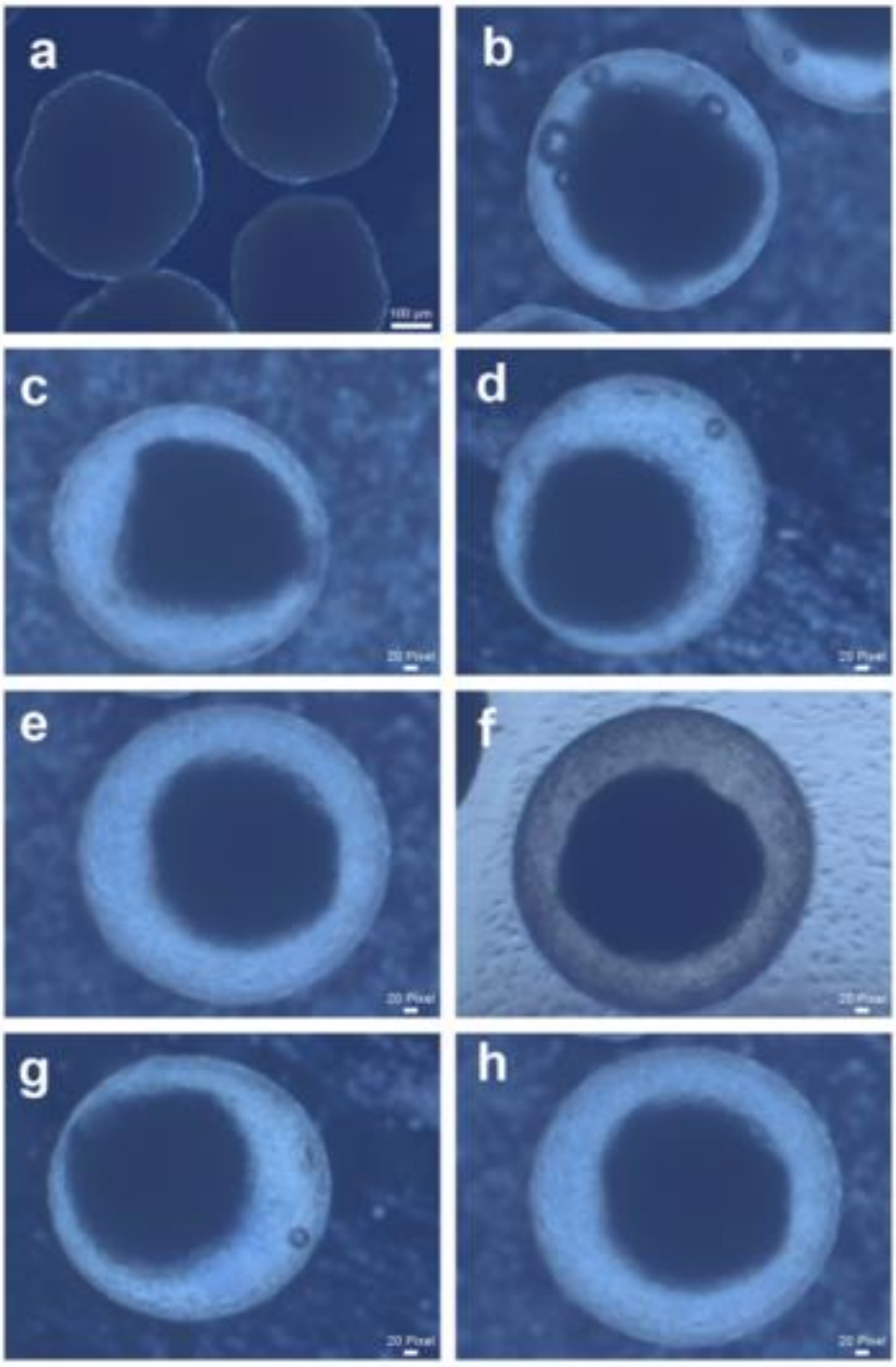


Figure 5.37. Photomicrographs of floating pellets of formulation EC/ERLRS/2 in 0.1N HCl: (a) 0 min; (b) 5 min; (c) 10 min; (d) 30 min; (e) 2 h; (f) 4 h; (g) 8 h and (h) 20 h.

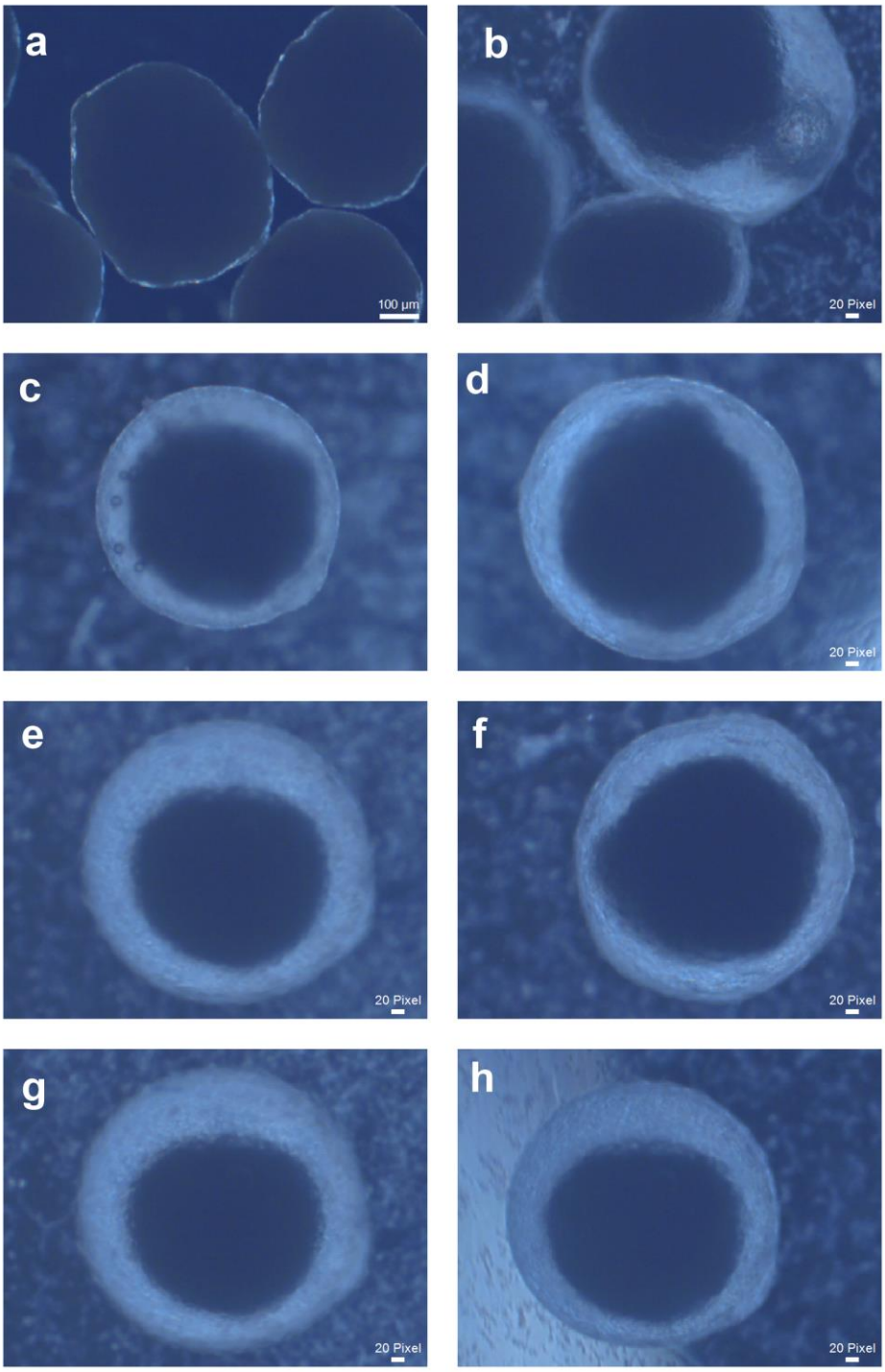


Figure 5.38. Photomicrographs of floating pellets of formulation EC/ERL100/2 in 0.1N HCl: (a) 0 min; (b) 5 min; (c) 10 min; (d) 30 min; (e) 2 h; (f) 4 h; (g) 8 h and (h) 20 h.

5.3.12. Stability Studies

The results of stability studies carried out on the designed formulations at different condition of temperature and humidity like controlled room temperature (CRT: 25 ± 2°C/ 60 ± 5% RH) and at accelerated condition (AT: 40 ± 2°C/ 75 ± 5% RH) are shown in Table 5.19. At refrigerated condition (FT: 5 ± 2°C) all the designed formulations were stable for entire study period (12 months). Hence the data has not been given for this condition. The log percent drug remaining to be degraded versus time profiles were linear for all designed formulations at various storage conditions indicating first order degradation kinetics. Low values of AIC and R values close to 1 demonstrated the first order kinetics of drug degradation (Table 5.19).

At accelerated condition, the maximum degradation rate constant for the LD and CD were found to be 60.77×10^{-4} $month^{-1}$ ($T_{90\%}$ = 17.28 months) and 56.77×10^{-4} $month^{-1}$ ($T_{90\%}$ = 18.50 months) respectively for formulation EC/ERLRS/2. The minimum degradation rate constant of 49.16×10^{-4} $month^{-1}$ was obtained for LD in case of formulation EC/ERL/2 whereas 49.20×10^{-4} $month^{-1}$ for CD in case of formulation EC/ERL100/2. These values were almost comparable to degradation rate constant and $T_{90\%}$ values of pure LD (46.12×10^{-4} $month^{-1}$, $T_{90\%}$ = 22.77 months) and pure CD (41.19×10^{-4} $month^{-1}$, $T_{90\%}$ = 25.49 months) obtained during preformulation studies [(Table 4.8) section 4.3.3b of chapter 4]. In vitro drug release profile of the aged samples was similar to zero time profiles for all the designed formulations (data not given). All the formulations were stable for entire study duration (6 months) with no apparent change in physical characteristics, in vitro release and floating behavior (Table 5.20).

In the formulations stored at CRT, the maximum degradation rate constant for the LD was found to be 29.96×10^{-4} $month^{-1}$ ($T_{90\%}$ = 35.05 months) for formulation EC/ERL100/2 and CD was 31.82×10^{-4} $month^{-1}$ ($T_{90\%}$ = 33.00 months) for formulation EC/ERL/2. The minimum degradation rate constant observed was 28.09×10^{-4} $month^{-1}$ with a predicted $T_{90\%}$ values of 37.38 months for LD in case of formulation EC/ERLRS/2 whereas 27.90×10^{-4} $month^{-1}$ with predicted $T_{90\%}$ = 37.64 months for CD in case of formulation EC/ERL100/2.

Table 5.19. First Order Degradation Kinetic Parameters of the LD and CD in Designed Formulations Evaluated for Stability Studies

Storage conditions	Formulation code	LD				CD			
		$K_d \times 10^4$ (month^{-1})[a]	$T_{90\%}$ (months)	R^2[b]	AIC[c]	$K_d \times 10^4$ (month^{-1})[a]	$T_{90\%}$ (months)	R^2[b]	AIC[c]
CRT (25 ± 2°C/ 60 ± 5% RH)	EC/ERL/2	28.80	36.37	0.9824	-4.68	31.82	33.00	0.9997	-20.89
	EC/ERLRS/2	28.09	37.38	0.9956	-0.48	28.67	36.62	0.9920	-7.91
	EC/ERL100/2	29.96	35.05	0.9983	-3.89	27.90	37.64	0.9921	-8.14
AT (40 ± 2°C/ 75 ± 5% RH)	EC/ERL/2	49.16	21.36	0.9907	-3.10	55.77	18.83	0.9965	-6.05
	EC/ERLRS/2	60.77	17.28	0.96.72	3.81	56.77	18.50	0.9774	1.71
	EC/ERL100/2	57.65	18.21	0.9724	2.63	49.20	21.34	0.9857	-1.29

[a] First order degradation rate constant
[b] Regression coefficient
[c] Akaike information criterion

Table 5.20. Stability Study Results for Designed Formulations Stored at Different Storage Conditions

Storage conditions	Formulation code	Floating lag time (min)	Pellets floating at 20 h (%)	$t_{80\%}$ (h)
Initial	EC/ERL/2	6.23 ± 1.25	91 ± 2	10.14
	EC/ERLRS/2	6.25 ± 1.00	90 ± 1	14.38
	EC/ERL100/2	7.50 ± 1.75	93 ± 1	9.55
CRT (25 ± 2°C/ 60 ± 5% RH)	EC/ERL/2	7.26 ± 1.25	88 ± 2	10.35
	EC/ERLRS/2	7.30 ± 1.75	85 ± 2	13.26
	EC/ERL100/2	8.69 ± 1.25	87 ± 3	8.26
AT (40 ± 2°C/ 75 ± 5% RH)	EC/ERL/2	6.96 ± 2.50	86 ± 2	9.56
	EC/ERLRS/2	7.63 ± 1.25	85 ± 2	14.23
	EC/ERL100/2	7.93 ± 1.23	85 ± 1	8.56

These values were almost comparable to degradation rate constant (27.27×10^{-4} $month^{-1}$) and $t_{90\%}$ (38.65 months) values of pure LD (30.25×10^{-4} $month^{-1}$, $T_{90\%}$ = 34.72 months) and pure CD (22.49×10^{-4} $month^{-1}$, $T_{90\%}$ = 46.68 months) obtained during preformulation studies [(Table 4.8) section 4.3.3b of chapter 4]. All the formulations were stable for entire study duration (12 months) with no apparent change in physical characteristics, in vitro release and floating behavior. The floating behavior of the aged samples were found to be similar to the fresh samples (Table 5.20). This implied absence of physical and chemical interaction between drug and selected formulation excipients.

5.4. Conclusions

The designed modified release floating pellets of LD and CD were found to possess good physical characteristics indicating suitability of manufacturing technique adopted for manufacture of the pellets. The assay values of all the designed formulations was found to be highly satisfactory. Acceptable values of friability, crushing strength, pellet shape and pellet size further confirmed suitability of the selected process parameters and adopted method. The designed formulations were found to be stable for 12 months when stored at CRT. This indicated that excipients, process and packaging materials adopted were appropriate. Further, method used for manufacturing was found to be relatively simple and can easily be adopted in conventional formulation manufacturing units on a commercial scale.

Drug release and floating properties of modified release floating pellets was affected by polymer nature and proportion, polymer combination and flexible nature of polymer. In present study, formulations extending release of LD and CD from 10 to 24 h were prepared. From almost all formulations, LD was released by polymer relaxation and erosion mechanism. Further, LD was released by first order releases kinetics from almost all formulations. Sustained release polymer (EC and Kollicoat® SR30D) coatings showed considerable influence on the LD release.

Designed pellet formulations were found to possess good floating behavior. Floating behavior of the designed formulations was affected by amount of effervescent agent coated, type, flexibility, hydrophilicity and porosity of gas entrapment polymer. Use of TA as effervescent layer along with $NaHCO_3$ layer showed additional benefits over the

formulations coated with $NaHCO_3$ layer alone. Formulations prepared by coating of both TA and $NaHCO_3$ layer as gas generating agents showed desired floating behavior irrespective of dissolution media pH.

Formulation showing extension of drug release between 10 - 12 h with desired floating properties were considered for further studies. Although formulations prepared by coating single effervescent agent ($NaHCO_3$) retarded LD release, it failed to show expected floating behavior in pH 3.0. Therefore, formulations prepared by coating single effervescent layer ($NaHCO_3$) were not considered for further studies. Formulation finally selected for in vivo studies were LC6 (immediate release pellets) and EC/ERL100/2 (modified release floating pellets).

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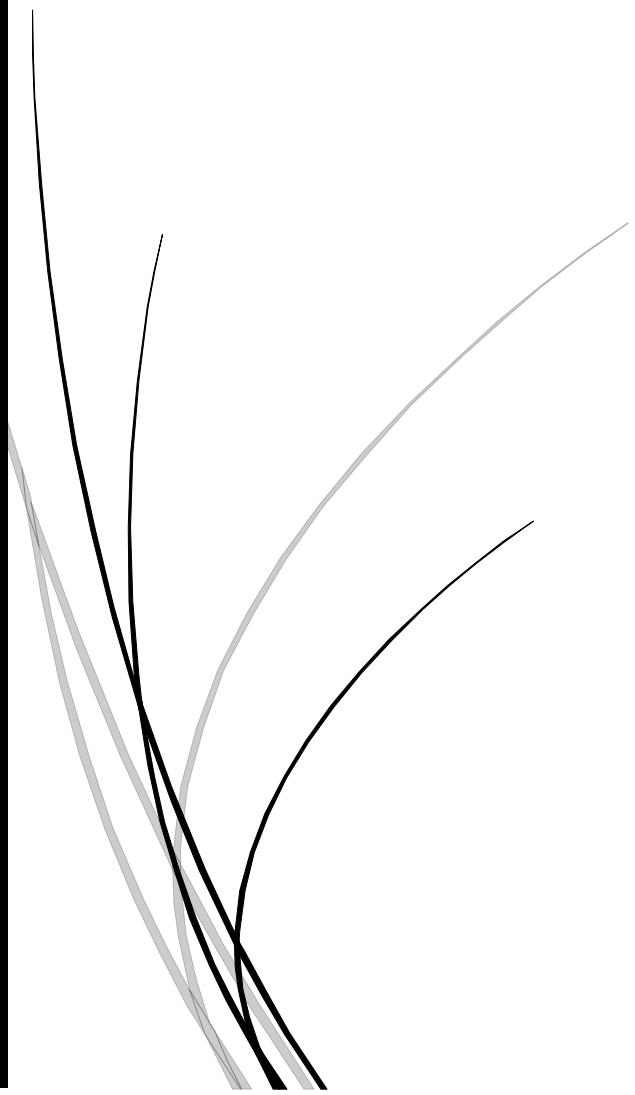
6

PHARMACOKINETIC AND PHARMACODYNAMIC STUDIES



6.1. Introduction

The fundamental objective of any drug delivery system is to eventually provide effective therapeutic concentration at site of action for intended duration to produce desired pharmacological effects with minimal or no undesirable or toxicological effects. Although the therapeutic advantage of the drug regimen is principally attributed to its intrinsic activity, the concentration level and duration for which it is maintained at site of action are critical for successful therapy. After administration of the dosage form, the conventional drug delivery systems (CDDS) undergoes processes such as release of free drug from dosage forms, absorption of free drug in to systemic circulation through various biological membranes, distribution to various body tissues including site of action and subsequently providing therapeutic effect. Further free drug also undergoes metabolism and excretion from the body. The main problem associated with CDDS is fluctuating plasma level for drugs having short half-life and narrow absorption window (NAW) in upper gastrointestinal tract (Singh and Kim, 2000). For such drugs, controlled release (CR) drug delivery systems can be suitable option to overcome the problems of CDDS like fluctuating plasma level. However, CR systems fail to provide constant supply of drug above absorption site which leads to wastage of drug and fluctuating plasma level. Further, fluctuating plasma level may leads to recurrence of disease symptoms.

On contrary, modified release gastroretentive delivery systems (MRGRDS) are capable to supply drug at site of absorption. In addition, MRGRDS releases drugs at controlled rate there by stable plasma concentration of the drugs can be maintained. Moreover, stable plasma concentration would reduce dosing frequency and therefore patient compliance can be promoted. Thus, increase in rate and extent of absorption due to supply of complete dose of drug at site of absorption would eventually improve the efficiency of drug delivery system leading to better therapeutic performance with minimal untoward effects and better patient compliance (Singh and Kim, 2000; Talukder and Fassihi, 2004; Chavanpatil et al., 2006; Rao et al., 2013).

From regulatory point of view, an important consideration for the development of any drug delivery system (DDS) is the quality of evidence needed under particular circumstances to substantiate the proof of therapeutic effectiveness. The concept of improved therapeutic efficacy by using MRGRDS is based on the assumption that GRDDS are capable of

retaining in stomach and releasing drug at controlled rate above absorption site (Dhaliwal et al., 2008; Shakya et al., 2013; Sharma et al., 2015). Considering the fact that, the in vivo fate of MRGRDS is significantly different in comparison to CDDS, detailed in vivo investigation to estimate biological fate of designed MRGRDS is essential (Streubel et al., 2006; Tadros, 2010). Moreover, MRGRDS has capability to modulate rate and extent of drug absorption by delivering drug at controlled rate and therefore, pharmacokinetic profiles of MRGRDS and CDDS may not be identical (Zhang et al., 2012). Thus, it is important to have complete understanding of the relationship between drug concentration and therapeutic effect or response, in preclinical animal model before exploring its final clinical benefits. Thus, effectiveness of the developed MRGRDS as a new drug product cannot be demonstrated without complete pharmacokinetic studies (Singh and Kim, 2000; Arora et al., 2005).

Although, FDA has not released any specialized guidelines for pellet formulations, pharmacokinetic requirement for novel drug delivery system (NDDS) covered under federal register are extended to these formulations (21 CFR part 314). As of now these regulations indicate that pharmacokinetic data obtained from plasma concentration time profile of a drug under investigation is sufficient for its regulatory approval as MRGRDS. Although the purpose of the pharmacokinetic study is to verify safety and efficacy of the candidate drug, application of pharmacokinetic principles in the design and development of MRGRDS provides the rational for designing effective and better delivery system. Moreover, pharmacokinetic study aid in understanding the complex relationship of these unit biological processes to modulate the intensity and time duration of therapeutic and adverse effect of the drug. In addition, pharmacodynamic studies in suitable animal model present a valuable information about therapeutic efficacy of active pharmaceutical ingredient. Gastroretentive delivery systems have capability to improve therapeutic efficacy by modulating rate and extent of drug absorption. In vivo pharmacokinetic evaluation of MRGRDS systems have already been reported in variety of animal models like rabbits (Joseph et al., 2002; Ali, 2007; Zhang et al., 2012), rats (Elmowafy et al., 2009; Zhu et al., 2014) and dogs (Klausner et al., 2003a; Patel et al., 2009). Studies have also been reported in humans (Sato et al., 2004; Xu et al., 2006; Goole et al., 2008; Shakya et al., 2013). In literature, pharmacodynamic studies for pure drugs and novel formulations

have also been reported using rat (Stocchi and Olanow, 2004; Schmidt et al., 2008; Ren et al., 2011).

This chapter presents in vivo oral pharmacokinetic and pharmacodynamic studies of selected batches of designed modified release floating pellets, immediate release pellets and pure drug. The pharmacokinetic parameters of LD, CD and 3-OMD (metabolite of LD) following administration of designed formulations were assessed in rats. Further, pharmacodynamic evaluation of designed formulation was also performed using 6-OH dopamine (6-OHDA) rat model in order to predict therapeutic effectiveness of designed formulations.

6.2. Experimental

6.2.1. Materials

Xylazine and ketamine used for inducing anesthesia to rats were purchased from local markets. 6-OH-dopamine hydrochloride (6-OHDA.HCl) (purity ≥ 97.0%) desipramine hydrochloride (purity ≥ 98.0%) and apomorphine hydrochloride (purity ≥ 98.0%) were purchased from Sigma-Aldrich® Corporation, India. The other materials and reagents used were same as mentioned in section 3.2 and section 5.2.1 of Chapter 3 and Chapter 5, respectively.

6.2.2. Formulation and Sample Preparation

The immediate release pellets containing LD and CD were manufactured by extrusion spheronization technique using Avicel® PH101 as spheronization aid and water as granulating liquid. In addition, PVPK30 was used as binder. Dissolution studies were also performed in order to verify immediate release of LD and CD from the designed formulations. Fresh batches of LC6 (Chapter 5, Table 5.1) and EC/ERL100/2 (Chapter 5, Table 5.6) were manufactured before experiment. Further, all the quality control tests mentioned in Chapter 5 were carried out for immediate release and freshly prepared modified release floating pellets before proceeding with animal dosing.

6.2.3. Oral Pharmacokinetic Studies

6.2.3.1. Animal Model

Adult male Wistar rats (weight 200 ± 20 g) were supplied by Central Animal Facility of BITS Pilani (India). Animal studies were performed as per the protocol (Protocol No. IACE/RES/18/22) approved by Institutional Animal Ethics Committee (IAEC), BITS Pilani and under the supervision of registered veterinarian. Animals were issued 15 days before commencement of the study and were kept under standard environmental conditions with free access to standard laboratory food and water ad libitum. All experimental procedures including euthanasia and disposal of carcass were carried out in accordance with the guidelines set by the IAEC, BITS Pilani.

6.2.3.2. Administration of Pure Drugs and Formulations

Animals were kept on fasting overnight with free water access before initiation of the study. Rats were randomly divided in to three groups (9 rats per group). Solution of LD and CD (mg equivalent to the 56 mg/kg and 14 mg/kg of LD and CD respectively) was orally administered to the first group of rats whereas second and third group of rats orally received immediate release pellets (IRP, LC6) and modified release floating pellets (MRFP, EC/ERL100/2) at same dose respectively. After administration of formulation, 2 mL water was administered to ensure complete swallowing of formulation.

6.2.3.3. Blood Sample Collection

Rats were anaesthetized in diethyl ether chamber and blood (400 µL) was withdrawn from the retro orbital plexus in to chilled poly propylene tube containing 40 µL anticoagulant solution (mixture of 10%, w/w Na_2EDTA and 5%, w/w $Na_2S_2O_5$ in water). The blood samples were withdrawn at 0.25, 0.5, 0.75, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0 and 18.0 h post dosing. The tubes containing blood samples were centrifuged (Eppendorf centrifuge - 5702R) immediately at 12000 rpm for 10 min at 4°C. The resulting supernatant plasma layer was collected carefully and stored at -80°C until further processing for analysis.

6.2.3.4. Analysis of Plasma Samples

Plasma sample obtained from each animal at respective time point were processed independently. Concentration of drugs present in the plasma was determined by validated bioanalytical method discussed in Chapter 3.

6.2.3.5. Rat Plasma Sample Preparation

Extraction of LD, CD and 3-OMD from the plasma samples was carried out using simple protein precipitation method. Plasma protein precipitation was carried out using TCA. To the each aliquot of plasma sample (200 µL), fixed amount of internal standard (IS, catechol) was added and vortex mixed for 5 min. Resulting sample was further vortex mixed with 195 µL of TCA (10%, w/w in water) for 5 min to ensure complete precipitation of plasma protein. Resulting sample was then centrifuged at 17000 rpm for 20 min at 4°C (Eppendorf centrifuge - 5702R) and obtained clear supernatant was analyzed using HPLC coupled with electrochemical detector (method discussed in Chapter 3, Section 3.5.3).

6.2.3.6. Data Analysis

Drug concentration in plasma at different time intervals was analyzed by the non-compartmental analysis method using WinNonlin® standard edition, version 2.1 (Pharsight Corporation, Mountain view, CA, USA) to estimate various pharmacokinetic parameters such as area under curve (AUC), area under the moment curve (AUMC) and mean residence time (MRT). The maximum drug concentration (C_{max}) and time to reach maximum concentration (T_{max}) were determined by model independent method. Finally results of oral pharmacokinetic studies were assessed using suitable statistical tests at 5% level of significance.

6.2.4. Pharmacodynamic Studies

6.2.4.1. Animal Model

Adult male Wistar rats (weight= 250 ± 20 g) were supplied by Central Animal Facility BITS Pilani (India). Before commencement of animal studies prior approval (Protocol No. IACE/RES/18/22) was obtained from Institutional Animal Ethics Committee (IAEC), BITS Pilani. Animal studies were performed with respect to the guidelines provided by the

Institutional Animal Ethics Committee and under the supervision of a registered veterinarian. Animals were issued 15 days before study and kept in standard cages under environmentally controlled conditions (ambient temperature: 25 ± 2°C; RH: 50 ± 5 %) on a 12 h light/dark cycle with free access to standard laboratory food and water ad libitum.

6.2.4.2. Induction of Experimental Parkinson's Disease by 6-OHDA Administration

Unilateral nigrostriatal lesions were produced by injecting 6-OHDA in to rat medial forebrain bundle (MFB). Rats were anesthetized using ketamine (100 mg/kg, i.p) and xylazine (5 mg/kg i.p) and placed into a stereotaxic frame (Inco, Ambala). As 6-OHDA damages both dopaminergic and noradrenergic axons in the MFB, desipramine (25 mg/kg i.p.) was administered to rats in order to prevent the damage to noradrenergic neurons. A midline sagittal incision was made in the scalp and bregma was located. A dental drill was used to make a hole through the skull. An infusion of 6-OHDA (4 mg/mL) was prepared by dissolution of 6-OHDA.HCl in saline containing 0.02%, w/v ascorbic acid. All the rats except vehicle control were infused with 16 µg of 6-OHDA (4 µL) unilaterally into the right MFB using following coordinates: - 4.4 mm posterior to bregma; 1.2 mm lateral to sagittal suture and - 7.8 mm ventral from the surface of the brain (Swanson, 2004; Paxinos and Watson, 2007). The neurotoxin was injected for 4 min at rate of 1 µL/min with help of 25 µL Hamilton syringe. Syringe was left in place for 4 min after the injection was finished. Immediately after surgery, the rats were injected with gentamicin (5 mg/kg, i.p) and housed individually in polypropylene cages for a week and then they were re-grouped in their home cages. Apomorphine (3 mg/kg, i.p) induced rotation were used to select animals with complete lesions in MFB. Two weeks after the 6-OHDA injections, rats exhibiting stable apomorphine-induced rotational asymmetry of at least 7 contralateral rotations per min were selected for the further studies.

6.2.4.3. Experimental Design

All valid PD rats were grouped in to five groups (9 rats per group) viz. group 1**:** Disease control (6-OHDA) (n = 9); group 2**:** vehicle control (n = 9); group 3**:** LD + CD (pure drug) (n = 9); group 4: IRP (LC6) (n = 9); group 5**:** MRFP (EC/ERL100/2) (n = 9). Experimental schedule is represented in Figure 6.1.

Rats in the group 1 (i.e. 6-OHDA treated) (n = 9) and group 2 (n = 9), were treated with vehicle (water) daily for 3 weeks. Rats in the group 3 were treated with LD (56 mg/kg, oral) plus CD (14 mg/kg, oral) in the form of solution daily for 3 weeks. Rats in the group 4 were treated daily for 3 weeks with IRP (LC6) containing LD plus CD at same dose orally. Rats in the group 5 were also treated on daily basis for 3 weeks with MRFP (EC/ERL100/2) containing LD (56 mg/kg, oral) plus CD (14 mg/kg, oral). After administration of formulation, 2 mL water was administered to ensure complete swallowing of formulation.

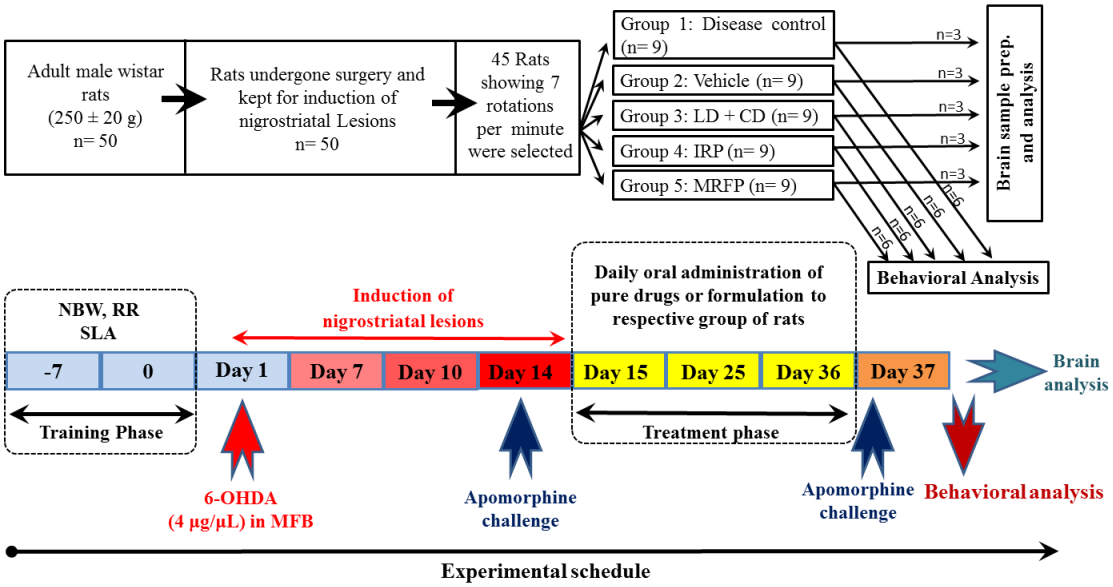


Figure 6.1. Schedule of the experimental performance.

[NBW= narrow beam walk test, RR= rotarod activity, SLA= spontaneous locomotor activity]

Behavioural analysis was carried out by estimation of apomorphine-induced rotations, abnormal involuntary movements (AIM), narrow beam walk test (NBW), rotarod activity and locomotor activity. Moreover, rat brains (three rats from each group) were collected for estimation of levels of dopamine, 3-OMD and DOPAC. These levels were analyzed in left and right lobe of each brain sample separately.

6.2.4.4. Evaluation of Behavioral Parameters

a. Apomorphine Induced Rotations

Apomorphine show a characteristic contralateral turning behavior when the supersensitive receptors in the lesioned side of the brain are activated and the rats starts showing turning behavior to the contralateral side. On test days, after administration of apomorphine (3 mg/kg i.p), apomorphine-induced rotations were counted for 30 min (Sharma et al., 2015).

b. Measurement of Abnormal Involuntary Movements (AIM)

Rats were monitored for AIMs using a procedure similar to that described by (Yang et al., 2012). On test days, rats were individually placed in plastic trays 5 min before drug treatment. Following injections, each rat was assessed for exhibition of axial, limb, and orolingual (ALO) and locomotive movements. At every 20 min intervals (i.e., 20, 40, 60, 80 min etc.), AIMs were rated for 60 s for each rat for a total of 2 hours, during which a severity score between 0 and 4 was assigned for each AIMs category. For each AIMs category, the scores for each time point were summed.

c. Narrow Beam Walk Test

Gait abnormalities and foot slip count was measured by narrow beam walk apparatus as per method described in literature (Sharma and Deshmukh, 2015). Briefly, the apparatus consists of a horizontal narrow beam (1 cm × 100 cm) suspended 1 m above a foam-padded cushion. A black box was placed at the end of the beam as finish point. A lamp (with 60 watt light bulb) was used to shine light above the start point and serves as an aversive stimulus. Time taken to cross the beam was manually measured by using stopwatch. During testing, the rats were given 1 min to traverse the beam. The latency to cross the beam along with their number of foot slips was recorded. If the rats did not complete the task or if they fell off from the beam or freeze, then they were assigned a maximum latency of 60 s to cross the beam and maximum 5 foot slips.

d. Rotarod Activity

Motor coordination was assessed using an automated rotarod apparatus. Rota rod motor training was performed at the beginning of the experiment. The apparatus (Inco, Ambala,

India) consists of a metal rod of 4 cm in diameter, 75 cm in length with 6 equally divided sections. Each rat was tested on the rotarod apparatus. Rats had to keep their balance on a rotating rod set at a speed of 15 rpm for a maximum of 300 seconds. The latency to fall from the rod was recorded (Sharma et al., 2015).

e. Locomotor Activity

Each animal was tested for spontaneous locomotor activity. Each animal was observed over a period of 10 min in a square closed arena (30 cm × 30 cm) equipped with infrared (IR) light sensitive photocells using a digital actophotometer (INCO, India) (Sharma et al., 2015). The number of times the IR photobeams of light were interfered, was recorded for each animal.

6.2.4.5. Collection of Brain Sample and Processing

Rats were anaesthetized in diethyl ether chamber and sacrificed by cervical dislocation. Immediately after cervical dislocation, heart was exposed and brain was then perfused through the aorta with ice-cold saline containing ascorbic acid (200 µg/L). After perfusion, right lobe (lesioned side) and left lobe of rat brain were removed quickly from the rat skull and stored at -80°C separately until further processing.

6.2.4.6. Brain Sample Analysis

Left lobe and right lobe of rat brain were processed and analyzed individually. Brain samples were homogenized in ice-cold solution (1 g/mL) containing mixture of 100 ng/mL of catechol, 0.1M of HCLO4, 0.4 mM of $Na_2S_2O_4$ and 1 mM of Na_2EDTA in water using tissue homogenizer. Resulting brain homogenate was centrifuged at 17000 rpm for 20 min at 4°C (Eppendorf centrifuge-5702R). The supernatant obtained was injected in HPLC system coupled with ECD for analysis (method discussed in Chapter 3, Section 3.5.4).

6.2.4.7. Statistical Analysis

Results of the behavioral studies were assessed using one way ANOVA test followed by tukey's multiple comparison post-hoc test at 5% level of significance.

6.3. Results and Discussion

6.3.1. Oral Pharmacokinetic Study

Oral pharmacokinetic study was carried out for LD and CD combination administered orally in the form of solution, IRP and MRFP. The plasma concentration versus time profile of LD, CD and 3-OMD following administration of various formulations are shown in Figure 6.2, 6.3 and 6.4 respectively. Further, summary of various pharmacokinetic parameters estimated by non-compartmental data analysis is represented in Table 6.1.

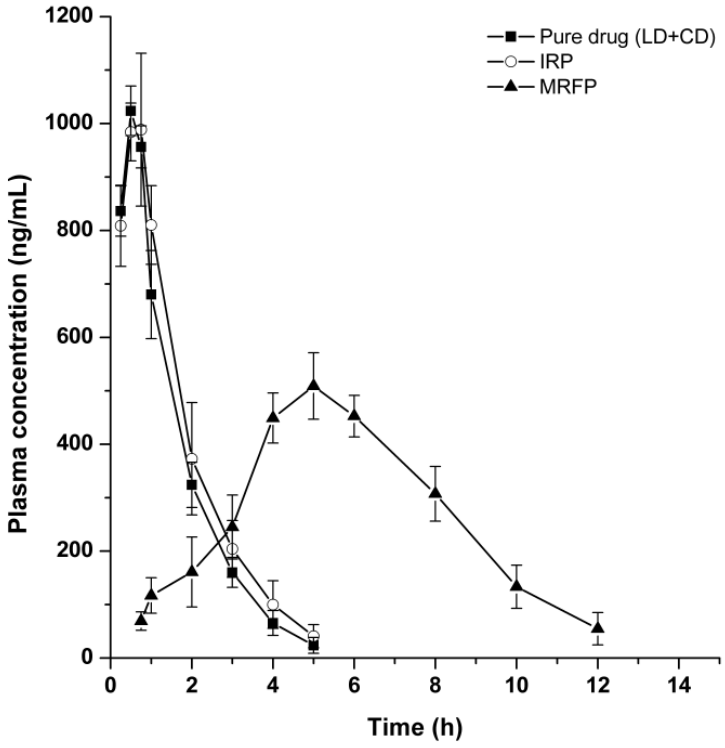


Figure 6.2. Plasma concentration time profile of LD for oral solution of pure drug, IRP and MRFP in rat plasma (Each value represents mean of three independent determinations with standard deviation). (IRP = LC6; MRFP = EC/ERL100/2).

On administration of oral solution of pure drug, LD and CD showed maximum concentration (C_{max}) of 1023.68 ± 46.43 ng/mL at 0.50 h and 124.62 ± 22.86 ng/mL at 0.75 h respectively. In addition, $AUC_{0-\infty}$ values for LD and CD following administration of oral solution of pure drug were found to be 1721.02 ± 127.58 ng.h/mL and 250.47 ± 39.83 ng.h/mL respectively (Table 6.1). Further, the C_{max} and T_{max} values observed for LD and

CD following oral administration of IRP were compared with that obtained after oral administration of oral solution of pure drug. Difference in C_{max} and T_{max} values for LD in case of oral solution of pure drug and IRP was not observed which can be attributed to the immediate absorption of LD in both cases. In addition, similar MRT and $AUC_{0-\infty}$ values for LD in case of oral solution of pure drug and IRP were observed which could be due to similar rate and extent of LD absorption.

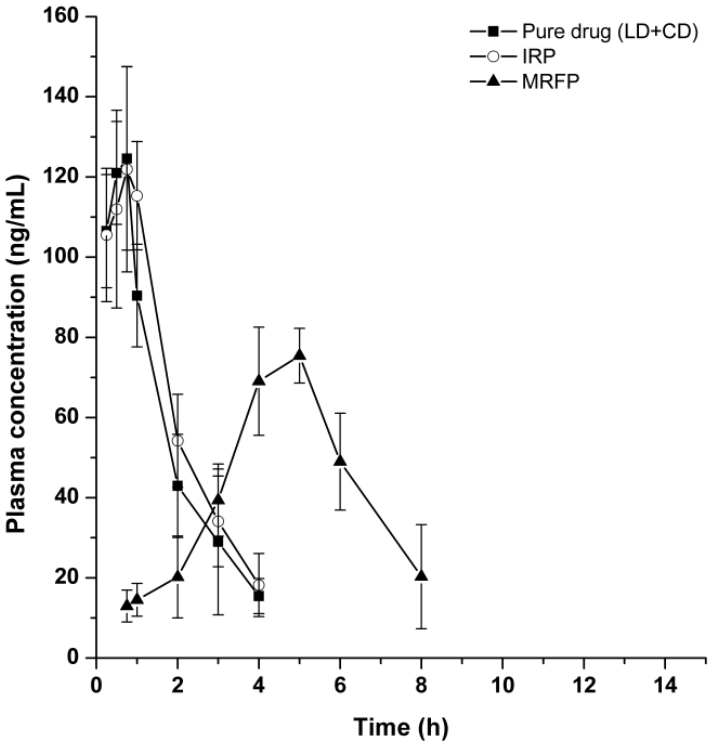


Figure 6.3. Plasma concentration time profile of CD for oral solution of pure drug, IRP and MRFP in rat plasma (Each value represents mean of three independent determinations with standard deviation) (IRP = LC6; MRFP = EC/ERL100/2).

Pharmacokinetic profiles of LD obtained following administration of MRFP were compared with that of IRP. $AUC_{0-\infty}$ values for both LD and CD were found to be significantly (5% level of significance) higher following administration of MRFP compared to IRP and oral solution of drug ($F_{(2, 6)}$ = 19.91) (Table 6.1). This could be because of controlled release of LD and CD. In addition, low C_{max} (LD = 509.04 ± 62.11 ng/mL, CD = 75.42 ± 6.81 ng/mL) and high T_{max} values (LD and CD= 5.00 h) were

observed for both LD and CD in case of MRFP in comparison to the IRP and oral solution of pure drug which may attributed to the slower release of LD and CD from the MRFP extending the absorption phase. Further, MRT values for LD observed for MRFP (6.22 ± 0.14 h) was higher than the MRT values obtained for IRP (1.56 ± 0.21 h). Higher MRT values might be due to the slow release of drugs from the MRFP. The observed results were found to be similar to the previous reports suggesting increase in AUC and MRT by sustained release formulations compared to immediate release formulations (Harder et al., 1995).

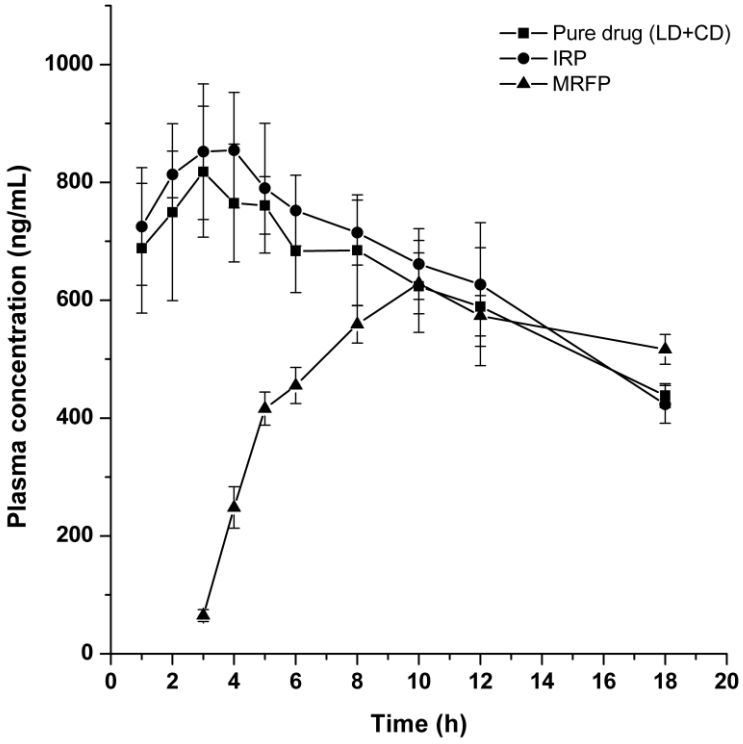


Figure 6.4. Plasma concentration time profile of 3-OMD for oral solution of pure drug, IRP and MRFP in rat plasma (Each value represents mean of three independent determinations with standard deviation) (IRP = LC6; MRFP = EC/ERL100/2).

The pharmacokinetic profiles of 3-OMD obtained following administration of MRFP, oral solution of pure drug and IRP is shown in Figure 6.4. Higher $AUC_{0-\infty}$ for 3-OMD was observed for MRFP (27980.10 ± 4599.91 ng.h/mL) compared to oral solution of pure drug (19543.60 ± 193.92 ng.h/mL) and IRP formulation (21244.88 ± 2295.10 ng.h/mL). This

can be attributed to slower release of LD from MRFP. During the sampling period used in the study 3-OMD concentration were found to decline relatively at slower rate than LD. Further, the $AUC_{0-\infty}$ of 3-OMD was found to be higher than the LD in all types of formulation supporting the fact that 3-OMD has higher mean residence time than LD (Bredberg et al., 1994). The observed results were found to be in line with the previous study reports demonstrating the superiority of controlled release gastroretentive formulations over Sinemet® CR preparation studied in healthy human volunteers (Klausner et al., 2003a; Klausner et al., 2003b).

These finding provides the evidence of the potential of MRFP for enhancing bioavailability. Although the $AUC_{0-\infty}$ for LD increased, there was no immediate absorption phase, instead gradual absorption occurred. In order to avoid such gradual buildup of absorption profile, modified release floating formulation has to be administered either with immediate release component which would act as loading dose for immediate onset of action or delivery system has to be further optimized for faster release rate of drugs. Further, various studies have already been reported indicating that formulations showing good in vitro floating behavior, had showed prolonged gastroretention in vivo (Iannuccelli et al., 1998; Singh and Kim, 2000). Therefore, in this study gastroretention studies has not been performed and pharmacokinetic studies were carried out based on assumption that designed formulation would show similar results for gastroretention in in vivo conditions. However, gastroretention studies should be carried out in human subjects to evaluate in vivo floating ability of the designed formulations.

Table 6.1. Pharmacokinetic Parameters Obtained Following Oral Administration of Oral Solution of Pure LD+ CD, IRP and MRFP in Rats

Formulation	Analyte	Pharmacokinetic parameters				
		C_{max}[a] (ng/mL) (Mean ± SD, n = 3)	**T_{max}[b] (h) (Mean ± SD, n = 3)**	**$AUC_{0-\infty}$[c] (ng.h/mL) (Mean ± SD, n=3)**	**$AUMC_{0-\infty}$[d] ($ng.h^2$/mL) (Mean ± SD, n=3)**	**MRT [e] (h) (Mean ± SD, n = 3)**
Pure drug Solution	LD	1023.68 ± 46.43	0.50	1721.02 ± 127.58	2425.17 ± 371.71	1.40 ± 0.16
	CD	124.62 ± 22.86	0.75	250.47 ± 39.83	457.46 ± 139.07	1.81 ± 0.36
	3-OMD	818.04 ± 111.01	3.00	19543.60 ± 193.92	391271.62 ± 40392.40	20.03 ± 2.27
IRP	LD	988.53 ± 142.90	0.75	1956.54 ± 315.88	3093.91 ± 909.57	1.56 ± 0.21
	CD	121.94 ± 25.62	0.75	287.72 ± 40.82	561.07 ± 193.86	1.92 ± 0.46
	3-OMD	854.67 ± 98.00	4.00	21244.88 ± 2295.10	514555.86 ± 90984.39	24.13 ± 1.68
MRFP	LD	509.04 ± 62.11	5.00	3244.34 ± 433.42	20215.08 ± 3174.89	6.22 ± 0.14
	CD	75.42 ± 6.81	5.00	377.29 ± 66.03	2172.82 ± 976.78	5.59 ± 1.47
	3-OMD	628.66 ± 51.73	10.00	27980.10 ± 4599.91	1262908.03 ± 374199.71	44.64 ± 6.04

[a] Peak plasma concentration
[b] time at C_{max}
[c] Area under curve from time 0 to ∞
[d] Area under first moment curve from time 0 to ∞
e Mean residence time

6.3.2. Pharmacodynamic Study

LD remains the most effective drug in treatment of the PD. However, long term treatment with LD has been reported to result in Levodopa induced dyskinesias (LIDs) and other abnormal involuntary movements (AIMs) in PD patients as well as in experimental animals (Hornykiewicz, 2002; Picconi et al., 2003; Factor, 2007; Picconi et al., 2008). In present study, the effect of chronic administration of LD along with CD in the form of different formulations in 6-OHDA treated rats was studied. The significant behavioral motor deficits, dyskinesia and other AIMs in rats were observed. In house manufactured IRP and MRFP of LD and CD were investigated for their therapeutic potential in LIDs.

Motor deficit and dyskinesia in rats is evidenced by the decreased dopamine level, development of AIMs, increased latency to cross the narrow beam, decreased latency to fall from rotating rod and decreased locomotor activity (Figure 6.5, Figure 6.7, Figure 6.8 and Figure 6.9). The present findings are in line with earlier reports, demonstrating similar disabling dyskinesias after chronic LD and CD treatment following 6-OHDA infusion in rats (Giorgi et al., 2008).

Administration of 6-OHDA is known to produce decrease in the density of tyrosine hydroxylase (TH^+) neurons indicating degeneration of the dopaminergic neurons in the nigrostriatal pathway thereby causing decrease in the dopamine levels, an important neurotransmitter involved in the motor functions (Sharma et al., 2015). In the present study, 6-OHDA treated rats showed significant reduction in striatal dopamine levels as compared with vehicle treated animals (Figure 6.5).

In addition, level of DOPAC was found to be less at lesioned side compared to non-lesioned side which can be attributed to decrease in the density of tyrosine hydroxylase neurons (Table 6.2). In addition to this, level of 3-OMD was found to be lower at non lesioned side (left lobe) compared to lesioned side (right lobe) in rats treated with different formulations suggesting presence of active tyrosine hydroxylase neurons in left lobe of brain (Table 6.2).

Chronic LD and CD treatment produced significant reduction in motor co-ordination and functions. The rats treated with either pure drug or IRP showed almost similar dyskinetic pattern. However, the animals treated with MRFP showed significant anti-dyskinetic effect and reduction in AIMs (Figure 6.6a).

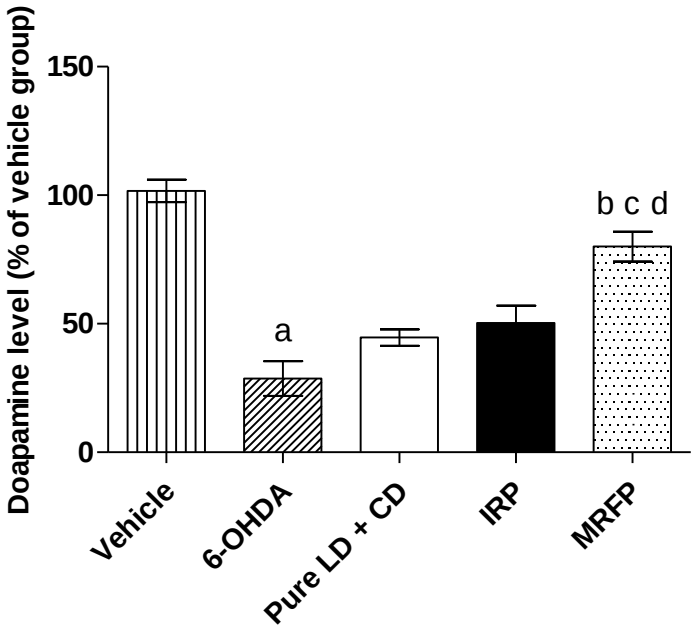


Figure 6.5. Effect of different formulations (oral solution of pure drug, IRP and MRFP) on DA level at lesioned side (right lobe) in 6-OHDA treated rats. (Each value represents mean of three independent determinations with standard deviation). $^{a}P < 0.001$ vs vehicle; $^{b}P < 0.001$ vs 6-OHDA; $^{c}P < 0.01$ vs pure LD and CD; $^{d}P < 0.05$ vs IRP (IRP = LC6; MRFP = EC/ERL100/2).

Table 6.2. Effect of LD and CD Treatment in the Form of Different Formulations (Oral Solution of Pure Drug, IRP and MRFP) on 3-OMD, and DOPAC Levels in Rat Brain.

Treatment	3-OMD (ng/g) (Mean ± SD, n = 3)		DOPAC (ng/g) (Mean ± SD, n = 3)	
	Lesioned side (right lobe)	Non lesioned side (left lobe)	Lesioned side (right lobe)	Non lesioned side (left lobe)
Vehicle	Not detected	Not detected	6.23 ± 0.23	8.23 ± 1.25
6-OHDA	Not detected	Not detected	7.84 ± 0.36	10.23 ± 1.25
IRP	531.56 ± 2.36	240.36 ± 4.56	8.25 ± 2.23	17.23 ± 2.12
Pure LD + CD	422.23 ± 3.23	221.25 ± 5.26	9.63 ± 2.56	18.23 ± 1.96
MRFP	933.42 ± 2.36	413.56 ± 2.56	8.25 ± 2.63	33.25 ± 2.56

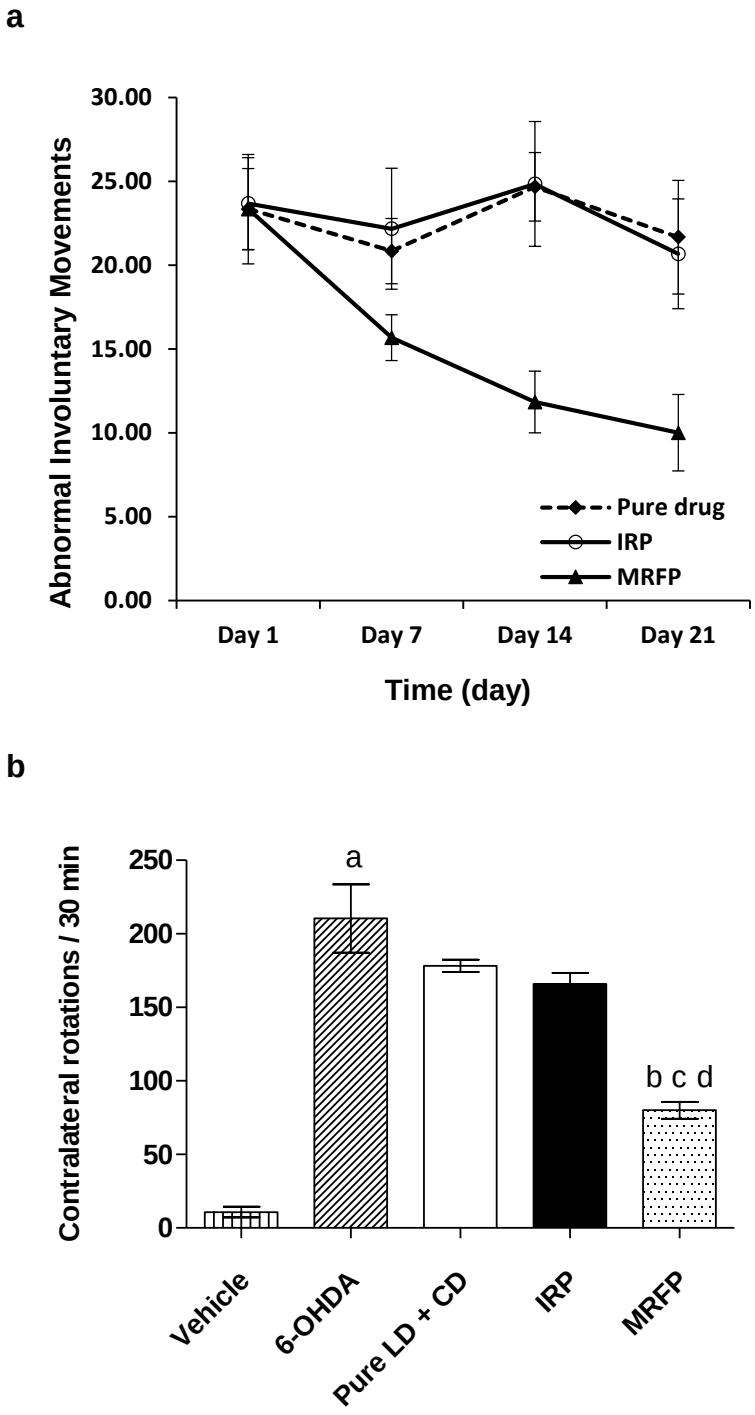


Figure 6.6. Effect of different formulations (oral solution of pure drug, IRP and MRFP) on (a) abnormal involuntary movements and (b) apomorphine induced contralateral rotations in 6-OHDA treated rats. (Each value represents mean of six independent determinations with standard deviation). [a]P < 0.001 vs vehicle; [b]P < 0.001 vs 6-OHDA; [c]P<0.01 vs pure LD and CD; [d]P < 0.01 vs IRP (IRP = LC6; MRFP = EC/ERL100/2).

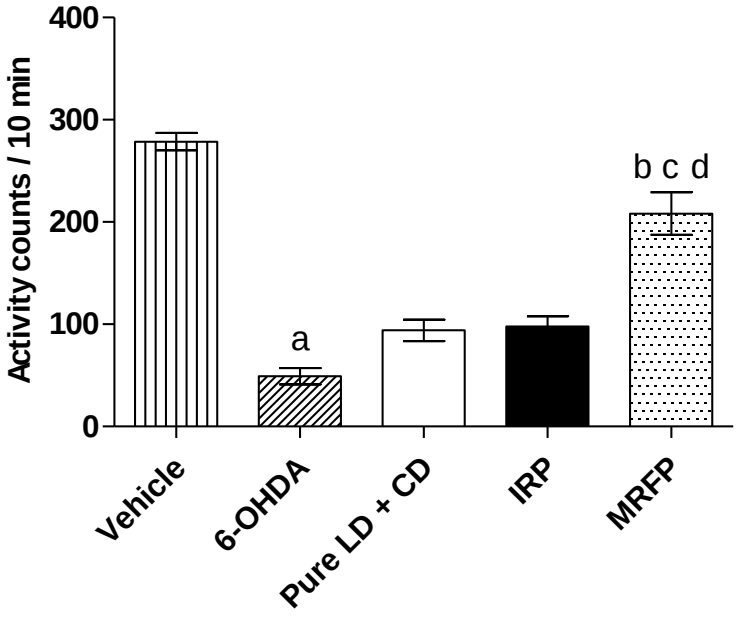


Figure 6.7. Effect of LD and CD treatment in the form of different formulations (oral solution of pure drug, IRP and MRFP) on locomotor activity in 6-OHDA treated rats. (Each value represents mean of six independent determinations with standard deviation). $^{a}P < 0.001$ vs vehicle; $^{b}P < 0.001$ vs 6-OHDA; $^{c}P<0.001$ vs pure LD and CD; $^{d}P < 0.001$ vs IPR (IRP = LC6; MRFP = EC/ERL100/2).

The apomorphine induced rotation were counted to compare the motor co-ordination in rats treated with different formulation (Figure 6.6b). The rats treated with either pure drug or IRP showed significantly higher contralateral rotations in comparison to the rats treated with the MRFP ($F_{(2, 17)}.= 80.87$) ($P < 0.05$)

The overall locomotor activity of dyskinetic rats was evaluated in actophotometer. The rats treated with either pure drug or IRP showed less locomotor activity. In contrast, the rats treated with MRFP showed significant improvement in locomotor activity ($F_{(2,17)}= 19.65$) ($P < 0.05$) (Figure 6.7).

The motor co-ordination of rats was assessed using the rotarod apparatus. The animals treated with either pure drug or IRP showed significant reduction in motor co-ordination and a decreased latency to fall from the rotating rod. However, the animals treated with MRFP showed significant increase in latency to fall ($F_{(2, 17)} = 29.59$) ($P < 0.05$) (Figure 6.8). This could be because of progression of the disease due to fluctuating dopamine level

in animals treated with oral solution of pure drug and IRP. In addition, the animals treated with MRFP took significantly lesser time to cross the narrow beam as compared with pure drug and IRP ($F_{(2, 17)} = 33.58$) ($P < 0.05$) (Figure 6.9).

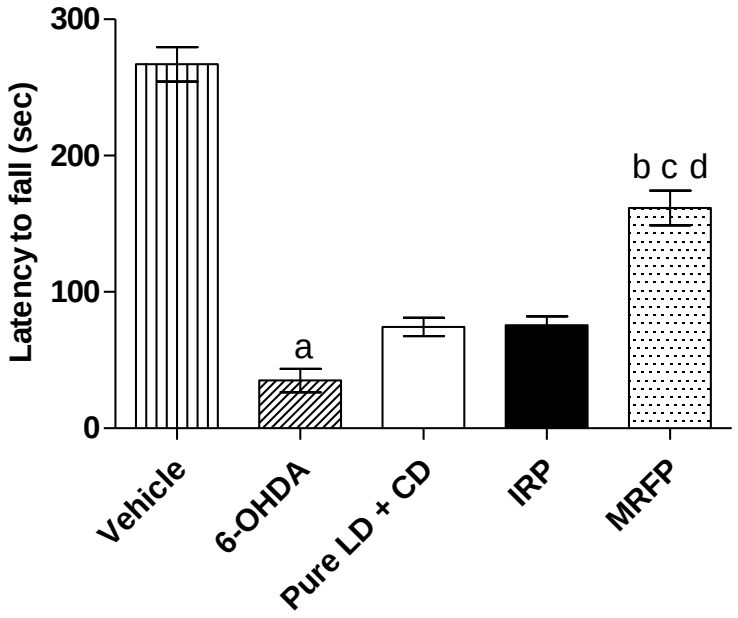


Figure 6.8. Effect of LD and CD treatment in the form of different formulations (oral solution of pure drug, IRP and MRFP) on rotarod activity in 6-OHDA treated rats. (Each value represents mean of six independent determinations with standard deviation). [a]$P < 0.001$ vs vehicle; [b]$P < 0.001$ vs 6-OHDA; [c]$P<0.001$ vs pure LD and CD; [d]$P < 0.001$ vs IPR (IRP = LC6; MRFP = EC/ERL100/2).

The fluctuating dopamine levels in the brain could be the possible explanation for the observed AIMs and dyskinetic movements. Similar AIMs have also been reported in previous studies with chronic LD treatment (Yang et al., 2012). In the present study, MRFP provided sustained LD release which could be responsible for reduction in fluctuating dopamine levels. In addition comparatively higher brain dopamine level was observed at lesioned side in case of rats treated with MRFP than IRP. Reduction in dopamine fluctuations could be responsible for the anti-dyskinetic effects and improved motor performance in rats treated with MRFP. Thus, these findings suggested that, designed MRFP can be useful to treat PD successfully with reduced expression of dyskinesia

especially in case of LD induced dyskinesia. Thereby, LD treatment duration can be extended for longer period with the help of designed MRFP.

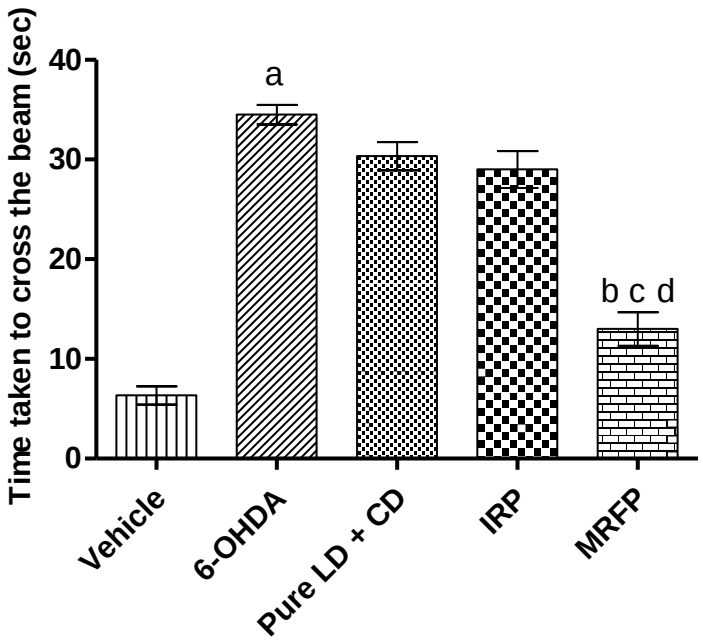


Figure 6.9. Effect of LD and CD treatment in the form of different formulations (oral solution of pure drug, IRP and MRFP) on narrow beam walk test in 6-OHDA treated rats. (Each value represents mean of six independent determinations with standard deviation). $^{a}P < 0.001$ vs vehicle; $^{b}P < 0.001$ vs 6-OHDA; $^{c}P < 0.001$ vs pure LD and CD; $^{d}P < 0.001$ vs IPR (IRP = LC6; MRFP = EC/ERL100/2).

6.4. Conclusions

In vivo studies of selected MRFP, IRP and oral solution of pure drug were conducted in rats. MRFP exhibited considerable increase in bioavailability of LD in comparison to the oral solution of pure drug and IRP. The enhancement of bioavailability of LD can be attributed to the site specific delivery of drug in controlled manner. In addition, pharmacodynamic studies indicated the potential benefits of the designed MRFP compared to oral solution of pure drug and IRP in LD induced dyskinesia. Hence, it can be concluded that the designed MRFP would be promising approach for the drug absorbed from upper

GIT. In addition it may provide more predictable and stable plasma concentration compared to conventional marketed formulations.

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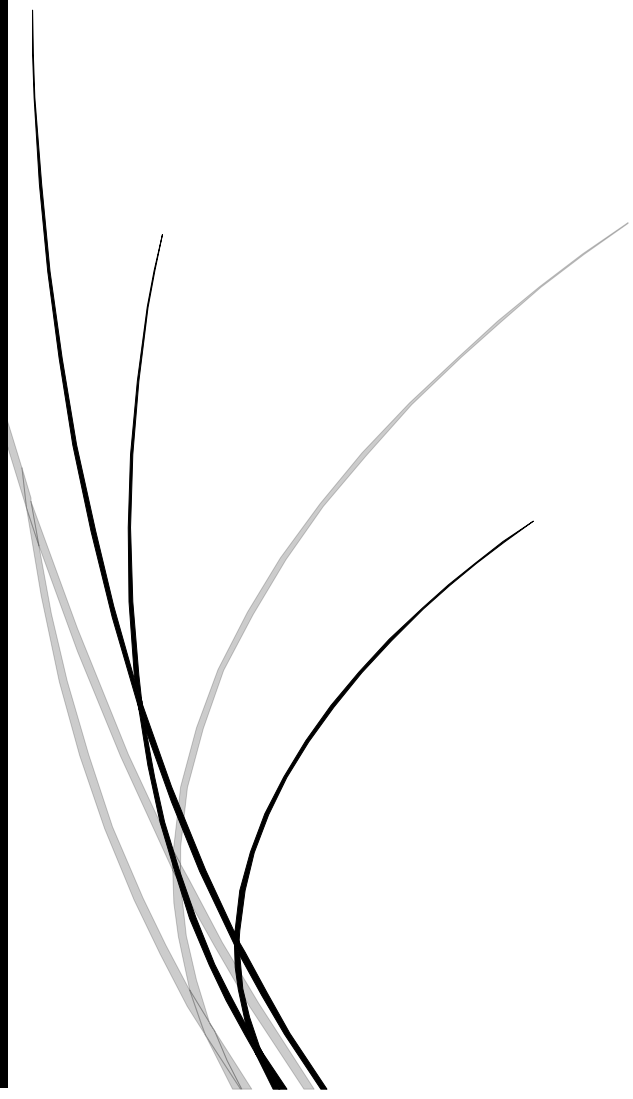
7 CONCLUSIONS



7. Conclusions

Over the last few years, modified release floating pellets based on multilayer structure have emerged as a novel drug delivery system for enhancing the therapeutic efficacy and patient compliance. In the current research, studies were carried out to design and characterize modified release gastroretentive floating pellets based on gas generation technique to enhance therapeutic efficacy of LD and CD by controlled release specifically to its absorption site.

As analysis is an integral part of pharmaceutical product development, new analytical methods were developed and validated for various studies. The developed thin layer and liquid chromatographic methods were found to be selective in the determination of LD and CD in bulk and formulation. These validated analytical methods were successfully used for the various preformulation and formulation development studies. The developed bioanalytical methods were also found to be selective and sensitive for the determination of the LD and CD in rat plasma. In addition, bioanalytical method was also developed for estimation of dopamine along with LD, 3-O-methyl dopa and 3, 4-dihydroxy phenyl acetic acid in rat brain. These validated bioanalytical methods were successfully employed for in vivo pharmacokinetic and brain estimation studies for the pure drug, immediate release pellets and modified release floating pellets. The developed methods were found to have various advantages over already reported methods.

Preformulation studies indicated that both LD and CD demonstrated a charge dependent solubility profile with increasing solubility towards acidic pH. In liquid state, both LD and CD were found to be most stable at pH 1.2 and sensitive towards basic pH conditions. Both drugs were more susceptible for basic and oxidative stress conditions. The results of the drug-excipient compatibility studies demonstrated insignificant interaction of drugs with various excipients used in formulations.

Extrusion spheronization technique was used for the manufacture of LD and CD loaded core pellet. Multiple coating layers were applied on LD and CD loaded core pellet by using fluidized bed processor. Pellets characteristics such as friability, mechanical crushing strength, pellet size and shape were found to be dependent on various formulation variables.

The optimized experimental conditions provided good quality modified release multiparticulate drug delivery system. The particle shape and microscopic imaging revealed that the optimized modified release floating pellet formulations were of spherical in shape. In addition, optimized coating parameters provided uniform coating around the core of drug loaded pellets. In vitro drug release from the designed formulations was controlled over 10-12 h and it could be well explained by the first order release kinetics. Furthermore, optimized formulation showed immediate floating and remains buoyant for more than 20 h. The various parameters such as coating levels of sustained releases and gas entrapped polymer layer found to have impact on sustained release properties of designed formulations. In addition, coating levels of effervescent layer, composition of effervescent agents in effervescent layer and coating levels of gas entrapped polymeric layers were found to have impact on floating properties of designed formulations.

In vivo pharmacokinetic studies in rats indicated that the designed modified release floating pellets have potential for effective delivery of LD and CD with enhanced bioavailability and sustained drug release under in vivo conditions. Area under drug concentration curve in plasma with modified release floating pellets was found to be more. Similarly increase in mean residence time indicated increase in drug residence time in the systemic circulation. Studies also indicated good compatibility of developed formulations in rats without any immediate undesirable effects. In addition, pharmacodynamic studies in 6-OHDA treated rats demonstrated the therapeutic potential of modified release floating pellets especially in LD induced dyskinesia compared to immediate release and oral solutions of LD and CD.

Thus, delivery of LD and CD using modified release floating pellets approach would be advantageous over the available conventional formulations with specific delivery to its site of absorption over extended duration. Moreover, it may offer benefit of reduced dose or dosing frequency leading to decreased untoward effects, improved patient compliance and increased therapeutic efficacy. Collectively these results demonstrated that LD and CD loaded modified release floating pellets has great potential to enhance bioavailability and effective treatment of Parkinson’s disease.

The optimized modified release particulate system can be easily scaled up and can be tested clinically in human's volunteers for final proof of concept. In order to establish the benefit risk ratio, further studies need to be carried out for the developed formulations in large number of diseased subjects. Designed MRFP may maintain stable plasma concentration resulting in avoidance of motor complications due to fluctuations in plasma concentration associated with current delivery systems. Multiple and chronic dosing pharmacokinetic studies would address the effectiveness of the modified release multiparticulate drug delivery system in such cases. Furthermore, in vivo pharmacokinetic and pharmacodynamic studies need to be carried out in human subjects to establish clinical effectiveness of the designed formulations over conventional and other novel drug delivery systems. Also in vivo gastroretentive study with the help of gamma scintigraphy in humans should be carried out in order to investigate the floating ability of designed formulations and to confirm improvement in the area under curve of LD was solely because of gastroretention of designed pellets formulation.

APPENDIX

List of Publications and Presentations

Publications from Thesis

1. **Raut P. P.**, Charde S.Y., 2014. Simultaneous estimation of Levodopa and Carbidopa by RP-HPLC using a fluorescence detector: its application to a pharmaceutical dosage form. Luminescence: The Journal of Biological and Chemical luminescence. 29, 762-771.
2. **Raut P. P.**, Charde S.Y., 2015. Design of modified release multi particulate drug delivery system for antiparkinsons drugs. Pharmaceutica Analytica Acta 6 (1) 96.
3. **Raut P. P.**, Charde S.Y., Bishnoi P., Simultaneous estimation of Levodopa, Carbidopa and 3-Oxymethyldopa in rat plasma using HPLC-ECD. Biomedical Chromatography DOI 10.1002/bmc.3713
4. **Raut P. P.**, Charde S.Y., Supekar A.P., Design of modified release multi-unit particulate floating drug delivery system of Levodopa and Carbidopa. Under review (Expert Opinion on Drug Delivery).
5. **Raut P. P.**, Jaipal A., Charde S.Y., Adhokshaja CSS, Design of modified release multiparticulate delivery system for Levodopa and Carbidopa combination: In vitro and in vivo evaluation. Under review (Colloids and Surfaces B: Biointerfaces).
6. **Raut P. P.**, Charde S.Y., Valavalkar V.V, Application of developed HPTLC techniques in preformulation studies of Levodopa and Carbidopa. Under review (Journal of Pharmaceutical Analysis).

Poster Presentations at International Conferences from Thesis

1. **Raut P. P.**, Charde S.Y., Design of modified release multi particulate drug delivery system for Antiparkinsons drugs. 2015. 5th International Conference and Exhibition on Pharmaceutics and Novel Drug Delivery Systems, Dubai, UAE.
2. **Raut P. P.**, Charde S.Y., Adhokshaja CSS, Supekar A.P. Modified release pellets for stomach site specific delivery of Levodopa and Carbidopa combination. 2015. 14th International Symposium of Controlled Release Society Indian Chapter on Advances in Technology and Business Potential of New Drug Delivery Systems, Mumbai, India.

3. **Raut P. P.**, Charde S.Y., 2015. Formulation and optimization of gastroretentive delivery system for Levodopa and Carbidopa combination using response surface methodology. AAPS-NUS 10th PharmSci@Asia and Annual Pharmacy Research Awareness Symposium. Singapore.
4. Charde S.Y., **Raut P. P.**, Design of modified release multi-particulate floating drug delivery system for Levodopa and Carbidopa combination based on a multilayer structure. 2015. 42nd Annual Meeting & Exposition of the Controlled Release Society. Edinburgh, Scotland.

Publications outside Thesis

1. **Raut P.P.**, Sharma A., Agarwal G., Charde S.Y., 2013. Quantitative estimation of Artesunate and Amodiquine HCl by high-performance thin-layer chromatography method in bulk and pharmaceutical formulations. Journal of Liquid Chromatography and Related Technologies. 37, 1568-1582.
2. Jaipal, A., Pandey, M.M., Charde, S.Y., **Raut, P.P.,** Prasanth, K.V., Prasad, R.G., 2014. Effect of HPMC and Mannitol on Drug Release and Bioadhesion Behavior on Buccal Discs of Buspirone Hydrochloride: In-vitro and In-vivo Pharmacokinetic Studies, Saudi Pharmaceutical Journal. 23, 315-326.
3. Dubey S.K., Deshpande S., Kumar S., **Raut P.P.**, Jain A., 2010. A high performance liquid chromatographic method for quantification of Trandolapril using UV spectrometric detection. International Journal of Biomedical Research. 1, 133-140.
4. **Raut P.P.**, Charde S.Y., Savani H., Bishnoi P. Design of pediatric sprinkle formulation for delivery of Artesunate and Amodiaquine Hydrochloride combination. Communicated to Malaria Journal.

Poster Presentations at National and International Conferences outside Thesis

1. **Raut, P.P.,** Charde, S.Y., Desai A.A., Sonawane, S., Gupta D.D. Design of modified release multi-unit particulate delivery systems of Amodiaquine Hydrochloride for pediatrics. 2012. 12th International Symposium on Advances in Technology and Business Potential of New Drug Delivery Systems, Mumbai, India.
2. **Raut, P.P.,** Jaipal, A., Manish, K., Charde, S.Y. Design and evaluation of pH independent sustained release tablet of Ondansetron Hydrochloride. 2010. 10th International Symposium on Advances in Technology and Business Potential of New Drug Delivery System, Mumbai, India.
3. Pandey, M. M., **Raut, P.P.,** Jaipal, A., and Charde, S.Y. Development of a high performance liquid chromatographic method for the estimation of Felodipine in bulk and formulation. 2011. 5th International Conference on Clinical Pharmacology-Discovery, Development and Beyond, Mumbai, India.
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