Design, Characterization and Pharmacokinetic Evaluation of Solid Lipid Nanoparticles of Ganciclovir for Improved Oral Absorption

THESIS

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Dedicated to

My Family

&

All My Teachers

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Garima Balwani

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List of Abbreviations/Symbols

%	Percentage
% RSD	Percentage relative standard deviation
% w/v	Percentage weight per volume
% w/w	Percentage weight per weight
α	Alpha
β	Beta
β’	Beta prime
µ	Micron
µg	Microgram
µL	Microliter
γ	Gamma
σ	Standard deviation of y-intercept
≈	Almost equal to
<	Less than
>	Greater than
°C	Degrees celsius
λ_{ex}	Excitation wavelength
λ_{em}	Emission wavelength
ACN	Acetonitrile
AFM	Atomic force microscopy
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
AUC	Area under the curve
BCS	Biopharmaceutical Classification System
BBB	Blood brain barrier
C_{max}	Maximum drug concentration in pharmacokinetic study
CMC	Critical micellar concentration
CMV	Cytomegalovirus
C_o	Concentration at time zero in partition coefficient studies
Conc.	Concentration
CPCSEA	Committee for the Purpose of Control and Supervision on Experiments on Animals
CrCl	Creatinine Clearance
C_t	Concentration at time t in partition coefficient studies

Da	Daltons
DCM	Dichloromethane
DL	Drug loading
DLS	Differential light scattering
DMSO	Dimethyl sulphoxide
DNA	Deoxy nucleic acid
DSC	Differential scanning calorimeter
EDTA	Ethylenediaminetetraacetic acid
EE	Entrapment efficiency
ELS	Electrophoretic light scattering
EPR	Enhanced permeation and retention
F	Absolute bioavailability
f1	Dissimilarity factor
f2	Similarity factor
FDA	Food and Drug Administration
F_{rel}	Relative bioavailability
FTIR	Fourier transformed infrared
GB	Glyceryl behenate
GDS	Glyceryl distearate
GIT	Gastrointestinal tract
GMS	Glyceryl monostearate
h	Hour
^{1}H-NMR	Proton nuclear magnetic resonance
HETP	Height equivalent to theoretical plate
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
HQC	High quality control
i.v.	Intravenous
i.m.	Intramuscular
i.e.	that is
IAEC	Institutional Animal Ethics Committee
ICH	International Conference on Harmonization
IPA	Isopropyl alcohol
IR	Infrared

IS	Internal standard
k	Resolution
K_{deg}	First order degradation rate constant
kg	Kilogram
kV	Kilo volt
LD	Lased diffraction
LOD	Limit of detection
LOQ	Limit of quantification
LLOQ	Lower limit of quantification
LQC	Lower quality control sample
M	Molarity
mbar	Millibar
MDR	Multi drug resistant
mg	Milligram
min	Minutes
mL	Millilitre
mm	Millimeter
mM	Millimolar
MQC	Medium quality control
MRT	Mean residence time
N	Normal
NDDS	Novel drug delivery system
ng	Nanogram
nL	Nanolitre
nm	Nanometer
o/w	Oil in water
P	Probability
P_{app}	Apparent partition coefficient
PCS	Photon correlation spectroscopy
PDI	Polydispersivity index
PEG	Polyethylene glycol
PF-68	Poloxamer 188/Pluronic F68
P-gp	P-glycoprotein
pK_a	Dissociation constant
ppm	Parts per million

QC	Quality control
R^2	Regression coefficient
RES	Reticulo-endothelial system
R_f	Retention factor
RH	Relative humidity
RP-HPLC	Reverse phase high performance liquid chromatography
rpm	Rotations per minute
RT	Room temperature
s	seconds
S	Slope of the calibration curve
s.c.	Subcutaneous
S_a	Standard deviation of intercept
S_b	Standard deviation of slope
SD	Standard deviation
SEM	Scanning electron microscope
SLN	Solid lipid nanoparticle
$S_{y/x}$	Standard deviation of residual
t	Time
$t_{1/2}$	Biological half-life
$T_{50\%}$	Time taken for 50% of the drug to release
$t_{90\%}$	Time taken for 90% of the drug to remain undegraded
TCA	Trichloroacetic acid
TEM	Transmission electron microscope
T_f	Tailing/asymmetric factor
t_{max}	Time to achieve maximum drug concentration
TPGS	d-alpha tocopheryl polyethylene glycol 1000 succinate
t_R	Retention time
USP	United States Pharmacopeia
UV	Ultraviolet
V_d	Volume of distribution at steady state
v/v	Volume by volume
w/o	Water-in-oil
w/o/w	Water-in-oil-in-water
XRD	X-ray diffraction

Abstract

The objective of the present research work was to design and evaluate solid lipid nanoparticulate drug delivery systems to improve the oral bioavailability of the antiviral drug ganciclovir. Ganciclovir is an antiviral drug used in immunocompromised people for prevention and treatment of infections caused by cytomegalovirus. It is the first line therapy for such infections and requires long term administration. Conventionally, ganciclovir is delivered via intravenous route which suffers from several drawbacks, like high cost, catheter-related infection and sepsis and requirement of professional staff for dosing, leading to patient incompliance. Delivery via oral route is limited and meant for preventive therapy and maintenance treatment only. This is due to the large oral dose of ganciclovir administered in a day as a result of its low oral bioavailability. Thus design of novel oral delivery system to overcome these drawbacks is necessary. Following specific studies were performed to achieve the objective.

As per the need of present work, analytical methods like spectrometric and HPLC methods were developed and validated. The proposed methods were accurate, precise, selective and sensitive and were employed for estimation of ganciclovir in various samples obtained during preformulation, formulation and in vivo studies. Solubility of ganciclovir was found to be high in aqueous while poor in non-aqueous solvents. It was pH dependent, with maximum solubility at both extreme pH. Consistent with the solubility results, partitioning of ganciclovir into organic phase was very poor and negative values of log P were obtained. At all the pH studied, the degradation was found to follow first order rate kinetics and degradation rate was less and at alkaline pH the degradation was found to be minimal. As confirmed by the DSC and FTIR studies, ganciclovir alone and in combination with different excipients, was stable for more than 6 months in controlled room temperature and refrigerated conditions.

Formulation development of solid lipid nanoparticles carried out was carried out by varying formulation variables with the aim of achieving low particle size and high drug entrapment. Nanoparticles were prepared using a double-emulsion solvent evaporation technique using different lipids, glyceryl monostearate, glyceryl distearate and glyceryl behenate. The in vitro characters of the designed nanoparticles were found to depend on the type and proportion of lipid and stabilizer. The nanoparticles showed good physical properties indicating the suitability of the method of preparation of formulation. The particle size analysis and morphological assessment demonstrated that the optimized

formulations had small and uniform particle size with spherical shape. Drug release was found to depend on both amount of lipid and surfactant in the nanoparticulate matrix. The drug release was extended upto 8 hous and release mechanism was described by the Baker Lonsdale model for spherical particles. The optimized batches showed drug entrapment of upto 48% and also depicted good stability and redispersibility in freeze-dried state.

Pharmacokinetic and biodistribution studies were conducted to study the in vivo behaviour of the developed formulations. A multi fold increase in oral bioavailability of ganciclovir and an altered distribution character was observed when administered as solid lipid nanoparticles. The absolute bioavailability of ganciclovir was increased by approximately 4-8 times on administration as nanoparticles. An increased uptake of solid lipid nanoparticles was seen in brain, heart and lungs which may prove beneficial in severe infestations like encephalitis, endocarditis and pneumonitis, respectively. A decreased uptake in liver, spleen and kidneys was seen probably due to the stealth effect of the hydrophilic coating of surfactants. Histopathological studies of the rat intestine did not reveal any local toxicity or deformity in structure of cells when nanoparticles were administered. Visual monitoring of animals post dosing also did not show any undesirable effect.

Collectively, these results indicated that solid lipid nanoparticles are promising delivery systems to be developed to enhance the oral bioavailability of ganciclovir, so that the dose and administration frequency of the current oral therapy can be reduced, the inconvenience of intravenous administration can be avoided and overall the patient compliance can be improved.

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1. Introduction

1.1. Introduction

With advancement in medical science and technology, expectations of mankind towards pharmacotherapy has increased. In recent years, there has been a decrease in the number of new drugs entering the market and as a consequence, a shift in the focus of research on design of better delivery systems of already existing drugs is evident. Conventional delivery of drugs suffers from several drawbacks like poor solubility, poor absorption, high first pass metabolism, low systemic bioavailability, administration of large doses, frequent administration, fluctuating drug levels in plasma due to attainment of peak and trough concentration, non-selective distribution, increased risk of occurrence of adverse effects and many more. All these shortcomings can result in missed doses, made-up doses and patient non-compliance with the therapeutic regimen, causing failure of treatment. To cater to these problems, pharmaceutical companies have started investing in design of better delivery systems for improved therapeutic efficacy and better patient compliance. Last two decades have seen many alternative and better formulations delivered by same or alternative routes with improved effectiveness. The advantages offered by these novel drug delivery systems (NDDS) include sustained and consistent blood level within therapeutic window, enhanced bioavailability, targeted delivery, reduced side effects, decrease in dosing frequency and in turn an improved patient compliance [1].

NDDS have found their application in all the routes of drug administration including, parenteral, oral, transdermal, nasal, ocular and other mucosal routes. However, owing to its unique facets, oral route is the most commonly employed route of drug delivery and a major area of research also. Ease of administration, patient compliance, flexibility in dosage form design, low cost of manufacturing and simplified regulatory approval process are some of the distinct advantages of this route. Despite its significant benefits, this route suffers from several drawbacks leading to limited oral bioavailability of compounds due to various reasons which include the existence of various pH affecting the solubility of drugs administered, first pass metabolism, enzymatic and permeability barrier [2]. The introduction of the Biopharmaceutical Classification System (BCS) has provided a basis to categorize drugs depending on two major parameters governing drug absorption via oral route – solubility and permeability. Class I compounds exhibit high solubility and permeability, class II have low solubility and high permeability, class III have high solubility and low permeability, while class IV depict low solubility and low

permeability [3]. Several NDDS, specifically for class II, III and IV compounds, have been designed to improve the aqueous solubility and/or permeability of drugs, and also are in investigation in the current scenario of pharmaceutical research. These NDDS include oral controlled release systems, mucoadhesive and transdermal drug delivery systems and targeted drug delivery systems like micro and nanoparticles. Among the NDDS, nanoparticulate systems can be correctly envisioned as the future of drug delivery technology owing to their potential to become useful therapeutic tools due to several distinct advantages.

1.2. Nanoparticulate Drug Delivery Systems

In the past decade research with emphasis on nanotechnology has been on surge as nanoparticulate pharmaceutical carriers have shown to enhance the in vivo efficiency of several drugs [4]. Nanoparticles are colloidal particles of less than 1 μm diameter that are prepared from natural or synthetic polymers. A wide variety of drugs, especially those with above mentioned unfavourable physicochemical and biopharmaceutical properties can be delivered using nanoparticulate carriers via a number of routes. At nanoscale, materials exhibit different, more desirable physical, chemical, and biological properties. The advantages of nanoparticulate systems include sustained and controlled release of drugs, increased membrane permeation, modified selective and site specific delivery and enhanced cellular uptake and protection of drugs from harsh environments at both the extracellular and intracellular levels. The use of innovative nano-scale drug delivery technologies like nanosuspensions: polymeric or solid lipid based, liposomes, niosomes etc, has shown to be promising for the delivery of drugs.

Nanosuspensions are colloidal dispersions in nano-range of drug based particles stabilized by a suitable stabilizer. Nanosuspensions have the ability to overcome solubility problems and bioavailability problems of drugs and can be generally applied to all poorly soluble drugs. The drug can be transformed to drug nanoparticles leading to an increase in saturation solubility, dissolution velocity and an increased adhesiveness to surfaces. Production techniques such as media milling and high-pressure homogenization have been successfully employed for large-scale production of nanosuspensions. The disadvantages of nanosuspensions include particle growth and instability issues due to drug recrystallization [5-7].

Liposomes are spherical vesicles of nanometer size composed of phospholipid bilayers surrounding an aqueous core. They are structurally versatile and thus have the ability to incorporate both lipophilic and hydrophilic drugs. Lipophilic drugs are embedded in the lipid layers and hydrophilic drugs are solubilized into the aqueous core. The most favourable character of liposomes is the biocompatibility of the lipids used, an important advantage over polymeric nanoparticles [8, 9]. Other aspects include the capability to modify size, composition, characters, surface charge and bilayer fluidity which alters the in vitro and in vivo behaviour of liposomes. The ability to carry cell-specific ligands on their surface gives liposomes the potential to be used in targeted drug delivery. Liposomes have been studied experimentally as carriers in cancer chemotherapy, antimicrobial therapy and vaccine delivery [10, 11], with few products reaching the market. Nonetheless, liposomes suffer from many drawbacks like chemical and physical instability, drug leakage, low drug loading and high cost.

Polymeric nanoparticles are solid colloidal particles composed of biocompatible polymers of synthetic, semi-synthetic or natural origin. They could be in the form of water-soluble polymer drug conjugates, polymeric nanocapsules or nanospheres, where drugs could be dissolved, entrapped in the nanoparticle, or adsorbed onto the surface, respectively. Utilization of a variety of formulation techniques, pre-existing polymers or formulation of novel in situ polymeric material is possible. The different fabrication methods include monomer polymerization, nanoprecipitation, emulsion diffusion, solvent evaporation or salting out and the selection primarily depends on the nature of the drug and polymer to be used [12]. Polymeric nanoparticles have revolutionized the scenario of research in drug delivery as they have shown significant improvement in therapeutic effectiveness over the traditional oral and intravenous (i.v.) systems. Their application is widespread and ranges from cancer and antimicrobial chemotherapy to protein and gene delivery [13]. Their advantages are increased stability of the pharmaceutical agents and the ability to be easily formulated with many desired adaptions for specific applications. These include surface modifications that can be done either to achieve long circulation times, mucoadhesion, modified or selective distribution or to target specific cells in the body. For example, in vivo long circulation time is achieved by coating the nanoparticle surface with hydrophilic polymer or surfactant, while active targeting to specific tissue can be achieved by incorporating a target-directed ligand, like peptide or monoclonal antibody, etc. in the formulation [14, 15]. Issues that

need to be addressed during manufacture of polymeric nanoparticles include drug loading capacity, in vivo fate of the polymers used, targeting capacity, acute and chronic toxicity of the polymers and residual solvents, cost and feasibility of scale up and physical and chemical stability on storage [16]. Because of these several problems, only few polymeric nanoparticulate products have reached the market till today.

1.3. Solid Lipid Nanoparticles

Solid lipid nanoparticles (SLNs) are another type of colloidal drug carriers that consists of solid lipid based particles dispersed in a size range of 10 to 1000 nm. SLNs were introduced in the early1990s as alternative colloidal systems to liposomes and polymeric nanoparticles as they combine the advantages of these colloidal systems while simultaneously avoid their disadvantages [17, 18]. The major advantage of SLNs over polymeric nanoparticles is their perfect biocompatibility as the lipids that are used in preparation are internal components of the body so are well tolerated, while their increased stability makes them superior to liposomes. Due to their favourable characters, SLNs have been shown to have applications in i.v., intramuscular (i.m.), oral, rectal, ophthalmic, dermal and other routes of administration [19]. Some of the proposed advantages of using SLNs include:

- Increased in vitro or in vivo stability of drugs and lack of drug leakage due to reduced mobility of the entrapped drug
- Enhanced oral bioavailability of drugs
- Controlled release and drug targeting
- High drug payload
- Reduced toxicity
- Minimal use of organic solvents
- Feasibility to incorporate both hydrophilic and lipophilic drugs
- Ease of scale up and cost-effective large scale production
- Better patient compliance

1.3.1. Components of SLN Formulations

The general components of SLNs include solid lipids and stabilizers. The lipid core in which drug is embedded can be hard fats or fatty acids of different carbon chain length (e.g. stearic acid, palmitic acid, behenic acid), triglycerides (e.g. tristearin, tripalmitin),

partial or mixed glycerides (e.g. glyceryl monostearate, glyceryl palmitostearate, glyceryl monooleate), steroids (e.g. cholesterol) and waxes (e.g. cetyl palmitate). The choice of lipid is critical to achieve sufficient stability and efficient drug loading capacity in the SLNs. Selection of lipids is based on the degree of crystallinity, fatty acid chain length and drug loading capacity of lipid, which in turn depends on the solubility of drug in the lipid and the polymorphic state and structure of lipid [20]. The lipid should be selected such that the solubility of the drug in lipid should be higher than that required because it decreases on solidification of the lipid. Solubilizers/surfactants may be used to enhance the solubility of drug in the lipid and for ease of preparation. Lipids consisting of mono- and diglycerides also act as self-solubilizing agents.

The structure and crystallinity of lipid is another important parameter for consideration as it also governs drug loading. Lipids with a perfect crystalline lattice, e.g. triglycerides, tend to cause drug expulsion, whereas partial glycerides or glycerides of different fatty acid form imperfect crystals and provide more space for drug accommodation. Waxes such as beeswax and cetyl palmitate also have ordered crystal arrangement which causes expulsion of drug outside the lipid core on storage [21, 22].The nature of the lipid also has a great influence on particle size of SLNs. It has been seen that the average particle size of SLNs is higher in case of lipids with higher melting point.

Emulsifiers or surfactants are other important excipients used in SLN formulation, as they are used to disperse the lipid phase into the aqueous phase uniformly. They stabilize the lipid-water interface by reducing the interfacial tension, leading to decrease in particle size and increase in surface area. The desired properties of emulsifier used to prepare SLNs include non-toxicity, non-irritancy, compatibility with drug and lipids and ability to form nanoparticles at low concentrations. The concentration of emulsifier used is crucial in designing SLNs as too low concentrations lead to particle aggregation and high concentrations cause decreased entrapment of drugs, fast release and toxicity [18]. Hence, optimization of emulsifier concentration is important.

Emulsifiers widely used as stabilizers in SLNs include non-ionic surfactants (polyoxyethylene-polyoxypropylene copolymers, e.g. Poloxamers, polyoxyethylenesorbitan fatty acid esters, e.g. Tweens), phospholipids (e.g. phosphatidylcholine, soyabean lecithin) and bile salts (e.g. sodium cholate, sodium glycocholate, sodium taurocholate). Particle size of SLNs dispersions stabilized with nonionic surfactants is generally larger than those obtained with ionic surfactants. The

combination of two or more emulsifying agents appears to produce mixed surfactant films at the interface. These mixed surfactants cover the surface efficiently as well as produce sufficient viscosity to promote the stability [20].

The stabilizers also alter the surface properties of the SLNs, which decide their in vivo fate after administration in the body. On reaching the systemic circulation, a major portion of SLNs is taken up by the organs of the reticulo-endothelial system (RES), which needs to be avoided to ensure long circulation of drug-containing SLNs in the blood stream and targeting to other sites. Several surfactants have been identified that lead to a decreased uptake of particulate delivery systems by these RES cells. The classical example of this phenomenon is coating of polyethylene glycol (PEG) or 'PEGylation' on nanoparticulate surface [23, 24]. These PEG chains at the surface avoid interaction with blood proteins, therefore resist RES uptake. Apart from PEG, several alternative surfactants have been studied and it has been found that certain non-ionic surfactants like poloxamers, Tween 80, etc, impart this property. These surfactants act by sterically stabilizing the nanoparticles. Besides, providing escape from the RES, this surfactant coating also hinders degradation by gastric lipases which SLNs encounter in the stomach after oral administration [25, 26]. Moreover, using specific surface modifications, the passage of SLNs through certain barriers such as the blood brain barrier (BBB) appears feasible. For example, particles coated with Tween 80 are able to cross the BBB and deliver drugs through them. Hence, selection of stabilizer is critical and is done based on the desired purpose. Thus it is possible to use SLNs for targeting specific organ or tissues or achieving selective distribution.

1.3.2. Methods of Preparation of SLNs

Apart from the ingredients used for the preparation, the method of preparation also greatly influences the properties of the SLNs. A number of methods of preparation of SLNs have been extensively described in the literature [19, 27]. The choice of method for preparation of SLNs depends on the properties of the drug as well as the formulation parameters such as concentration of lipid and type of stabilizers used. Some of the widely used methods of preparation are described below

1.3.2.1. High Pressure Homogenization

High pressure homogenization is a reliable, powerful and easy to scale up technique for preparation of SLNs. Initially utilized for preparing nanoemulsions for total parenteral

nutrition, this technique was adopted to prepare SLNs by Mueller and Lucks in 1996 [28]. In this method, the dispersions are forced through a gap of very small size under high pressure (100-2000 bar). The fluid accelerates over a very short distance and upto very high velocity and consequently, a reduction in particle size is seen due to the shear stress and cavitational forces acting at the narrow aperture. Some of the advantages of using high pressure homogenization process include narrow particle size distribution, better dispersion of formulations with higher lipid content, avoidance or low volumes of organic solvents, acceptability of homogenization equipment by the regulatory authorities and feasibility of scale up for large scale production [19]. High pressure homogenization could be carried out under hot or cold conditions.

In hot homogenization technique, the lipids are first melted by heating them at temperatures 5-10°C above their melting points. The drug is then dissolved or dispersed in the hot melted lipids. The drug loaded lipid melt is poured in a hot aqueous surfactant solution to form a coarse pre-emulsion that is subsequently homogenized at a temperature above the melting point of the lipid, till desired average particle size is obtained. SLNs are obtained on cooling the hot o/w nanoemulsion to room temperature. When a heterogeneous system is cooled down, the average droplet size increases due to a phenomenon called Ostwald's ripening, i.e., the redeposition of the diffused particles over small evaporating droplets to form larger droplets [29]. The hot homogenization method of SLN preparation thus highly relies on the efficiency of the emulsifiers to stabilize the cooled mixture against agglomeration or Ostwald's ripening. In hot homogenization technique, small particles size is obtained due to decreased viscosity of the dispersed phase, however, an accelerated degradation rate may be seen for both thermolabile drugs and carriers [19].

In case of cold homogenization technique, the drug is dissolved or dispersed in the molten lipid, same as in hot homogenization. The drug lipid melt is then rapidly cooled using liquid nitrogen or dry ice resulting in formation of a solid solution of drug in lipid matrix. This solidified product is milled to yield microparticles (50-100 µm), which are dispersed in cold aqueous surfactant solution and subjected to the high pressure homogenization at or below room temperature. The main advantage of the cold homogenization technique is the prevention of temperature-induced drug degradation, however, exposure of the drug to temperature cannot be completely avoided due to solubilization of the drug in melted lipid and also temperature generated during homogenization process. This

method also prevents drug leaching into aqueous phase during homogenization, so is particularly useful for loading hydrophilic drugs [27]. The disadvantage of cold homogenization is production of SLNs with large particle size and broader distribution, when compared to hot homogenization process.

1.3.2.2. High Shear Homogenization and Ultrasonication

High shear homogenization and ultrasonication are few other simple and widely used high energy dispersion techniques used for the production of SLNs. The procedure starts with placing the lipid, excipients and the water phase into a rotor stator homogenizer or ultrasonicator with a sonotrode followed by applying high shear mixing (5000 to 25000 rpm) or ultrasonic mixing (> 20 kHz). The formed hot nanoemulsion is then cooled to form solid particles [30-32]. Although these are very simple procedures, the properties of the final product are usually poor and large portions of microparticles with high polydispersity index (PDI) are detected. Another disadvantage of ultrasonication method is the possibility of metal contamination by the probe, which may affect product quality.

1.3.2.3. Microemulsion Technique

The technique of spontaneous microemulsion formation on mixing of lipophilic phase and aqueous surfactant phase was first adopted by an Italian scientist M. R. Gasco for preparation of SLNs [33]. In this method, the lipid (generally a low melting lipid) is melted and the drug is dispersed in it. To this lipid melt, an aqueous mixture of surfactant and co-surfactant which is heated to a temperature at least equal to the melting temperature of the lipid is added under mild stirring to obtain a transparent microemulsion. This microemulsion is then dispersed in cold water (2°C to 10°C) in the ratio between 1:25 and 1:50, under mild mechanical stirring. SLNs are produced due to rapid recrystallization of lipid on dispersion in cold aqueous medium.

1.3.2.4. Solvent Emulsification/Evaporation Method

SLNs are also produced by a nanoprecipitation method comparable to the production of polymeric nanoparticles by solvent emulsification and evaporation method. In this method, the drug and lipid are dissolved in a water-immiscible organic solvent and this solution is emulsified in an aqueous phase containing surfactant. The solvent is evaporated leading to precipitation of SLNs [34]. This method offers the advantage of avoidance of thermal degradation of drugs, however disadvantages of using organic solvents prevail.

1.3.2.5. Solvent Diffusion Method

In this technique, the drug and lipid are dissolved in a partially water-miscible solvent and emulsified into aqueous surfactant solution. This emulsion is then transferred to water under continuous stirring, during which the droplets of dispersed phase solidify as lipid nanoparticles due to diffusion of the organic solvent [35].

1.3.2.6. Solvent Injection Method

This method is similar to solvent diffusion method but involves a completely miscible organic solvent. The lipid and drug are dissolved in the polar organic solvent and then injected in aqueous solution of surfactant under stirring. This causes a rapid migration of the organic solvent in the water and precipitation of the lipid nanoparticles [36]. The more polar the solvent, the lesser is the particle size of the SLNs obtained.

1.3.2.7. Double Emulsion Method

Incorporation of hydrophilic drugs in SLNs prepared using the above mentioned techniques results in low encapsulation due to low lipid solubility of the drug. A double emulsion method was first utilized for loading hydrophilic drugs into lipid matrix as lipospheres [37]. In this the aqueous drug solution is added to the lipid melt containing lipophilic stabilizer to form a primary w/o emulsion, which is then dispersed in an aqueous phase containing hydrophilic stabilizer. SLNs are subsequently formed by cooling the emulsion and can be separated by centrifugation or filtration. The primary emulsion may also be formed in a lipid solution in organic solvent and further processing is done in a manner similar to that of solvent emulsification/evaporation method [38]. In the recent years much research is being done on delivering hydrophilic drugs and macromolecules like peptides by SLNs prepared using this technique [39-41].

1.3.3. Drug Incorporation Models

Based on the distribution of drug in the lipid matrix, three models of drug incorporation in the SLNs are described in literature [27]. These are the homogenous matrix or solid solution model, drug-enriched shell model and drug-enriched core model. These three models represent the ideal type, although there can also be mixed types which are developed. The structure of SLNs formed depends on the chemical nature of drug and excipients and the interaction between them and also on the processing conditions employed for formation of SLNs.

When the drug is distributed molecularly in the lipid matrix, a matrix type solid solution model is formed. It is produced when SLNs are prepared by cold homogenization technique or when highly lipophilic drugs are loaded into SLNs using the hot homogenization technique. As discussed above, in the cold homogenization method, the solidified drug-lipid mixture is grinded and then mechanically broken down to nanoscale by high pressure homogenization, forming nanoparticles having the homogeneous matrix structure. This structure also forms when the drug and lipid crystallize together without any phase separation on cooling after hot homogenization process. These nanoparticles show different physicochemical properties than pure drug.

When phase separation is seen during the cooling process that leads to formation of solid particles, a core-shell model forms. When the lipid crystallizes out first, a lipid core is formed and the concentration of drug in the remaining liquid lipid increases continuously. Finally, towards the end of the process, the drug also crystallizes and a drug-enriched shell model is developed. A fast drug release is seen with this model, which is desirable when increased drug penetration is required on application of SLN to the skin [42-44].

A core enriched with drug is formed when the opposite occurs, the drug starts precipitating first and the shell is composed primarily of the lipid. This happens when the amount of drug added is close to the saturation solubility of the drug in the molten lipid. When cooling is induced, due to supersaturation, drug starts precipitating first. Due to further cooling recrystallization of lipid takes place, surrounding the drug core as a membrane. This leads to controlled dug release governed by the Fick's law of diffusion [42].

1.3.4. Characterization of SLNs

SLNs are characterized with respect to particle size and size distribution, zeta potential, particle shape and morphology, crystallinity and lipid modification, drug content and entrapment efficiency and in vitro drug release.

1.3.4.1. Particle Size and Zeta Potential

Photon correlation spectroscopy (PCS), also known as differential light scattering (DLS) and Laser Diffraction (LD) are the frequently used techniques for determination of particle size of nanoparticles. In DLS, a laser beam is applied to the sample and time-dependent fluctuations in the scattering intensity caused due to the Brownian motion of

dispersed particles are measured. Using the Stokes-Einstein equation and the data from these fluctuations, the hydrodynamic diameter of the particles can be calculated. This equation relates the velocity of a particle to its hydrodynamic diameter. DLS is very sensitive and can detect particles even below 1 nm, however, larger particles (> 5-10 µm) are not suitable for analysis by this technique. On the other hand, LD technique can measure sizes in the range of nanometers to several millimeters. Particle size determination by LD is based on measuring the degree of diffraction from the surface of particles when laser beam is passed through a dispersed particulate sample. The data collected by LD is used to calculate the equivalent sphere diameter of particles according to Mie scattering theory. Particle size distribution is also generated by both the techniques, either in graphical form or as PDI [45].

Both the techniques of DLS and LD do not calculate direct particle size but they use light scattering and diffraction to compute diameter, assuming that the particles are spherical. Therefore, misleading data may be obtained for particles which are not spherical and are of irregular shape. Concentration of particles in the dispersions also governs the results, hence the information obtained should be confirmed with another suitable method like microscopy.

Zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. It is an important parameter that is related to nanoparticle stability or aggregation in a dispersion, and can have significant implications on product performance. Zeta potential depends on the surface charge and the presence of any adsorbed layer at the interface. Dispersions with zeta potential values of above +30 mV and below -30 mV are considered to be stable as these higher values of charge provide electrical repulsion between the particles preventing flocculation and coalescence. Zeta potential is determined by measuring electrophoretic light scattering (ELS) used to determine particle velocity in electric field [46]. Modern instruments typically combine the analytical methods for particle size measurement by DLS and zeta potential by ELS.

1.3.4.2. Particle Shape and Morphology

Electron microscopic techniques like scanning electron microscope (SEM), transmission electron microscope (TEM) and atomic force microscopy (AFM) are used to study the particle shape and morphology of SLNs. In addition, these techniques can also be utilized

to estimate the size of the particles. SEM and TEM utilize electrons to visualize particles in the nanometer range. In SEM, back scattered electrons from the exterior of the particles are detected and the surface of the SLNs is studied, whereas in TEM, electrons transmitted through the specimen are detected and the internal structure of particles is studied [47]. In SEM, the sample needs to be conductive, hence gold or platinum coating is done for non-conducting samples. TEM allows achievement of high resolution images after staining of sample with phosphotungstic acid. Both these techniques are lengthy and sophisticated with respect to sample preparation (dehydration, staining, conductive coating, etc.) and processing (vacuum, heating, etc.) rendering them expensive and not suitable for routine measurement. AFM is another technique which measures the force that acts between the surface of the particles in the sample and a probing tip. The method is more appropriate for surface analysis and provides good resolution without the use of vacuum and does not involve sample coating or staining. The only disadvantage is the need to immobilize the sample by removal of solvent or dehydration which may lead to shrinkage or crystallization of the lipid [48].

1.3.4.3. Measurement of Crystallinity and Lipid Modifications

Lipids display polymorphism i.e., crystal modifications or different degree of crystallinity. For example, triglycerides have three main crystal modifications, namely α, β′ and β form. The α-modification is the most random and loosely packed, followed by β′ form which is more closely packed whereas the β-modification is highly ordered with most closely packed molecules. The α and β′ forms, when formed on recrystallization of lipid on processing of SLNs, have the tendency to be transformed into the most stable β form [49]. If this transformation takes place during storage, the initial nanoparticulate structure may be lost, leading to aggregation or drug leakage, owing to a reduction of amorphous regions in the carrier lattice. Sometimes, cooling of nanaoemulsions may not result in crystalline structure of lipid but it may exist as supercooled melts. These supercooled melts may form liposomes or other colloidal structures [50]. Hence, characterization of the state of lipid is important to fully understand the formulations. Techniques employed to detect crystal forms of lipids include differential scanning calorimetry (DSC), X-ray diffraction (XRD), infrared and raman absorption and proton nuclear magnetic resonance (^{1}H-NMR).

1.3.4.4. Drug Content and Entrapment Efficiency

During the preparation of SLNs there is an interaction between drug and lipid which leads to the drug incorporation and this is based on the characteristics of both the lipid and the drug. Therefore, estimating the amount of drug actually present in the SLNs is important and is expressed in two terms, i.e. drug loading and entrapment efficiency.

Drug loading (DL) expresses the amount of drug in the particles divided by the weight of total carrier system (all ingredients taken together), while entrapment efficiency (EE) is the amount of the drug incorporated in the particles divided by its overall amount in the formulation. The drug content is nanoparticles is determined by extracting the drug from the SLN preparation by completely digesting the SLN structure with help of organic solvent. In order to estimate the amount of free drug, techniques like dialysis, ultracentrifugation, gel filtration or membrane filtration are utilized. Drug concentration is measured either in the separated aqueous media or directly in the particles.

1.3.4.5. In Vitro Drug Release

The most preferred and widely used practice for determining drug release from colloidal dispersions is the use of dialysis membranes of suitable cut-off diameter (usually 12000-14000 kDa). In this method, the SLN dispersion is placed in a dialysis tubing which is hermetically sealed to form a bag. This bag is then placed in a dissolution medium which maintains sink condition for the drug. The free drug diffuses out from the bag while the movement of SLNs with encapsulated drug is hindered. The amount of drug released with time is estimated using suitable analytical technique [51, 52]. Reverse dialysis method can also be used to determine drug release from SLNs. In this, a dialysis bag containing dissolution medium is placed in SLN dispersion and drug content is estimated in the medium inside the bag. Another technique is by using Franz diffusion cells, in which the donor and the receiver compartments are separated by a cellophane membrane of suitable molecular weight cut-off size. The SLN dispersion is placed at the donor side and drug released is analyzed by sampling from the receiver chamber. The in vitro release can provide only an estimate of in vivo performance of the SLNs because it does not take into account the possible enzyme degradation of SLNs or their interaction with cell organelles and lipid membranes in the body.

1.3.5. Stability of SLNs

Typically SLNs should follow the general stability aspects of formulations meant for drug delivery. The physico-chemical stability of the lipid carriers show variations due to their composition and structure [53]. The assessment of long term physical stability of SLNs during storage is done by measuring particle size and distribution, zeta potential, drug content and encapsulation efficiency. All these parameters depend on the type of lipid used and the storage conditions, mainly temperature. Chemical instability due to change in polymorphic form of lipid should also be studied during storage period, as transformation from one form to other with perfect crystalline lattice can cause drug expulsion or leakage.

Stabilization of the particles can be achieved by removal of the water as the powders are generally more stable than the suspensions. For this purpose, the most commonly used technique is freeze-drying or lyophilization, which is the removal of water from a frozen sample by sublimation and desorption under vacuum [54]. SLN dispersions are freeze-dried to obtain dry products which can easily be stored and reconstituted before use by addition of an aqueous medium. The freeze-drying process may modify the size and shape and the protective properties of the surfactant in the SLNs. Drug expulsion and change in the zeta potential are also possible during the process. Inclusion of suitable cryoprotectant is mandatory before lyophilization. Glucose, mannose, maltose, trehalose, sucrose are some of the cryoprotectants that are used in SLNs. These cryoprotectants prevent lipid adhesion after freeze-drying by forming a hydrophilic protective sheath and allow reconstitution into dispersions [55, 56].

Spray drying is another process that is used to produce powders from SLN dispersions [57]. In involves the use of high temperature, so it is preferable that the lipids in the formulation have melting points higher than 70°C. Carbohydrates and ethanol mixtures instead of pure water are used during the drying process to optimize the powder properties after redispersion.

Microbial stability is essential for SLNs meant for parenteral administration and is achieved using different sterilization techniques. Membrane filtration, autoclaving and sterilization with γ- radiation are used for this purpose. SLNs with small particle size (< 200 nm) and narrow size distribution can be filtered using 0.22μ aseptic filters, but is not suitable because it involves application of high pressure during filtration. γ- sterilization

can be used but it sometimes causes chemical breakdown of the phospholipid structure. The most popular method is by steam sterilization. The SLN structure is presumed to be reformed on cooling after the sterilization cycle is complete. The process of autoclaving may or may not have effect on physical stability and particle size of SLNs [58]. It may also cause degradation of the lipid and/or drug used in the formulation due to the high temperature. Hence, it becomes necessary to re-evaluate the properties of the SLNs after sterilization. Aseptic processing can also be applied but is very expensive and complex.

1.3.6. Routes of Administration of SLNs

Owing to their unique advantages, SLNs have been explored as drug carriers for various drugs and delivered via different routes of administration including parenteral, oral, transmucosal and topical.

1.3.6.1. Parenteral Administration

SLNs can be administered by i.v., i.m. or subcutaneous (s.c.) route. SLNs are usually used to target a specific a tissue or organ after i.v. administration. The particle size of i.v. administered SLNs must be below 5µm to avoid blocking of fine blood capillaries leading to embolism. It is seen that due to the small size and hydrophobic surfaces, SLNs are rapidly cleared from the circulation by the organs of the RES, particularly liver and spleen. This passive targeting to RES is undesirable except in some cases of liver diseases and physiologic disorders. Consequently, it becomes necessary to avoid such recognition of SLNs by the RES and form particles having long circulation times. To achieve this, 'stealth SLNs' were prepared by coating of nanoparticle surface by hydrophilic substances such as PEG, poloxamers, Tween 80, etc. which prevent recognition of SLNs by the proteins and macrophages of the RES. SLNs may also act as controlled release or depot formulations when administered by i.m. or s.c. route. The release depends on the nature and amount of lipid, surfactant, particle size and drug incorporation in the SLNs. Delivery of drugs incorporated into SLNs by parenteral route has been extensively reviewed by Wissing et al. [59].

1.3.6.2. Oral Administration

SLNs have been explored as an effective delivery system to overcome the challenges associated with oral delivery of drugs that have low solubility, poor permeability, degradation and instability in the GIT and presystemic metabolism. Several mechanistic approaches are proposed for the absorption of SLNs when administered via peroral route

[60]. The possible routes of absorption of nanoparticles are intercellular or paracellular transport (particles < 500nm), and intracellular uptake by the M-cells of the Peyer's patches in the gut. M-cells are specialized epithelial cells located above the lymphoid follicles of the gastrointestinal tract and transfer antigens from the lumen to the systemic circulation through intestinal lymphatics via thoracic lymph duct [61]. Another possible route of uptake of SLNs is similar to the absorption process of the dietary lipids, i.e, by lipase mediated chylomicron formation into the lymphatic system. This direct transport to the lymphatic system of drugs incorporated in SLNs offers the advantage of bypassing the liver first pass effect and the efflux transporters expressing on the intestinal epithelium. In addition, targeting to lymph may be beneficial in the treatment of lymphatic cancers and infections such as leishmaniasis, malaria and acquired immunodeficiency syndrome (AIDS).

Factors which affect the absorption of SLNs after oral administration include their particle size and surface characters. Particle size, in the submicron range, is a very critical parameter in the absorption of SLNs [62]. The M-cell uptake of nanoparticles is found to be size-dependent, i.e., smaller the size, higher the uptake. Larger particles are retained in the Peyer's patches, while smaller ones are released, facilitating the absorption into the lymphatic system. Surface properties, like hydrophobicity and surface charge influence bio-adhesion and interaction with lipases present in the gastrointestinal tract (GIT). SLNs with higher hydrophobicity exhibited relatively higher accumulation in the Peyer's patches [62] and exhibited lesser degradation by the lipases in the GIT. Similarly, surface modification of SLNs also alter their absorption process. Ligands such as lectins, when attached to the surface of SLNs, increase the adhesion of particles to the mucous of GIT and increase the uptake by M-cells [63]. Surface modification of SLNs with surfactants like polysorbates or poloxamers prevents the enzymatic degradation of the particles due to the creation of a steric barrier [64].

Several categories of drugs such as anti-cancer [65], antibiotics [66], antiviral [67], antihypertensives [68], etc have been loaded into SLNs and studied for oral delivery. The advantages achieved include enhanced bioavailability, improved stability and a reduction in toxicity.

1.3.6.3. Transmucosal and Pulmonary Administration

Transmucosal routes are increasingly gaining interest among pharmaceutical scientists as a means of delivering drugs through various absorptive membranes. These include buccal, sublingual, nasal, ocular, vaginal and rectal routes. Advantages of these routes include possible avoidance of first pass effect and avoidance of metabolism or degradation of drug within GIT. The use of nanocarriers with mucoadhesive properties represents a challenging but promising drug delivery strategies. Mucoadhesive drug delivery systems prolong the residence time of the dosage form at the site of absorption while the nanoparticulate carrier facilitates the enhanced permeation through the mucosal barrier, thus contributing to improved therapeutic response of the drug. Though there are few reports of SLNs in buccal, vaginal and rectal delivery, applications via ocular route has been exploited extensively. Cavalli and co-workers prepared SLNs incorporating tobramycin of particle size < 100 nm and studied its ocular bioavailability in rabbits [69]. On instillation in eye of rabbit, the SLNs produced a 1.82 times higher concentration and 4.2 times increased amount of tobramycin in aqueous humour, when compared to the tobramycin standard eye drops. Additionally, increased residence time of 6 h and no sign of ocular irritation was seen in case of SLNs. Cationic SLNs are also developed for enhancing the corneal adhesion and improved permeation by mechanisms like phagocytosis by cornea epithelial cells [70]. This longer adhesion leads to lesser frequency of administration.

Nasal administration of SLNs of drugs like ondansetron [71], budesonide [72], alprazolam [73], and many others have been studied. SLNs have also been evaluated as drug carriers for direct nose-to-brain delivery of certain CNS active compounds [74]. In recent years only, pulmonary delivery of SLNs has been exploited both for treatment of airways diseases and systemic disorders as it is a non-invasive route, has large surface area of alveolar epithelium, rich vasculature and circumvents the first pass effect [75]. The particle size required to reach the inner airways is between 1-5 µm, so the SLN dispersions are converted into composite microparticles by spray or freeze drying using suitable powder carriers. SLNs loaded with paclitaxel for direct targeting to lung lymphatics have been developed and in vivo efficacy was studied in tumor bearing mice model. SLN aerosols were administered using an inhalation chamber connected to the nebulizer [76]. It was seen that the SLNs inhalation groups presented an efficiency

superior to that observed with commercial i.v. Taxol formulation, with a decrease in metastases numbers per animal and no adverse effects were reported.

1.3.6.4. Topical Administration

SLNs have also found their application via the dermal route and several cosmetic preparations based on SLNs have reached the market [42]. Pharmaceutical agents that have been incorporated in SLNs and delivered by the dermal route include several antifungals [77], antibiotics and steroids [78] that are frequently used in the treatment of skin disorders. Due to small particle size and in turn increased surface area, SLNs ensure close contact to stratum corneum and thereby increases penetration of encapsulated drug into the skin. Sustained release property of SLNs supplies the drug to the skin over a prolonged period and the occlusive nature results in film formation on the skin, which reduces transdermal water loss. Chemically labile vitamin-based products like vitamin E are protected against degradation by incorporating them into SLNs and also show better permeation [79].

1.3.7. Applications of SLNs

It has been more than 20 years since SLNs have been studied as drug delivery carriers and have been used to incorporate a broad variety of drugs with modified pharmacokinetic and manifold pharmacologic actions. Few important areas of application of SLNs include tumour targeting in cancer chemotherapy, brain targeting in treatment of central nervous system disorders and in protein, peptide and gene delivery.

Targeting of anti-cancer drugs to tumor location using SLNs has been widely investigated and promising results are obtained [80]. These nanostructures have the ability of passive targeting to tumor cells due to enhanced permeation and retention (EPR) effect. The leaky vasculature of the tumor paves the way for SLNs to accumulate into the tissue, while the normal tissues are escaped, leading to selective distribution. Active targeting to a particular tumor site is also possible by surface modification of the SLNs. Due to preferential accumulation of SLNs at the tumor site, the toxicity of anti-cancer drugs decreases, which is the most beneficial characteristic of nanoparticles in cancer chemotherapy. It is also possible to deliver drugs across the BBB by means of SLNs [81, 82], which facilitate the transport of molecules across the BBB on the account of their nano size and their lipophilicity. SLNs also help in overcoming the efflux of drug molecules from cancer and brain cells leading to multi-drug resistance (MDR)

phenomenon [83, 84]. It is also seen that polysorbate coated SLNs show enhanced permeation to the brain [85]. This effect is seen due to adsorption of a serum protein, apolipoprotein E to polysorbate present on the surface of SLNs. This protein aids in the transport of lipids into the brain via a low density lipoprotein receptor on the BBB.

SLNs also act as an attractive drug delivery system for biopharmaceuticals, like protein and peptide based drugs, particularly through non-parenteral routes as needle-free alternatives. Their ability to bypass gastric and intestinal degradation, controlled release behavior and the unique uptake mechanisms through the intestinal mucosa make them a promising carrier for oral protein delivery. However, protein incorporation into SLNs is not an easy task on the account of the high hydrophilicity and instability during handling of these biomolecules. Several researchers have regularly published promising results concerning the incorporation of several peptides and proteins in SLN particulate carriers. These results are highlighted in a review by the scientists Almeida and Souto in 2007 [86]. Gene therapy is a rapidly advancing field with great potential for the treatment of genetic and acquired systemic diseases. Advantages offered by SLNs for gene delivery include their rapid uptake by cells and protection of the incorporated compound against chemical degradation. Further preparation techniques involving less mechanical force are suitable as they do not damage the nucleic acid strands [87]. Cationic surfactants are usually preferred for gene delivery, because a positive surface charge will promote the interaction with the negatively charged cell membrane [88].

1.4. Antiviral Therapy and Role of SLNs

Viral infections are ubiquitous in the world and require specific antiviral therapy to combat the symptoms. The antiviral therapies that are currently available are developed for the treatment of infections due to human immunodeficiency virus (HIV), herpes viruses, hepatitis B and C and influenza viruses. These agents include small molecular weight drugs that inhibit viral replication in the body. Most of them are meant for oral use and suffer from several drawbacks that reduce their efficacy, like poor solubility, short half-life and low bioavailability necessitating the administration of large and frequent doses causing side effects. This leads to patient incompliance and failure of adherence to therapy and recurrence of symptoms. The long term use of antiviral agents also causes development of resistant strains and emergence of new viruses, subsequently leading to failure of therapy.

Apart from being time consuming and expensive, development of new antiviral agents is a big challenge because there are only limited viral specific sites that can be targeted without harming the host cells. Hence, reformulating the available antiviral agents into NDDS has been taken up to improve their therapeutic efficacy [89]. The advantages offered by SLNs as drug delivery systems have been exploited in the delivery of antiviral drugs and have shown promising results.

Acyclovir is the most efficacious drug used against infections caused by the herpes simplex viruses. Its delivery presents major challenges like poor solubility and permeability, leading to administration of large and frequent doses. SLNs for acyclovir have been developed for improving its dermal and ocular penetration by different research groups [90, 91]. In one of such study, SLNs loaded with acyclovir were prepared using double emulsion process and evaluated in vitro for skin permeation using rat and human cadaver skin model [92]. It was seen that there was 15.17 and 17.65 fold accumulation of acyclovir in dermal tissues of rat and human cadaver skin, respectively, as compared with the commercial formulation. In the same study histopathological observation suggested no cutaneous toxicity of SLNs.

SLNs for delivery of antiretroviral drugs have also been formulated to target HIV. The passive uptake of nanocarriers by macrophages is particularly beneficial here as the macrophages and T-cells are the primary targets of HIV. Several antiretroviral agents like zidovudine [93], lamivudine [94], combination of stavudine, delavirdine and saquinavir [95], etc have been loaded into SLNs to target the infection site, thereby increasing the therapeutic effectiveness of the drugs. Aji Alex *et al*. studied the possibility of intestinal lymphatic targeting of lopinavir using SLNs. Lopinavir is a protease inhibitor which has become an important component of the combination chemotherapy of HIV and has shown poor oral bioavailability due to both first pass metabolism and P-glycoprotein (P-gp) efflux. The SLNs were formed using glyceryl behenate and were studied for lymphatic uptake by performing in vivo oral pharmacokinetic study in rats. A significant enhancement in the bioavailability of lopinavir was observed after drug-loaded SLNs were administered with a 2.13 fold increase in mean plasma concentration of lopinavir than that obtained after administration of an aqueous dispersion of drug. This result was attributed to the ability of SLNs in reaching the oral lymphatic region (approximately 5 times higher than aqueous dispersion) which ultimately delivers the drug to the systemic circulation [96].

Thus, it can be seen that SLNs could improve the treatment efficacy of antiviral drugs by reducing the occurrence of adverse effects and resistance. It is also possible to reduce the dose and frequency of administration and shorten the time of treatment, rendering the therapy more cost effective and patient compliant.

1.5. Definition of Problem and Objectives of the Research

Ganciclovir is an antiviral drug effective against cytomegalovirus (CMV), a strain of herpes viruses [97]. CMV is a virus which is global and most people in the world are exposed to CMV in their lifetime, but typically only those with a weakened immune system acquire the infection. Serious CMV infections can occur in people who have weakened immune systems due to AIDS, have undergone organ transplant, under chemotherapy or immunosuppressive drugs. Disease manifestations of CMV infection include GIT diseases (the most common), pneumonia (rare but most serious), retinitis and encephalitis [98]. CMV retinitis, a sight-threatening infection associated with AIDS, currently requires lifelong i.v. treatment with ganciclovir.

Ganciclovir was the first Food and Drug Administration (FDA) approved drug with significant activity against CMV and is still the primary drug of choice for these infections. Initial treatment with ganciclovir involves daily continuous i.v. infusions for several days, followed by oral or i.v. maintenance therapy [99]. The i.v. therapy suffers from limitations like patient inconvenience associated with i.v. administration, higher cost, incidence of needle- related infections and sepsis. Oral treatment is of choice but poor bioavailability (<10%) requires frequent administration of large dose per day (four capsules of 250 mg administered each time, thrice in a day) [100]. Ganciclovir is a BCS-class III drug having high solubility and low permeability due to its hydrophilic nature. It is mainly transported by paracellular route, where the limited surface area and the tight junctions present between the adjacent cells restrict the transport of the drugs. Few reports also suggest P-gp mediated efflux of ganciclovir from the enterocytes back to the intestinal lumen [101]. Furthermore, poor oral bioavailability of ganciclovir is associated with greater inter-subject variability of plasma concentrations, development of drug resistance and drug wastage. These shortcomings necessitate the need for better oral delivery systems for ganciclovir, particularly for increased absorption and selective distribution.

SLNs have been widely studied in past few years to enhance the oral bioavailability of numerous drugs. Considering the advantages offered by SLNs as drug carriers, the present research was aimed at the design and development of novel SLN based drug delivery systems, to improve oral absorption and bioavailability and attain modified distribution of ganciclovir. To achieve this, following objectives were laid down for the current research work

- Design and development of ganciclovir loaded SLNs using different excipients and methods of preparation
- Characterization of the developed formulations for particle size, PDI, morphology, zeta potential, DL and EE
- Optimization of various formulation parameters to achieve the desired product characters
- Study of in vitro release characters and stability of the designed formulations
- Perform pharmacokinetic and biodistribution studies of the optimized batches to assess the in vivo behaviour of the novel formulation

The proposed research work required analysis and estimation of drug at different levels of sensitivity at various stages and also some preformulation studies. Thus, suitable analytical and bioanalytical methods were also planned to develop and validate estimation of ganciclovir in various studies during the preformulation, formulation and characterization stages. The following methods were planned to develop:

- Ultraviolet (UV) Spectrometric method
- Spectrofluorimetric method
- High performance liquid chromatographic (HPLC) methods

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2. Drug Profile

2.1. Introduction

Ganciclovir is a synthetic nucleoside analog of 2'-deoxyguanosine and a potent inhibitor of the human herpes virus family. It was approved by FDA in 1989 as a drug with significant activity against CMV and still remains the first line treatment for CMV infection in immunocompromised people like patients with advanced HIV infection or who have received an organ transplant and are at risk of CMV infection [1, 2]. It works by preventing the spread of CMV infection or slowing the growth of CMV. It was shown to be 26 times more potent than its analogue acyclovir against the human CMV strain in vitro.

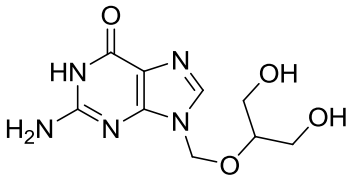
2.2. Physicochemical Properties

Name : Ganciclovir

Chemical name : 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine

Molecular formula : $C_9H_{13}N_5O_4$

Chemical structure :



Molecular weight : 255.23

Chemical Class : Guanine nucleoside analogue

Therapeutic Class : Antiviral

Description : Odourless, white to off-white crystalline powder

Melting point : 250-252°C

Solubility : It is a polar hydrophilic compound with a solubility of 2.6 mg mL^{-1} in water at 25°C

pK_a : 2.2 (pK_a1) and 9.4 (pK_a2)

Partition coefficient : 0.022 (n-octanol/water)

2.3. Therapeutic Indications and Dosage

Ganciclovir is indicated for the treatment of CMV retinitis in immunocompromised patients, including patients with AIDS. It is also indicated for the prevention of CMV disease in transplant recipients at risk for CMV disease [3]. Parenteral ganciclovir is used for induction and maintenance treatment of CMV retinitis, while oral ganciclovir for maintenance treatment of CMV retinitis in patients whose active retinitis has been resolved by i.v. induction therapy. Oral therapy with ganciclovir is also used for the prophylaxis of CMV disease in immunocompromised patients who are at risk for developing CMV disease [4]. The off-label indications for ganciclovir include CMV infections at various sites in the body like esophagitis, colitis, pneumonitis or CMV neurological disease. Ganciclovir is also used off-label in treatment of certain human herpes virus 8 diseases, including multicentric Castleman's disease (a disease of the lymph nodes) and varicella-zoster virus disease called progressive outer retinal necrosis [5].

For treatment of CMV retinitis, the recommended initial dosage for patients with normal renal function is 5 mg kg^{-1} (given i.v. at a constant rate over 1 h) every 12 h for 14 to 21 days. Following induction treatment, the recommended maintenance dose is 5 mg kg^{-1} given as a constant-rate i.v. infusion over 1 h once daily, 7 days per week or 6 mg kg^{-1} i.v. once daily for 5 days. For prevention of CMV disease in transplant recipients, the recommended initial dosage for patients with normal renal function is 5 mg kg^{-1} (given i.v. at a constant rate over 1 h) every 12 h for 7 to 14 days, followed by 5 mg kg^{-1} once daily, 7 days per week or 6 mg kg^{-1} i.v. once daily for 5 days [4].

Oral dose for maintenance therapy of CMV retinitis and for CMV prophylaxis is 1000 mg 3 times a day or 500 mg 6 times a day (every 3 h while awake) with food [6]. Patients who experience progression of CMV retinitis while receiving maintenance treatment should receive reinduction treatment.

For patients with renal impairment, the dose of ganciclovir is modified according to the level of impairment and for patients undergoing hemodialysis, the dose should not exceed 1.25 mg kg^{-1} 3 times per week, following each hemodialysis session [7].

For pediatric patients, the safety and efficacy of ganciclovir has not been established. Administration to pediatric patients should be undertaken only after careful evaluation and only if the potential benefits outweigh the risks. For patients with CMV retinitis and

more than 3 months of age, the induction therapy is given at 5 mg kg^{-1} i.v. every 12 h for 14 to 21 days, followed by maintenance therapy at 5 mg kg^{-1} i.v. once daily for 7 days or 6 mg kg^{-1} i.v. once daily for 5 days. For CMV prophylaxis in transplant recipients of age >1 year to 18 years, the induction therapy is 5 mg kg^{-1} i.v. every 12 h for 7 to 14 days followed by maintenance with 5 mg kg^{-1} i.v. once daily 7 days a week, or 6 mg kg^{-1} i.v. once daily, 5 days a week. Oral prophylactic dose is 10 mg kg^{-1} every 8 h with food for patients ≥ 6 months. For HIV-infected patients, the prophylactic dose for age group >1 month to 12 years is 5 mg kg^{-1} i.v. once daily and >12 years is equivalent to the adult dose [7].

2.4. Mechanism of Action

Human viruses that are susceptible to ganciclovir include herpes simplex viruses 1 and 2, human herpes virus type 6, human CMV, epstein-barr virus and varicella zoster virus. Ganciclovir has been shown to have significantly higher activity against the human CMV than other available antiviral agents. Ganciclovir inhibits viral DNA synthesis. It is converted to its active nucleotide i.e., ganciclovir monophosphate form intracellularly by a viral phosphotransferase encoded by the UL97 gene during CMV infection. Ganciclovir diphosphate and triphosphate are formed by cellular enzymes. The triphosphate form is a competitive inhibitor of deoxyguanosine triphosphate incorporation into DNA and preferentially inhibits viral rather than host cellular DNA polymerases. Incorporation into viral DNA causes eventual cessation of DNA chain elongation [8].

In vitro studies showed that, being a congener of acyclovir, ganciclovir exhibited comparable antiviral activity against herpes simplex virus and varicella zoster virus [9, 10], but was more potent in inhibiting human CMV [11, 12]. Ganciclovir was selectively activated to the triphosphate form in cells infected with CMV to levels at least 10-fold higher than those measured for acyclovir-triphosphate and up to as much as 100-fold higher than the levels found in uninfected cells [13]. It was found that in herpes simplex and varicella zoster virus, the initial step of phosphorylation of ganciclovir was carried out by the virus-specified thymidine kinase in a similar manner to that of acyclovir. It was also reported that human CMV lacks such a thymidine kinase activity in infected cells and the gene UL97 in HCMV encodes a protein that is homologous to protein kinases and bacterial phosphotransferases, which aids in the phosphorylation of ganciclovir into its monophosphate form [14, 15]. This unique phosphorylation

mechanism confers ganciclovir, the specificity of action towards CMV infected cells [16]. The further conversion of ganciclovir 5' monophosphate to di- and triphosphate forms is brought about by cellular kinases induced in cells infected with CMV.

2.5. Resistance

Although, ganciclovir has shown good and selective activity against CMV, its use in chronic antiviral therapy has been associated with the development of resistant virus [17-20], and resistant CMV has been associated with a poor response to therapy. Several research groups have isolated the resistant CMV strains and studied the mechanism involved in conferring resistance to ganciclovir [21-26]. Since the antiviral action of ganciclovir is dependent on its initial phosphorylation by a phosphotransferase encoded by the CMV (gene UL97) and the subsequent inhibition of viral DNA polymerase (gene UL54), any alteration or mutation in either of these genes can provide ganciclovir resistance.

2.6. Pharmacokinetic Properties

The pharmacokinetics of ganciclovir has been evaluated in immunocompromised patients with serious CMV disease. Dose independent kinetics was demonstrated over the range of 1.6 to 5.0 mg kg^{-1} when administered i.v. and up to a total daily dose of 4 g day^{-1} when administered orally [27]. At the end of a 1 h i.v. infusion of 5 mg kg^{-1}, total ganciclovir area under the concentration vs. time curve (AUC) ranged between 22.1 ± 3.2 (n=16) and 26.8 ± 6.1 µg h mL^{-1}(n=16) and maximum concentration (C_{max}) in serum ranged between 8.27 ± 1.02 (n=16) and 9.0 ± 1.4 µg mL^{-1} (n=16). The absolute bioavailability of ganciclovir following oral administration of ganciclovir capsules under fasting conditions was approximately 5% (n=6) and following food was 6-9% (n=32). When ganciclovir capsules were administered orally with food at a total daily dose of 3 g day^{-1} (500 mg 6 times daily or 1000 mg thrice daily), the AUC over 24 h and C_{max} at steady state, were similar following both regimens with an AUC of 15.9 ± 4.2 and 15.4 ± 4.3 µg h mL^{-1} and C_{max} of 1.02 ± 0.24 and 1.18 ± 0.36 µg mL^{-1}, respectively (n=16) [28].

In a phase I/II study the pharmacokinetics of oral ganciclovir in HIV infected persons was studied. Oral bioavailability ranged from 2.6% to 7.3%. The mean C_{max} achieved at 1000 mg every 8 h was 1.11 µg mL^{-1}, and mean trough level was 0.54 µg mL^{-1}. The time

to reach maximum drug concentration (t_{max}) in serum was 1.0–2.9 h, with a serum half-life ($t_{1/2}$) of 3.0–7.3 h, suggesting prolonged oral absorption [29].

Ganciclovir is widely distributed to all tissues and crosses the placenta, with volume of distribution (V_d) of approximately 0.74 L kg^{-1}. Although the distribution of ganciclovir into human tissue and fluid is not fully understood, it has been found that that i.v. administered ganciclovir concentrates in the kidneys, with lower concentrations in the lung, liver, brain, and testes. In individuals with renal impairment, distribution appears to be reduced. Ganciclovir crosses the blood-brain barrier with 41% of the plasma concentration detectable in cerebrospinal fluid. There is no marked accumulation in any of the tissue [2, 4].

Renal excretion of unchanged drug by glomerular filtration and active tubular secretion is the major route of elimination of ganciclovir. In patients with normal renal function, 91.3 ± 5.0% (n=4) of i.v. administered ganciclovir was recovered unmetabolized in the urine. Systemic clearance of i.v. administered ganciclovir was 3.52 ± 0.80 mL $min^{-1}kg^{-1}$ (n=98) while renal clearance was 3.20 ± 0.80 mL $min^{-1}kg^{-1}$ (n=47), accounting for 91 ± 11% of the systemic clearance (n=47). $t_{1/2}$ was 3.5 ± 0.9 h (n=98) following i.v. administration and 4.8 ± 0.9 h (n=39) following oral administration [4].

2.6.1. Special Populations

Ganciclovir pharmacokinetics, after i.v. administration, was studied in 27 neonates (aged 2 to 49 days) at two doses, 4 mg kg^{-1} (n=14) or 6 mg kg^{-1} (n=13). The pharmacokinetic parameters found were C_{max} of 5.5 ± 1.6 and 7.0 ± 1.6 µg mL^{-1}; systemic clearance of 3.14 ± 1.75 and 3.56 ± 1.27 mL $min^{-1}kg^{-1}$, respectively and $t_{½}$ was 2.4 h for both groups [30].

Ganciclovir i.v. pharmacokinetics was also studied in pediatric patients, aged 9 months to 12 years and it was found that the pharmacokinetic characteristics were the same after single and multiple i.v. doses (5 mg kg^{-1}). Also, the pharmacokinetics of both i.v. and oral ganciclovir in pediatric patients was found to be comparable to that observed in adults. [31, 32].

The pharmacokinetics following i.v. administration of ganciclovir was evaluated in eight CMV infected patients with various degrees of renal insufficiency [33]. The patients received 5 mg kg^{-1} as a 1 h infusion twice daily for periods of up to 2 weeks. The terminal elimination $t_{1/2}$ of ganciclovir was markedly increased (11.50 ± 3.90 h) as compared with

values obtained in patients with normal renal function (3.60 ± 1.40 h). In another similar study, the immunocompromised patients with renal impairment received i.v. doses ranging from 1.25 to 5.0 mg kg^{-1} based on the creatinine clearance (CrCl) values. The elimination $t_{1/2}$ of ganciclovir was increased from 4.6 ± 1.40 h in patients with CrCl values between 50 and 79 mL min^{-1} to 10.7 ± 5.7 h in patients with CrCl values < 25 mL min^{-1} [4]. Hemodialysis efficiently reduced levels of ganciclovir in plasma by about 50%, indicating that the drug should be administered after dialysis [33, 34]. These findings make it necessary to modify the dosage of ganciclovir in patients with renal impairment. No pharmacokinetic studies of ganciclovir have been conducted in adults older than 65 years of age.

2.7. Adverse reactions

The most common adverse events reported during ganciclovir therapy include neutropenia (with absolute neutrophil counts going as low as <500 μL^{-1}), thrombocytopenia (less than 50,000 platelets μL^{-1}), anaemia (haemoglobin counts as low as 6.5 grams $deciliter^{-1}$) and elevated creatinine [4]. Other less common adverse reactions include fever, rash, diarrhea, anorexia vomiting, confusion, seizures and neuropathy [35]. Catheter-related infection and sepsis are also frequently observed on prolonged i.v. dosing.

2.8. Drug Interactions

No formal drug interaction studies of ganciclovir and drugs commonly used in transplant recipients have been conducted, but an increase in serum creatinine level was observed when ganciclovir was administered with nephrotoxic drugs like cyclosporine or amphotericin B. The combination of ganciclovir and zidovudine (an anti-HIV agent) has been associated with an increased incidence of severe neutropenia, necessitating a reduction in the dose of zidovudine or its withdrawal or the addition of granulocyte colony-stimulating factor [36]. The combination of oral/i.v. ganciclovir and didanosine has been reported to result in a 72% increase in the AUC of didanosine when these drugs are administered simultaneously or 2 h apart, requiring monitoring of symptoms of peripheral neuropathy associated with didanosine [37, 38]. When coadministered with probenecid, the AUC of ganciclovir was increased by 53% due to an interaction involving competition for renal tubular secretion [38]. It is also possible that drugs that inhibit replication of rapidly dividing cell populations such as bone marrow,

spermatogonia and germinal layers of skin and gastrointestinal mucosa may have additive toxicity when administered concomitantly with ganciclovir.

2.9. Marketed Dosage Forms

Ganciclovir is available as a lyophilized powder for injection (500 mg), meant for i.v. route and as oral capsules (250 and 500 mg). An ophthalmic gel formulation for treatment of retinitis is also available.

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3. Analytical and Bioanalytical Methods

3.1. Introduction

Analytical method is an important and integral part in the process of drug discovery and development of pharmaceuticals [1, 2]. Each stage of the drug and formulation developmental process requires a suitable analytical method to authenticate the comprehensiveness and quality of the procedure. A series of analytical methods are developed for the specific needs of the particular development stage. For example, analytical methods are developed to aid in the process of drug synthesis, screen potential drug candidates, support formulation studies, monitor the stability of bulk and formulated products, and test final products for various in vitro and in vivo performance attributes. Since, analytical methods play very vital role, their quality and dependability is a major factor in the drug and formulation development programme. It is very necessary to ensure that the analytical method being employed provides accurate and reliable results. Therefore, the method which is developed for a particular purpose needs to be validated for certain parameters so that the method is effective in providing true, reliable and consistent results and is free from erroneous and false conclusions [3]. The validation parameters have been laid down by regulatory agencies like International Conference on Harmonization (ICH), FDA, United States Pharmacopoeia (USP), etc and typically include specificity, selectivity, sensitivity, linearity, accuracy, precision, robustness and ruggeddness [4-6]. The method of determination, sample size, statistical tests and interpretation of the results of these parameters are also specified in these guidelines. Thus, well developed and validated analytical methods became a pre-requisite for successful formulation development.

Ganciclovir is official in the USP 37 which describes its assay procedure by using HPLC [7]. However, for daily and routine analysis, HPLC is not preferred. An extensive survey of literature has revealed UV-visible spectrophotometric [8, 9] and HPLC methods [10-12] for determination of ganciclovir in bulk and pharmaceutical formulations. For estimation of ganciclovir in biological matrices like plasma, serum, urine and tissues, the literature is enriched with HPLC methods utilizing UV, fluorescence and mass spectroscopic detectors [10, 13-20]. Radioimmunoassay and enzyme-linked immunosorbent assay have also been reported for the quantification of this drug [21].

HPLC is the most extensively used technique for quantification of ganciclovir. The official method available in USP utilizes large proportion of organic modifier in the

mobile phase, which increases the cost of analysis at laboratory scale. Reported HPLC methods are mostly dedicated for estimation of ganciclovir in human plasma or serum. Some of these methods involved elaborate and prolonged procedures like gradient elution [15], amperometric detection [22], precolumn flurosecence derivatization [23] and ion exchange chromatography. Also, many methods used ion pairing agents in mobile phase which, apart from increasing the cost of analysis, cause deterioration of column during prolonged use and occurrence of interfering or ghost peaks during analysis. The reported HPLC methods for determination of ganciclovir in pharmaceutical formulations are simple and accurate but utilize volatile mobile phase which may lead to fluctuation in mobile phase characteristics like % composition, pH etc. leading to variation in estimation during long runs [10-12]. The reported spectrophotometric methods are either based on derivatization of drug with some agent [8, 9, 24-28] or utilize derivative spectroscopy to quantify the drug [9]. These techniques make the estimation of drug lengthy and costly.

For the present work, quantitative estimation of ganciclovir was required during several preformulation studies, in vitro characterization of the developed formulation and during in vivo pharmacokinetic and biodistribution studies of the optimized formulations. A simple, fast and accurate analytical method which could be used for routine drug assay, solubility and dissolution studies was desirable. For this purpose, a new simple UV spectrophotometric and a more sensitive spectrofluorimetric methods were developed. These methods were based on measuring the native absorbance and fluorescence respectively, hence were simple and rapid, eliminating the need of any pre-reaction or derivatization.

Since analysis of drug in presence of related substances and excipients is an intricate process, it was also imperative to develop a stability indicating method, which would efficiently separate the drug from impurities, degradants and interferences from the excipients used in formulation design. Though several bioanalytical methods are reported, none was considered suitable for estimation of ganciclovir in rat plasma and tissue samples due to complex sample preparation methods, use of concentrated protein precipitating agents and multi-component mobile phase that could cause column deterioration. Therefore, a stability indicating HPLC method for determination of ganciclovir in presence of related substances and degradation products was developed,

which was then extended to estimation of ganciclovir in rat biomatrices for accurate quantification of in vivo pharmacokinetic parameters.

The in-house developed methods were validated according to the current regulatory guidelines pertaining to analytical and bioanalytical methods using suitable statistical tests [29]. These methods were successfully applied for estimation of ganciclovir in different samples obtained during the formulation development. These methods' development and validation processes are presented in this chapter.

3.2. Materials

Ganciclovir (assay 99.6% w/w) was obtained as a gift sample from Ranbaxy Laboratories Limited (Gurgaon, India). Ultrapure water (Milli-Q Plus, Millipore®, India) was used throughout the analysis. HPLC grade methanol was purchased from Merck. All buffer salts were of analytical grade and procured from SDFine Chemicals Limited (Gujarat, India). Excipients such as lactose monohydrate, croscarmellose sodium, microcrystalline cellulose, colloidal silicon dioxide and magnesium stearate were obtained from IPCA labs Limited (Mumbai, India) as gift samples. Glyceryl monostearate (GMS), glyceryl distearate (GDS) and glyceryl behenate (GB) were obtained as gifts from Gatefosse (France). Poloxamer 188 (PF-68) and d-alpha tocopheryl polyethylene glycol 1000 succinate (TPGS) were obtained from BASF Inc. (Germany) while soy lecithin, Lipoid S75 from Lipoid (Germany). Commercial product Ganguard® capsules (Ranbaxy Laboratories Limited, India), labeled to contain 250 mg of ganciclovir, were purchased from local market. Ganciclovir loaded SLNs were prepared in-house.

3.3. Spectrometric Methods

3.3.1. Spectrometric Conditions

A double-beam Jasco (Japan) UV-Visible-Near Infrared spectrophotometer, model V570 connected to computer loaded with spectra manager software and a double-beam Shimadzu (Japan) UV-Visible spectrophotometer, model UV 1800 connected to computer loaded with UV probe software were used for the UV method development and for intermediate precision respectively. Both the instruments have an automatic wavelength accuracy of 0.1 nm and matched quartz cells of 10 mm path length. Absorption spectra were recorded from 190 nm to 400 nm at scanning speed of 400 nm sec^{-1} using medium response mode and 1nm bandwidth. Quantitative analysis was carried out at fixed wavelength mode.

A Shimadzu (Japan) spectrofluorophotometer Model RF-5301PC loaded with in-built software and equipped with 150W xenon lamp and 3mm quartz cells was used for the spectrofluorimetric method. Measurement parameters used were: excitation band width - 5 nm, emission band width - 5 nm, photo-multiplier tube response - high, slit width - 1 nm, λ_{ex}= 257 nm and λ_{em} = 374 nm.

3.3.2. Method Development

Two spectrometric methods namely, UV spectrophotometric and spectrofluorimetric methods were developed for quantitative estimation of ganciclovir. Different media were investigated to develop the methods based on the solubility of the drug, ease of sample preparation, stability and sensitivity of response and wide-ranging applicability of method. Absorbance and fluorescence of samples were measured at respective optimized wavelengths and sensitivity of the methods was calculated based on standard formulae.

3.3.3. Preparation of Stock and Calibration Standards

A stock solution of 100 µg mL^{-1} of ganciclovir was prepared by dissolving 5 mg of drug in 50 mL of water. For preparation of different concentrations, aliquots of stock solutions were transferred into a series of 10 ml standard flasks and volumes were made with the optimized media i.e., pH 1.2 hydrochloric acid buffer (USP). Nine different concentrations in the range of 3-27 µg mL^{-1} and eight concentrations in the range of 0.25 to 2 µg mL^{-1} of ganciclovir were prepared in the hydrochloric acid buffer for standard graph of UV method and spectrofluorimetric method respectively.

3.3.4. Validation of Methods

Selectivity of the methods was established by analyzing the drug in presence of excipients. Samples containing excipients with and without ganciclovir (placebo) were prepared and analyzed by the proposed methods and the responses obtained were compared with that of pure drug solution. In a separate study, samples were prepared independently from pure drug stock and commercial sample stock solution in the selected medium and analyzed. Paired *t*-test and variance ratio *F* test at 95% level of significance were performed to compare the means of percent recovery values.

To establish linearity of the proposed methods, nine replicates of the drug concentrations 3-27 µg mL^{-1} and 0.25 to 2 µg mL^{-1} were prepared from the stock solutions and analyzed by the UV and fluorescence methods, respectively. The data was subjected to least square regression analysis and predicted concentrations were calculated from the corresponding

equations generated. One way ANOVA at 95% level of significance was performed on the response values observed for each pure drug concentration during the replicate measurement of the standard solutions.

Sensitivity of the methods was expressed as the limit of quantification (LOQ) and limit of detection (LOD). The LOQ and LOD were calculated according to standard formulae as $10 \times \sigma/S$ and $3.3 \times \sigma/S$, respectively, where σ is the standard deviation of y-intercept of regression equation and S is the slope of the calibration curve.

As a part of determining accuracy of the proposed methods, different levels of drug concentrations namely, low quality control (LQC), medium quality control (MQC) and high quality control (HQC) were prepared from independent stock solution and analyzed. Accuracy was assessed as the % bias and mean % recovery for each of the quality control samples. To further validate the accuracy of the methods, recovery studies were performed by standard addition method. The pre-analyzed samples were spiked with three different levels of the standard ganciclovir, analyzed by the proposed methods and recovery of the drug was calculated at each of the different levels.

For precision studies, different levels of drug concentrations (LQC, MQC and HQC) were prepared for three different times in a day (intra-day variation) and for three different days (inter-day variation). One set of these samples were re-analyzed using Shimadzu instrument by the proposed UV method to establish inter-instrument precision. The % relative standard deviation (% RSD) of the predicted concentrations from the regression equations was taken as the measure of precision.

Robustness was examined by evaluating the influence of small variations in the experimental parameters on the analytical performance of the methods. It was determined by (a) changing pH of the medium by ± 0.2 units and (b) stability of ganciclovir in the selected medium at room temperature for 48 h. Three different concentrations (LQC, MQC and HQC) were prepared in the media with different pH and mean % recovery was determined for each sample.

3.3.5. Analysis of Formulations

Ten capsules were weighed, their contents were taken and thoroughly mixed. Amount of the powder equivalent to 50 mg of ganciclovir was taken, mixed with the selected medium to prepare a 100 μg mL^{-1} concentration, sonicated for 30 min and filtered through Whatman filter paper number 40. The filtrate was suitably diluted to prepare

concentrations in the range of calibration curves. The samples were then analyzed by the methods and the nominal content of ganciclovir in the capsules was calculated using the regression equations.

3.3.6. Results and Discussion

For the development of spectrometric methods, various buffers of pH ranging from 1-12 were investigated. Organic solvents were not used since ganciclovir has good solubility in aqueous media and all study samples were to be in aqueous media. Among the pH ranges tried, ganciclovir depicted absorbance at all the pH ranges with emission decreasing with increase in the pH above 3.0. This led to the selection of pH 1.2 hydrochloric acid buffer (USP) as the medium for method development as ganciclovir showed maximum and stable absorbance and native fluorescence intensities at this pH. The medium was easy to prepare and had good buffering capacity. Absorption and emission spectra of ganciclovir are shown in Figure 3.1. Ganciclovir showed absorption maxima at 255 nm in UV method and fluorescence intensities were measured at 374 nm (λ_{em}) after excitation at 257 nm (λ_{ex}). The calibration data of both the methods is presented in Table 3.1.

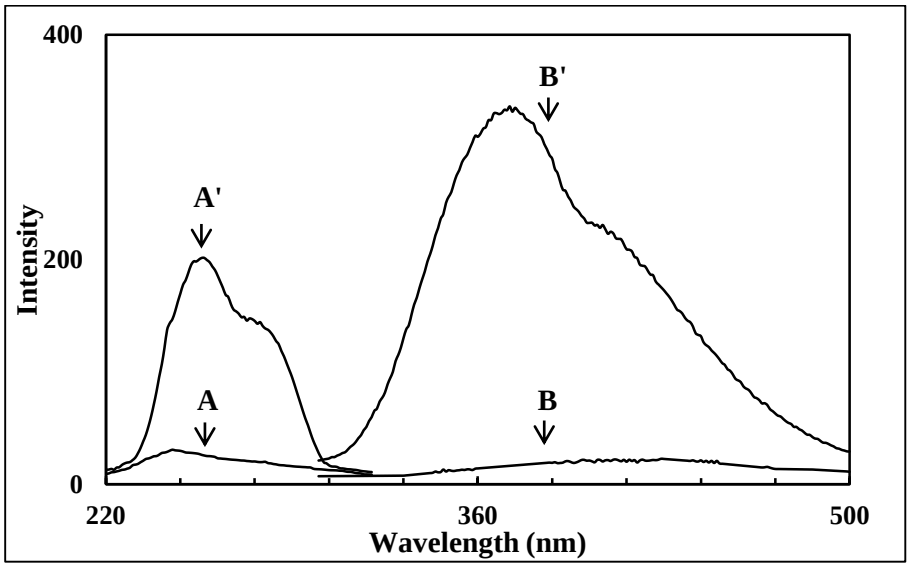

Figure 3.1. Absorption (A, A') and emission (B,B') spectra of blank hydrochloric acid buffer of pH 1.2 (A, B) and ganciclovir (A', B')

Table 3.1. Calibration data of ganciclovir by spectrometric methods

UV Spectrophotometric Method			**Spectrofluorimetric Method**		
Drug conc. (μg mL^{-1})	**Absorbance (± SD)**	**% RSD**	**Drug conc. (μg mL^{-1})**	**Fluorescence Intensity (± SD)**	**% RSD**
3.00	0.1457 ± 0.003	1.77	0.25	69.96 ± 2.71	3.87
6.00	0.2920 ± 0.006	1.18	0.50	136.48 ± 1.92	1.41
9.00	0.4343 ± 0.008	1.83	0.75	204.26 ± 2.47	1.21
12.00	0.5794 ± 0.011	1.87	1.00	271.66 ± 2.11	0.78
15.00	0.7211 ± 0.006	0.79	1.25	337.24 ± 2.28	0.68
18.00	0.8695 ± 0.006	0.68	1.50	403.05 ± 2.98	0.74
21.00	1.0152 ± 0.007	0.66	1.75	469.30 ± 3.93	0.84
24.00	1.1585 ± 0.006	0.55	2.00	538.47 ± 8.18	1.52

Each value represents average of nine individual determinations

The validity of the methods was checked by testing selectivity, linearity, sensitivity, accuracy, precision and robustness according to ICH recommendations.

The selectivity of the proposed methods was proven by their ability to determine ganciclovir without interference from the common excipients, both in placebo and formulation samples. The emission spectrum of pure drug sample was matching with the marketed formulation sample in the selected medium. The calculated *t*-value and variance ratio *F*-value were found to be lower than that of the critical values, indicating that statistically there was no significant difference between response obtained for solutions prepared from pure drug sample and commercial formulation sample (Table 3.2).

Using the above procedures, it was found that, there is a linear relationship between response and concentrations over the range of 3-24 μg mL^{-1} in UV method and 0.25–2 μg mL^{-1} in spectrofluorimetric method. Statistical evaluation of the regression data for ganciclovir was performed and standard deviation of the residual ($S_{y/x}$), standard deviation of the intercept (S_a) and standard deviation of the slope (S_b) for both the methods are given in Table 3.3. The very low values of these parameters point out to low scattering of the points around the calibration curve, thus, indicating the high accuracy and high precision of the methods. Goodness of fit of regression equation was supported by high regression coefficient value of 1.0 and less calculated *F*-value in the proposed methods. The LOD and LOQ values are also summarized in Table 3.3.

Table 3.2. Determination of ganciclovir in commercial formulation and statistical comparison with pure drug sample to establish selectivity

Conc.	% Recovery ± SD			
	UV Spectrophotometric method		Spectrofluorimetric method	
	Commercial capsules	Pure drug sample	Commercial capsules	Pure drug sample
LQC*	99.40 ± 0.84	100.52 ± 0.56	101.65 ± 1.22	97.84 ± 1.05
MQC*	100.53 ± 1.29	101.32 ± 0.49	100.29 ± 0.76	100.74 ± 0.59
HQC*	99.86 ± 0.48	100.40 ± 0.16	101.98 ± 0.04	101.94 ± 0.79
Mean	99.93 ± 0.57	100.75 ± 0.50	101.31 ± 0.90	100.17 ± 2.11
t	*1.87 (2.78)***		*0.84 (2.78)***	
F	*1.30 (19.0)***		*0.18 (19.0)***	

Each result is the average of three separate determinations

* LQC, MQC and HQC for UV method are 4, 13, 22 μg mL^{-1} and 0.4, 1.1 and 1.8 μg mL^{-1} for spectrofluorimetric method, respectively.

**Values between brackets are the tabulated *t* and *F* values, at p=0.05

Table 3.3. Performance data for the proposed spectrometric methods for determination of ganciclovir

Parameter	Results	
	UV Spectrophotometric	Fluorimetric
Concentration range (μg mL^{-1})	3.00-24.00	0.25-2.00
Regression coefficient (R^2)	1.00	1.00
Slope	0.0482	267.0000
Intercept	0.0009	3.5000
$S_{y/x}$	0.0016	0.9866
S_a	0.0011	0.7688
S_b	6.8×10^{-5}	0.6089
Calculated F value (critical F value)*	0.0014 (2.032)	0.0010 (2.032)
LOD (μg mL^{-1})	0.11	0.01
LOQ (μg mL^{-1})	0.33	0.03

where, $S_{y/x}$ Standard deviation of the residuals

S_a Standard deviation of the intercept

S_b Standard deviation of the slope

*Theoretical value of F(8,63) based on one-way ANOVA at P=0.05 level of significance

The mean recoveries of nearly 100% and low % RSD values obtained in accuracy studies (Table 3.4) revealed that any small change in the drug concentration in the solution can be accurately determined by these proposed methods. The validity and reliability of the methods were evaluated by standard addition method (Table 3.5).

Table 3.4. Evaluation of accuracy of the proposed sprectrometric methods for ganciclovir determination in pure form

UV Spectrophotometric Method				**Spectrofluorimetric Method**			
Conc. Taken (µg mL^{-1})	**Conc. Found (µg mL^{-1}) ± SD**	**% Recovery ± SD**	**% Bias***	**Conc. Taken (µg mL^{-1})**	**Conc. Found (µg mL^{-1}) ± SD**	**% Recovery ± SD**	**% Bias***
4	3.99 ± 0.05	99.87 ± 1.22	-0.13	0.4	0.406 ± 0.01	101.61 ± 2.41	1.61
13	13.16 ± 0.06	101.24 ± 0.43	1.24	1.1	1.117 ± 0.03	101.52 ± 2.78	1.52
22	22.07 ± 0.04	100.31 ± 0.18	0.31	1.8	1.817 ± 0.02	100.96 ± 1.31	0.96

Each result is the average of six separate determinations

*Accuracy is given in % bias = 100 × [(predicted concentration – nominal concentration)/nominal concentration)]

Table 3.5. Results of standard addition method for the spectrometric methods

UV Spectrophotometric Method				**Spectrofluorimetric Method**			
Preanalyzed amount (µg)	**Amount added (µg)**	**Recovered amount ±SD (µg)**	**% Recovery ± SD**	**Preanalyzed amount (µg)**	**Amount added (µg)**	**Recovered amount ±SD (µg)**	**% Recovery ± SD**
50.6	25	76.57 ± 0.64	101.28 ± 0.85	7.56	2	9.52 ± 0.11	99.54 ± 1.19
50.6	50	100.54 ± 1.18	99.94 ± 1.17	7.56	2.5	10.08 ± 0.12	100.24 ± 1.22
50.6	100	148.92 ± 0.37	98.89 ± 0.24	7.56	3	10.55 ± 0.09	99.93 ± 0.86

Each result is the average of three separate determinations

The results of the standard addition method showed high absolute recovery values. In the intra-day and inter-day precision study, the % RSD values were found to be very small indicating consistent repeatability. (Table 3.6).

In the robustness study, it was also found that none of the variables introduced in the experimental conditions significantly affected the performance of the methods as the recovery values were found to be close to 100% (Table 3.7). Also the ganciclovir solution in the selected medium exhibited no changes in response and spectra for 48 h when kept at room temperature. This provides an indication of the stability and dependability of the proposed methods during the routine analysis.

Table 3.6. Precision data for the proposed spectrometric methods

Conc.	**UV Spectrophotometric method**				**Spectrofluorimetric method**			
	Intra-day (% RSD) (n=3)			**Inter-day (% RSD) (n=9)**	**Intra-day (% RSD) (n=3)**			**Inter-day (% RSD) (n=9)**
	Day 1	**Day 2**	**Day 3**		**Day 1**	**Day 2**	**Day 3**	
LQC	0.96	1.21	1.34	0.98	2.30	1.51	1.03	1.51
MQC	0.38	0.43	0.57	0.75	2.71	0.35	0.86	1.38
HQC	0.30	0.18	0.78	1.32	1.29	0.71	0.13	1.02

Table 3.7. Robustness data for the proposed spectrometric methods

Conc.	**UV Spectrophotometric method**		**Spectrofluorimetric method**	
	% Recovery ± SD	**% RSD**	**% Recovery ± SD**	**% RSD**
pH 1.0				
LQC	98.65 ± 1.09	1.10	97.92 ± 0.68	0.70
MQC	98.60 ± 0.66	0.67	98.19 ± 1.37	1.40
HQC	98.17 ± 0.23	0.24	99.70 ± 0.61	0.61
pH 1.4				
LQC	98.89 ± 0.99	1.00	97.84 ± 1.05	1.07
MQC	97.78 ± 0.63	0.64	100.74 ± 0.59	0.59
HQC	99.59 ± 0.39	0.39	101.94 ± 0.79	0.78
Stability at room temperature after 48 h				
LQC	99.85 ± 0.76	0.76	101.74 ± 1.08	1.06
MQC	100.27 ± 0.86	0.85	101.99 ± 0.89	0.87
HQC	100.07 ± 0.21	0.21	100.74 ± 0.14	0.13

Each result is the average of three separate determinations

The proposed methods were successfully used in the estimation of ganciclovir in its commercially available capsules. The assay values of ganciclovir in the formulation ranged from 99.40-100.53% in the UV method and from 100.29-101.98% in the spectrofluorimetric method, with low standard deviation (SD) values, as shown in Table 3.2. The values were close to that of the label claim suggesting the applicability of the methods in determining ganciclovir in the presence of excipients matrix.

3.4. HPLC Analytical Method

3.4.1. Chromatographic Instrumentation and Conditions

A Shimadzu (Japan) HPLC system consisting of a pump system (LC10AT VP), integrated system controller with autosampler (SIL HTA), column oven (CTO-10 AS VP) and UV detector (SPD-10A VP) was used. Data acquisition and analysis was done using 21 CFR part 11 compliant LCSolutions software. Chromatographic separation was achieved on a reverse phase (RP) C_{18} end-capped column, Lichrospher® (E. Merck, Germany), of dimensions 250 × 4.6 mm and particle size 5 µm. The optimized mobile phase consisted of 10 mM phosphate buffer of pH 3.0 and methanol (92:8 v/v). The mobile phase was filtered through 0.22 µm filter and degassed by sonication before use and was delivered in isocratic elution mode at a flow rate of 1 mL min^{-1}. Injection volume was 50 µL and the wavelength of UV detector was set at 254 nm. Analysis was carried out at room temperature 25°C after baseline stabilization for at least 30 min.

3.4.2. Method Development

Different buffers (acetate, phosphate and citrate) of diverse pH range were tried as mobile phase with several proportions of organic modifiers (acetonitrile, methanol) to develop the RP-HPLC method. The objective was to achieve high peak height to area ratio, good peak symmetry, minimum, adequate separation from solvent and/or related substance peak(s) along with minimum damage to the column.

3.4.3. Preparation of Stock and Calibration Standards

A primary stock of 100 µg mL^{-1} was prepared by dissolving 5 mg ganciclovir in 50 mL water. A secondary stock of 10 µg mL^{-1} was prepared by diluting 1 mL of the primary stock to 10 mL using phosphate buffer pH 3, which was used as the diluting solvent. For preparation of different concentrations for calibration curve, aliquots of secondary stock were transferred to series of 2 mL click lock centrifuge tubes and volume was made with the diluting solvent. Seven calibration standards, in triplicate, containing 25, 50, 100,

250, 500, 1000 and 1500 ng mL^{-1} of ganciclovir were prepared and analyzed to generate the calibration curve.

3.4.4. Method Validation

The method was validated for selectivity, sensitivity, linearity, accuracy, precision and robustness. Forced degradation studies were performed to check the stability indicating potential of the method.

Selectivity was assessed by the placebo spiking technique. Placebo blend containing commonly used excipients, lactose, microcrystalline cellulose, croscarmellose sodium, colloidal silicon dioxide, magnesium stearate, GMS, GDS, GB, PF-68, TPGS and lecithin, was prepared and known concentrations of ganciclovir were spiked in it and analyzed under the above mentioned conditions. The chromatograms of the spiked placebo blends were then compared with those obtained with freshly prepared calibration standards. The chromatograms of the samples prepared independently from pure drug stock and commercial sample stock solution were also matched to study selectivity.

Linearity test solutions were prepared, in triplicate, in the range of 25–1500 ng mL^{-1} on three different days of validation, injected into the HPLC system and the peak area vs. concentration data was analyzed with least squares linear regression. Analysis of residuals, tests for slope and intercept and one way ANOVA were also performed on the data.

Sensitivity was presented in terms of LOQ and LOD, which were calculated using standard formulae, $10 \times \sigma/S$ and $3.3 \times \sigma/S$, respectively.

Accuracy was determined by analyzing different levels (LQC = 50 ng mL^{-1}, MQC = 250 ng mL^{-1} and HQC = 1000 ng mL^{-1}) of drug concentrations by the proposed method and calculating the % bias and % recovery. Further, to check the accuracy of the method, placebo spiking and standard addition methods were adopted, where three different levels of drug concentrations (20, 50 and 100% of labeled claim) were spiked into placebo and pre-analyzed formulation samples, respectively. The % recovery values were calculated to report the accuracy.

For precision studies, repeatability was established by checking intra-day variation in drug analysis and intermediate precision by checking inter-day variation. Different levels of drug concentrations (LQC, MQC and HQC) were prepared for three different times in

a day and on three different days and analyzed. The % RSD of the obtained assay values was taken as a measure of precision.

The robustness of the method was established by introducing small changes in the HPLC conditions which included change in % of methanol in the mobile phase (5% and 10%), pH of mobile phase (2.5 and 3.5) and buffer strength (5 and 15 mM). Robustness of the method was studied using six replicates at a concentration level of 1000 ng mL^{-1} of ganciclovir. The solution stability of ganciclovir was carried out by leaving the standard solutions in tightly capped volumetric flasks at room temperature for 48 h. Additionally, the stock solution was stored in a tightly capped volumetric flask at 4°C and assay was determined after regular intervals for a period of one month. System suitability parameters were recorded as a fundamental part of the procedure. These included various chromatographic performance factors like retention factor, resolution, asymmetric or tailing factor (T_f), number of theoretical plates and height equivalent to theoretical plate (HETP).

Further, forced degradation studies were performed to evaluate the stability indicating property of the method. Ganciclovir was subjected to acidic, alkaline, oxidative and thermal stress conditions. The drug solution (100 µg mL^{-1}) was prepared in 0.1N hydrochloric acid, 0.1N sodium hydroxide and 3% hydrogen peroxide and all samples were refluxed at 80°C for 2 h. For thermal degradation, sample of drug was placed in a temperature controlled oven at 100°C for 4 h. All samples were then cooled to room temperature, suitably diluted in diluting solvent and analyzed by the proposed method.

3.4.5. Analysis of Formulations

For commercial capsules of ganciclovir, contents of 10 capsules were weighed, mixed and pulverized. A quantity of powder equivalent to 10 mg of ganciclovir was taken and sonicated with water for 30 min. The volume was made upto 50 mL with water to obtain a stock solution of 100 µg mL^{-1}. The stock solution was then filtered using Whatman filter paper number 40. Aliquot of this primary stock solution was diluted to a concentration of 1000 ng mL^{-1} with diluting solvent and the samples were analyzed using proposed method.

To estimate the amount of ganciclovir in the in-house developed SLNs, 10 mg of the ganciclovir loaded freeze-dried nanoparticles were dissolved in 4 mL of methylene chloride. 10 mL of water was then added to the mixture and the contents were stirred

overnight to allow the ganciclovir to diffuse into the aqueous layer. The water phase was then retrieved from the mixture and tested for its ganciclovir content.

3.4.6. Results and Discussion

During optimization studies, main concern was laid on peak properties and response functions. These parameters were set by studying the effect of different pH buffers, organic modifiers and organic to aqueous ratio. Phosphate buffer (pH 3–7, strength 10-25 mM), citrate buffer (pH 3–5, strength 10-25 mM) and acetate buffer (pH 3–5, strength 10-25 mM) were studied and it was found that the peak properties of ganciclovir were not affected much by the different pH ranges and buffer strengths tried. The reason behind such observation may be the existence of ganciclovir in predominantly unionized form at all these pH ranges. Among the organic modifiers tried, methanol was suitable as it gave satisfactory separation and peak properties. Acetonitrile was also studied but it resulted in less symmetrical peak and is also more expensive as compared to methanol. Among the buffers, phosphate buffer pH 3 was selected because it gave the best resolution and sharp peak and it also shows good buffering capacity rendering it suitable for use in long runs as well. Thus the final optimized mobile phase consisted of methanol and phosphate buffer pH 3 (8:92 v/v), which was found to produce a retention time (t_R) of 6.5 ± 0.3 min (Figure 3.2a).

Selectivity of the method was proved by the absence of interference in the placebo samples near the drug peak (Figure 3.2b), indicating that the method may be used for estimation of ganciclovir in the presence of formulation excipients. The drug spiked placebo samples also did not show significant change in response and peak properties in comparison with calibration standards (Figure 3.2c). Also the chromatogram of the commercial sample matched with that of the pure drug standard (Figure 3.2d).

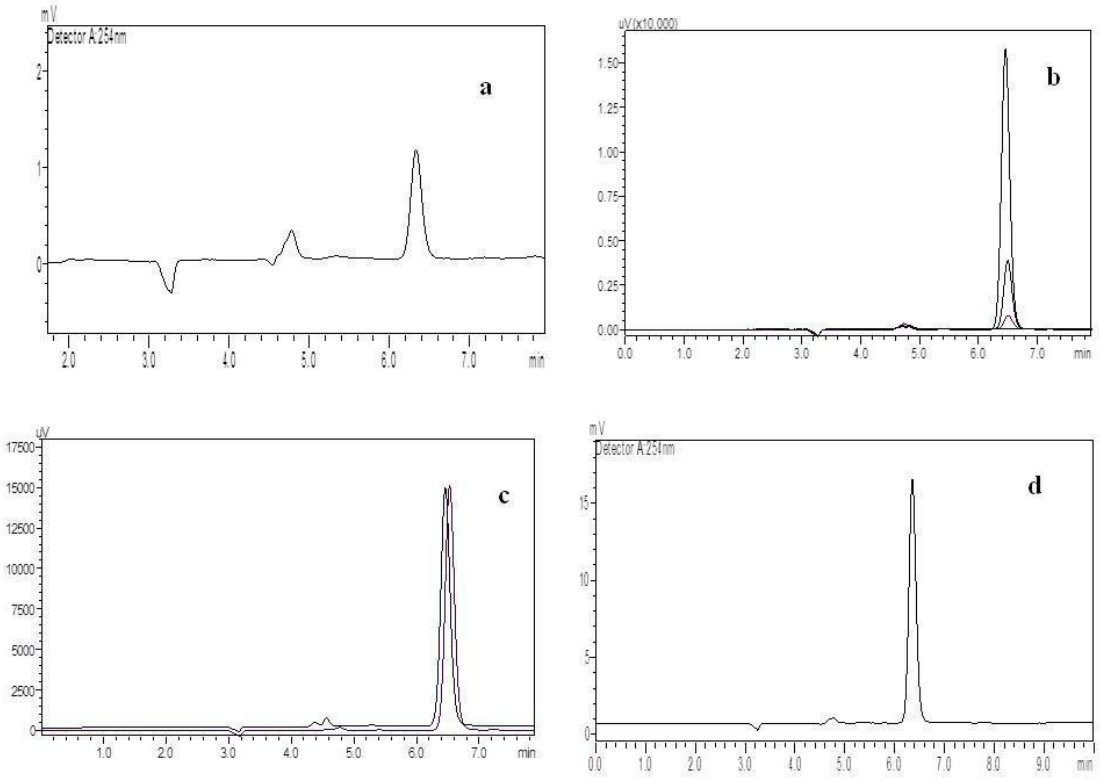

Figure 3.2. Representative chromatograms of ganciclovir depicting selectivity of the HPLC method. a) calibration standard at LOQ (25ng mL^{-1}), b) Placebo overlaid with standards (LQC, MQC and HQC), c) Spiked placebo overlaid with standard (1000 ng mL^{-1}) and d) test sample (commercial capsules).

The calibration data of the proposed HPLC method is given in Table 3.8. It was found that there is a linear relationship between peak area and concentrations over the range of 25-1500 ng mL^{-1}. After treating the data for the peak area vs concentration by linear regression analysis, R^2 value of 0.9999 was obtained. The best-fit linear equation obtained was average peak area (mV s) = 159.61 × concentration (ng mL^{-1}) + 165.76. The R^2 value close to one and the low value of standard error of estimate (773.5) points out to the goodness of fit of regression analysis, which was further, confirmed by the less calculated *F*-value than the critical *F*-value generated by applying one way ANOVA at 5% significance level. The LOD and LOQ values, as calculated from the standard formulae, were found to be 8.29 and 24.13 ng mL^{-1} respectively.

Table 3.8. Calibration data for the HPLC analytical method

Drug conc. (ng mL^{-1})	Peak Area (± SD)	% RSD
25	4072.11 ± 68.21	1.68
50	8024.78 ± 121.93	1.52
100	16118.56 ± 325.82	2.02
250	39845.67 ± 484.74	1.22
500	79830.44 ± 410.16	0.51
1000	161233.10 ± 931.30	0.58
1500	238697.10 ± 1259.51	0.53

Each value represents average of nine individual determinations

Accuracy and precision data of the method is given in Table 3.9. The % bias and % RSD values of the assay of ganciclovir were within ± 3% and 3%, respectively. These low values and the consistent and high absolute recoveries represent accuracy and precision of the method. Accuracy was further reinforced by the recovery studies conducted by placebo spiking and standard addition techniques (Table 3.10). These studies also indicated the suitability of method to analyze ganciclovir in presence of formulation excipients.

Table 3.9. Accuracy and precision data for the HPLC analytical method

Conc. Level	Predicted Conc. (ng ml^{-1})		% Recovery ± SD	% Bias*
	Mean ± SD	% RSD		
Intra-day				
LQC**	48.64 ± 0.86	1.76	97.29 ± 1.71	-2.71
MQC**	247.77 ± 2.43	0.98	99. 11 ± 0.97	-0.89
HQC**	1002.55 ± 1.89	0.19	100.26 ± 0.19	0.26
Inter-day				
LQC**	49.08 ± 0.46	0.93	98.16 ± 0.91	-1.84
MQC**	252.79 ± 5.14	2.03	101.12 ± 2.06	1.12
HQC**	995.59 ± 9.68	0.97	99.56 ± 0.97	-0.44

Each result is the average of three separate determinations

*Accuracy is given in % bias = 100 × [(predicted concentration – nominal concentration)/nominal concentration)]

** LQC, MQC and HQC are 50, 250 and 1000 ng mL^{-1} respectively

Table 3.10. Accuracy studies by placebo spiking and standard addition method

Technique	Amount of drug added (% of label claim)	Recovered amount ±SD (mg)	% Recovery ± SD
Placebo spiking*	20	49.73 ± 0.21	99.46 ± 0.43
	50	123.72 ± 1.82	98.97 ± 1.46
	100	251.01 ± 2.73	100.40 ± 1.10
Standard Addition**	20	309.46 ± 3.04	103.15 ± 1.01
	50	377.58 ± 3.73	100.69 ± 1.00
	100	501.37 ± 3.29	100.27 ± 0.66

Each result is the average of three separate determinations

*Placebo blend prepared equal to unit capsule weight

**Commercial capsule preparation containing 250 mg of ganciclovir per capsule

In robustness studies, the results obtained were not affected by varying the conditions and were in accordance with the results of original conditions. The recovery values determined for the samples under robustness conditions did not show any significant change from the samples under original conditions (Table 3.11). Further, none of the factors studied showed significant effect on the system suitability parameters indicating the ability of the method to estimate ganciclovir correctly. Stability experiments at all QC levels demonstrated that ganciclovir was stable up to 48 h at room temperature and 1 month at 4°C. Results were calculated in terms of % recovery which ranged from 97.61-100.34%.

Table 3.11. Robustness data of the proposed HPLC method

Parameter changed		% Recovery ± SD	% RSD
Ratio of Methanol : Phosphate Buffer pH 3	5:95	100.86 ± 0.67	0.67
	10:90	100.15 ± 1.06	1.06
pH of phosphate buffer	2.5	102.15 ± 1.30	1.27
	3.5	99.51 ± 0.86	0.87
Buffer Strength (mM)	5	101.03 ± 0.76	0.75
	15	101.21 ± 0.09	0.09

Each result is the average of three separate determinations

The method was found to be suitable in terms of system performance as obtained values for primary system suitability parameters such as retention factor ≈ 2.5, resolution ≈ 6.0, number of theoretical plates ≈ 8860 and HETP ≈ 17 µm were well acceptable. The

method also showed good peak symmetry ($T_f \approx 1.11$) and consistent injection repeatability. System suitability study confirmed that the method was specific, precise and stable for determination of ganciclovir.

Selectivity of the method was further verified by the ability of the method to separate the degradation products obtained on subjecting ganciclovir to stress testing conditions. The chromatograms of the samples undergone forced degradation are shown in Figure 3.3 (a-d). Ganciclovir remained stable in acidic, basic and thermal conditions, with recovery values ranging from 98.95-101.29%. The recovery of ganciclovir sample treated with hydrogen peroxide was 63.95% showing that ganciclovir is susceptible to oxidation induced degradation. In all the conditions, the peak of ganciclovir was not significantly shifted, depicting the stability indicating property of the proposed method.

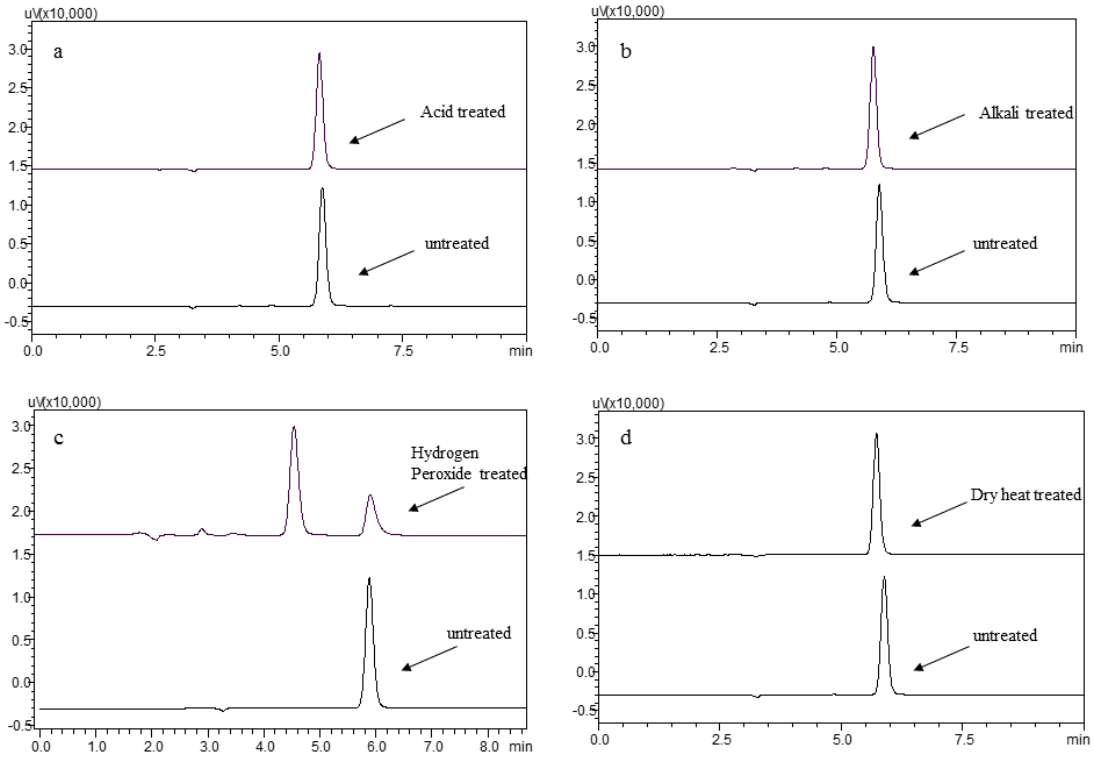

Figure 3.3. Chromatograms of forced degradation study. a) acid treated, b) alkali treated, c) hydrogen peroxide treated and d) dry heat treated samples

The proposed method was applied to the determination of ganciclovir capsules and the result of the assay yielded 101.29% recovery with a SD of 1.36, indicating that the method is selective for the measurement of ganciclovir without interference from the

excipients used in these capsules. The drug loading of ganciclovir in SLNs was also determined successfully, the results of which are shown in Chapter 5.

3.5. HPLC Bioanalytical Method

3.5.1. Chromatographic Instrumentation and Conditions

Same HPLC system as mentioned in section 3.4.1 was used. The chromatographic conditions were also same except the ratio of the components of mobile phase. The ratio of phosphate buffer pH 3 and methanol was changed to 97:3 from 92:8. Analysis was carried out at room temperature 25°C after baseline stabilization for at least 30 min.

3.5.2. Animals

Healthy male Wistar rats were obtained from Hisar Agricultural University, Haryana, India. Rats were housed in polycarbonate cages in a group of 3 and maintained in standard laboratory conditions with alternating light and dark cycle of 12 h each, with temperature 23±2 ºC and relative humidity conditions 62±5% RH in the housing unit and had free access to food (standard pellet chow feed) and filtered water ad libitum. Rats were treated according to the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA, Registration number 417/01/A/ CPCSEA) and all experiments were conducted in adherence to the approved protocol of the Institutional Animal Ethics Committee (IAEC) of Birla Institute of Technology & Science, Pilani, India (Protocol numbers IAEC/RES/13/12, IAEC/RES/13/12/REV/15/8, IAEC/RES/13/12/REV-2/17/13 and IAEC/RES/18/15).

3.5.3. Preparation of Drug Free Plasma Pool and Tissue Homogenates

For collection of plasma, blood samples were withdrawn from more than 15 healthy rats from the retro-orbital sinus using a microcapillary. The blood was collected into 2 mL polypropylene centrifuge tubes (Tarsons, India) containing 0.1 mL of 10% (w/v) solution of anticoagulant disodium ethylene diamine tetraacetic acid (EDTA). The blood was gently mixed with EDTA and then centrifuged at 12000 rpm for 15 min at 4°C using a cooling centrifuge (Remi cooling compufuge, model CPR24 with servo controlled voltage stabilizer, Remi Instruments, India). The supernatant plasma was pooled into fresh tubes and stored at –20°C and thawed before analysis.

Tissues were collected from six healthy animals by surgical process. Under diethyl ether anaesthesia, a midline abdominal incision was made in the animal to expose all the

visceral organs. Spleen, liver, kidneys, heart and lungs were then located and excised one by one. Brain was excised by laying the rat ventrally and making a midline incision over the skull. The animal was then sacrificed using standard protocol. These tissues were then transferred to a petridish containing ice cold phosphate buffer saline (PBS), cleaned gently and dried with Whatmann filter paper number 40. The organs were then weighed individually and transferred to 50 mL polypropylene centrifuge tubes placed on an ice bath. Two volumes (2 mL for 1 g tissue) of ice cold PBS was added to each tube and homogenized at 20000 rpm to get a fine suspension using a Kinematica™ Polytron™ PT 1600E Benchtop Homogenizer (Thermo Fisher Scientific Inc., USA). The tissue homogenates were then stored in clean tubes at –80°C and thawed before analysis.

3.5.4. Preparation of Stock Solution and Standards

A primary stock solution of 1 mg mL^{-1} of ganciclovir was prepared in water, from which a series of seven working stock solutions containing 0.5, 1, 2, 10, 20, 40, 100 µg mL^{-1} of ganciclovir were prepared by serial dilution, in water. A primary stock of 100 µg mL^{-1} internal standard (IS), acyclovir, was prepared in water and a working standard of 8 µg mL^{-1} was made from it. Seven analytical standards containing 25, 50, 100, 500, 1000, 2000 and 5000 ng mL^{-1} of ganciclovir were prepared freshly, in triplicate, by diluting 30 µL of each working stock to 500 µL with diluting solvent of HPLC method. Seven plasma standards containing 25, 50, 100, 500, 1000, 2000 and 5000 ng mL^{-1} of ganciclovir were prepared by spiking 10 µL of each working stock to 190 µL of blank rat plasma. This satisfies the limit of 5% addition of external aqueous solution to plasma. Each plasma standard was vortex-mixed using Spinix vortex mixer (Tarsons, India) for 1 min and allowed to equilibrate with the drug. Similarly, calibration standards in all the tissues were prepared by spiking the working stocks into blank tissue homogenates and vortex-mixed to equilibrate with the drug. All the stock solutions and standards were prepared fresh in five replicates on three different days of validation.

3.5.5. Sample Processing

Determination of ganciclovir and related drugs in biological matrices is a challenging task as it is a nucleoside analogue and bears structural resemblance to endogenous components. Therefore, for proper estimation, primary focus was laid on efficient extraction of drug from the complex biological matrices. For this purpose, various sample clean-up methods were explored for maximum recovery and a well resolved response in

the chromatogram. Based on the literature available on various similar drug extraction techniques and the physicochemical properties of ganciclovir, it was noted that a single step protein precipitation procedure would be the best one to execute. Liquid-liquid extraction was waived out due to poor partition coefficient of ganciclovir in organic solvents while solid phase extraction, though effective, would make the process expensive and lengthy. For protein precipitation, various solvents like acetonitrile, methanol, formic acid, perchloric acid, trichloroacetic acid (TCA), ammonium acetate, etc, in different ratios, alone and in combinations were investigated. Similarly, the ratio of mobile phase components was also optimized for better selectivity and resolution.

Processing of the drug spiked samples was done to extort the drug from the matrix, which was extracted efficiently by a one-step protein precipitation method. To 200 µL of drug spiked plasma standard, 50 µL of working standard of IS and 150 µL of freshly prepared 5% TCA were added, vortex mixed for 5 min. The concentration of IS in all samples was maintained at 1µg mL^{-1}. For tissue samples, IS was not used as the homogenates gave cleaner extracts which were better resolved and gave good and consistent recoveries. Thus for tissue samples, to 200 µL of drug spiked tissue standard, 200 µL of freshly prepared 5% TCA was added and vortex mixed for 5 min. Resulting solutions were centrifuged at 12,000 rpm for 20 min at 4°C and the clear supernatant was transferred to autosampler microvials for analysis. Blank plasma and tissue samples were processed in a similar manner.

3.5.6. Method Validation

The method developed in plasma was validated for selectivity, sensitivity, linearity, accuracy, precision, recovery and drug stability in plasma. For the methods developed in various tissues, partial validation was carried out which included selectivity, linearity, accuracy and precision. The method was also applied to test samples collected during animal experiments to determine in vivo pharmacokinetic and biodistribution characters of ganciclovir.

Selectivity of the method was assessed by examining the interference from various endogenous proteins and other impurities present in the biomatrix. Blood and tissue samples were collected from six different rats and processed for plasma and homogenates, respectively. Six blank samples (without drug and IS), six zero samples (with IS but without drug) and six non-zero samples with IS and drug at lower limit of

quantitation (LLOQ) level (25ng mL^{-1} for plasma) were processed individually and analyzed by the proposed method. The chromatograms obtained for the blank samples were compared against analytical and calibration standards for any interference at the retention time of the drug and IS. Also, the mean response at LLOQ level was compared with that of blank samples.

Linearity was determined by analyzing the calibration standards, prepared in plasma and tissues, at each concentration on three different days. Ratio between the peak area of ganciclovir and peak area of IS was plotted against the corresponding concentration of ganciclovir and the data was subjected to linear least square regression analysis. Calibration equation was generated to calculate the predicted concentrations and one-way ANOVA was performed on each replicate response.

Sensitivity of the method was given as LLOQ, which is the concentration at which the response is at least 5 times the response of the blank sample and is also reproducible with a precision of 20% and accuracy of 80-120%. The standards at LLOQ concentration were prepared in pentaplates and analyzed by the proposed method on three different days. Concentration of ganciclovir in the standards was calculated from calibration equation and % recovery, % bias and % RSD were determined.

The extraction efficiency of the method was obtained as the recovery of ganciclovir from spiked plasma and tissue samples. It was assessed by comparing the peak areas of the QC standards (LLOQ = 25 ng mL^{-1}, LQC = 50 ng mL^{-1}, MQC = 250 ng mL^{-1} and HQC = 2000 ng mL^{-1}) with those of the reference analytical standards (unextracted standards). It was expressed as % area ratio of the extracted ganciclovir relative to the area of the reference standard.

Accuracy and precision was determined by replicate (n=5) analysis of samples at four levels (LLOQ, LQC, MQC and HQC). Each QC standard was processed and analyzed on three different days of validation. Concentration of ganciclovir was calculated from the calibration equation and accuracy was expressed as % recovery and % bias while precision was given as % RSD

Stability determination of drug in biological fluids is an essential part of bioanalytical method validation to ensure the integrity of the drug at various storage and operational conditions. The stability studies included short-term stability, long-term stability, post-

preparative stability and freeze and thaw stability. Each of these studies was carried out at four QC levels in triplicate.

For short-term stability studies, the QC standards were kept at room temperature and each set was processed and analyzed at 0, 1, 6, 12, and 24 h post-spiking. Similarly, long term stability studies were conducted by storing the spiked QC standards at -20°C upto 60 days, with analysis time points of 7, 15, 30 and 60 days. In post preparative stability, the stability of ganciclovir and IS was checked in the final solution before injecting into the HPLC system. The QC standards were processed, loaded into the autosampler and analyzed at different time points of 6, 12, 24 and 48 h. Stability of ganciclovir was also checked by subjecting the QC standards to freeze and thaw cycles. The four QC standards were prepared in plasma and stored at –20°C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen under same conditions. These cycles were repeated and the samples were analyzed with the proposed method for three cycles. On each day of the stability study, freshly prepared QC standards were processed and analyzed with the stability samples and the stability results were found out by comparing the results for stored samples with those for freshly prepared samples. The results were expressed as % recovery and % bias.

3.5.7. Analysis of In vivo Samples

Samples of in vivo pharmacokinetic and biodistribution studies of pure drug and designed formulations were analyzed by above validated method. The process and results are presented in Chapter 6.

3.5.8. Results and Discussion

Through optimization of method parameters and chromatographic conditions, it was aimed to achieve good separation of drug peak from the endogenous interfering substances. A single step protein precipitation technique of drug extraction using TCA, provided good sample clean up and highest extraction efficiency as compared to those obtained by other precipitating agents used. The % composition of mobile phase was changed from 92:8 phosphate buffer pH 3 and methanol to 97:3 because it eluted the drug at a t_R away from that of interferences, yielding satisfactory resolution and other system suitability parameters. The same extraction protocol and chromatographic conditions showed good and consistent results in method development in tissue samples also.

The chromatograms of the blank, zero and non-zero standard (LLOQ) of rat plasma are shown in the Figure 3.4 (a-c). Six such sets of chromatograms were compared and non-interference in the results indicated the selectivity of the method. Absence of interference near the vicinity of the drug, in the real time pharmacokinetic samples, further confirmed the selectivity of the method (Figure 3.4d).

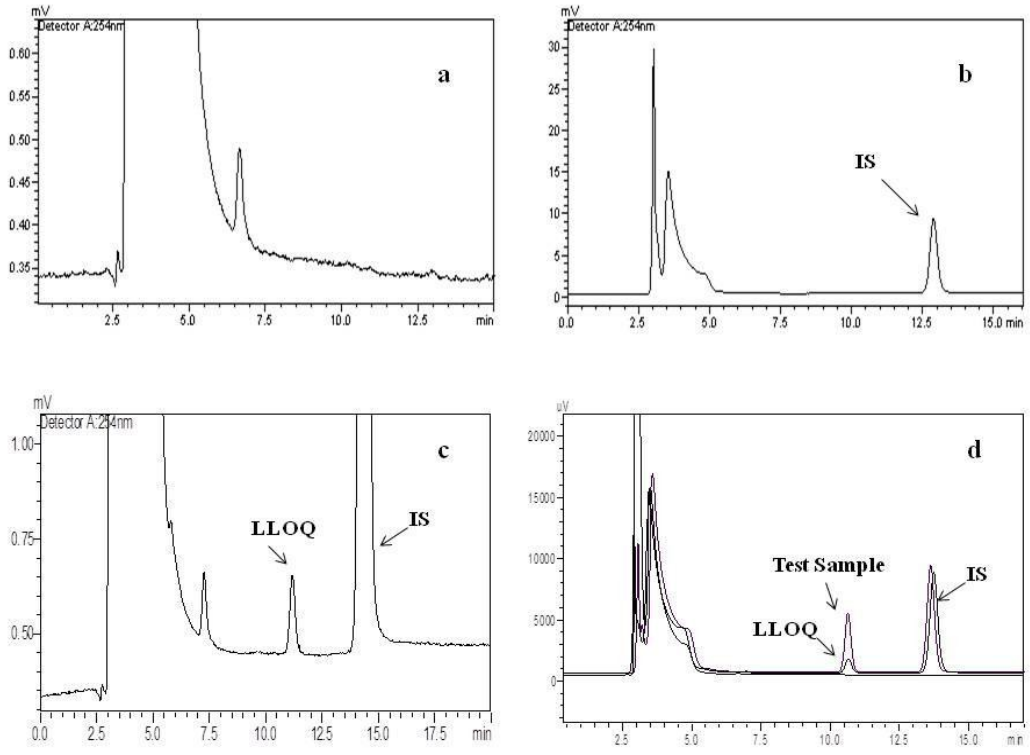

Figure 3.4. Representative chromatograms of a) blank plasma, b) blank plasma + IS (zero sample), c) spiked plasma standard at LLOQ (25ng mL^{-1}) + IS and d) in vivo test sample overlaid on blank plasma and LLOQ standard + IS.

The calibration data of the proposed method in rat plasma is given in Table 3.12. The linearity range was found to be 25-5000 ng mL^{-1}. The best-fit linear equation obtained was ratio of peak area of drug/IS = 0.0004 × concentration (ng mL^{-1}) + 0.0012. Linear regression analysis yielded high R^2 value of 0.9999 and low values of standard error of estimate (0.0084). Goodness of fit was also supported by the lower calculated *F* value obtained in the one way ANOVA test performed for peak area ratio obtained at individual concentration levels. Similarly, linear relationship also existed between the average peak

area and concentration of ganciclovir in the tissue samples. The results of the method development and partial validation in different tissues are given in Table 3.13.

Table 3.12. Calibration data for rat plasma standards

Drug conc. (ng mL^{-1})	**Peak area of drug/ Peak area of IS (± SD)**	**% RSD**
25	0.0094 ± 0.0009	9.18
50	0.0182 ± 0.0015	7.91
100	0.0376 ± 0.0037	9.56
500	0.2069 ± 0.0111	5.35
1000	0.3944 ± 0.0238	6.02
2000	0.8271 ± 0.0219	2.66
5000	2.0327 ± 0.1041	5.12

Each value is average of nine separate determinations

The lowest concentration on calibration curve of plasma (25 ng mL^{-1}) yielded a response which was five times more than that of blank response or background noise. The % recovery at this level was 89.34% (lying in the range 80-120%) and % RSD was 7.48% (< 20%), thus making it acceptable as the LLOQ. The LLOQ in kidney, liver and lungs was also 25 ng mL^{-1} while that in brain, heart and spleen was 50 ng mL^{-1}.

Extraction efficiency or recovery of the method in the plasma was found to be in the range of 88.09 – 93.55% with low % RSD (< 6%) at all QC levels (Table 3.14). The method also depicted acceptable and consistent recoveries of ganciclovir from different tissues.

In accuracy and precision studies, the % bias and % RSD values were found to be well within the limits for all the QC levels, illustrating the reliability and correctness of the method. The % bias and % RSD values of plasma standards are given in Table 3.15, while that of tissues are given in Table 3.13. These results also suggest the suitability of the method for estimation of ganciclovir in biological matrices.

Table 3.13. Linearity, accuracy and precision data for tissue standards of ganciclovir

Tissue	Linearity (ng mL^{-1})	Calibration equation	Validation Parameters			
			Conc. Level	Accuracy (% bias)	Precision (% RSD)	
					Intra-day	Inter-day
Brain	50-2000	Peak Area = 72.27 × conc. (ng mL^{-1}) – 1122.80 R^2 = 0.9998	LLOQ	19.18	2.03	2.40
			LQC	6.14	6.56	2.86
			MQC	-8.04	3.69	8.36
			HQC	0.44	4.96	5.85
Heart	50-5000	Peak Area = 69.50 × conc. (ng mL^{-1}) + 275.97 R^2 = 0.9998	LLOQ	-11.80	19.80	7.21
			LQC	-9.57	9.51	9.91
			MQC	-3.89	5.84	10.44
			HQC	2.47	1.50	0.74
Spleen	50-2000	Peak Area = 66.78 × conc. (ng mL^{-1}) + 1029.60 R^2 = 0.9999	LLOQ	-12.75	5.28	13.42
			LQC	2.22	3.40	6.53
			MQC	2.10	1.99	2.98
			HQC	-0.94	4.55	1.22
Kidney	25-2500	Peak Area = 75.07 × conc. (ng mL^{-1}) + 262.66 R^2 = 0.9999	LLOQ	-6.81	14.57	8.08
			LQC	-11.11	7.65	11.85
			MQC	1.33	1.14	1.79
			HQC	-0.07	1.81	2.98
Liver	25-2000	Peak Area = 65.12 × conc. (ng mL^{-1}) + 581.44 R^2 = 0.9996	LLOQ	-13.05	6.12	19.51
			LQC	-3.50	8.96	13.47
			MQC	9.01	1.58	1.39
			HQC	5.83	3.05	7.78
Lungs	25-2500	Peak Area = 71.35 × conc. (ng mL^{-1}) + 498.54 R^2 = 0.9999	LLOQ	-9.88	15.66	8.16
			LQC	2.57	9.86	2.52
			MQC	-3.29	0.86	1.90
			HQC	2.38	6.30	2.69

Table 3.14. Recovery of ganciclovir from plasma standards

Conc. Level	Mean absolute recovery ± SD	% RSD
LLOQ*	91.66 ± 4.67	5.09
LQC*	93.55 ± 4.90	5.24
MQC*	88.09 ± 2.72	3.08
HQC*	92.89 ± 1.58	1.70

Each result is the average of fifteen separate determinations

* LLOQ, LQC, MQC and HQC are 25, 50, 250 and 2000 ng mL^{-1} respectively

Table 3.15. Accuracy and precision of the proposed method in plasma standards

Conc. Level	Accuracy (% Bias*)	Intra-day Precision (% RSD)	Inter-day Precision (% RSD)
LLOQ**	-10.66	7.48	8.76
LQC**	-5.77	8.60	1.86
MQC**	6.08	0.64	1.81
HQC**	9.27	1.83	0.38

*Accuracy is given in % bias = 100 × [(predicted concentration – nominal concentration)/nominal concentration)]

** LLOQ, LQC, MQC and HQC are 25, 50, 250 and 2000 ng mL^{-1} respectively

The % recovery for short-term and long-term stability studies ranged from 93.16-107.61% and 92.05-104.91%, respectively, indicating that ganciclovir was stable up to 24 h at bench top conditions and for 60 days at – 20°C. In post preparative stability studies, the results demonstrated that the extracted ganciclovir samples were stable in the auto sampler at room temperature up to two days yielding acceptable % recovery values (89.34–109.27%), making it feasible to reanalyze the samples following a night in the auto injector. Ganciclovir also did not show any significant degradation during freeze-thaw cycles and gave recoveries in the range of 91.05-101.26%, making it suitable for storage at sub-zero conditions. The results obtained in all stability conditions, expressed in % bias, are shown in Figure 3.5.

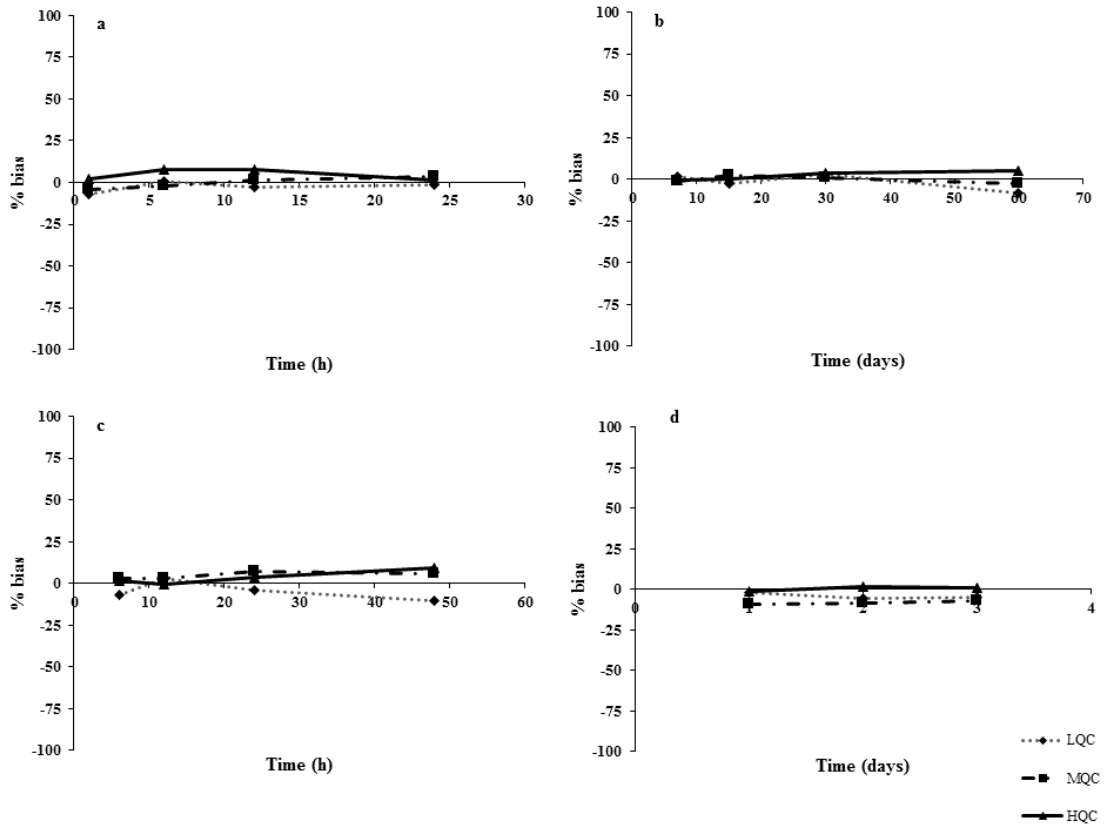

Figure 3.5. Stability of ganciclovir in rat plasma. a) short-term stability, b) long-term stability, c) post-preparative stability and d) freeze-thaw stability.

The validated method was applied successfully to study the pharmacokinetics and biodistribution of ganciclovir in rats. (Chapter 6).

3.6. Conclusion

The developed UV spectrophotometric and spectrofluorimetric methods were found to be selective, accurate, reproducible and robust. The simple composition of the medium used in these methods and measurement of drug's inherent responses (absorbance and fluorescence) rendered the methods rapid and cost effective. These methods were used for routine analysis of drug in bulk, pre-formulation and in vitro release samples.

The stability indicating RP-HPLC method was successfully developed and demonstrated selectivity using forced degradation studies. In addition, the method was fully validated and were found to be sensitive, accurate and precise with high and consistent recoveries at all the levels studied. The low analysis time of 8 min in HPLC and suitability in determining ganciclovir in the presence of degradation and/or related products were the added benefits of the method.

A simple and sensitive HPLC method has also been developed and validated for estimation of ganciclovir in rat plasma and tissues (brain, heart, spleen, kidney, lungs and liver). A single step protein precipitation process, used to extract the drug from the biological fluids, provided acceptable recovery values in all the matrices. The validation results suggested that the method was sensitive, accurate and reproducible and also yielded good recoveries in the extensive stability studies performed at various processing and storage conditions. The method was successfully employed for estimation of ganciclovir in bio samples collected during in vivo pharmacokinetic studies of pure drug and its formulations.

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4. Preformulation Studies

4.1. Introduction

Preformulation, as the name implies, refers to the information generated on the active pharmaceutical ingredient prior to formulation development. Preformulation studies are very critical in laying the foundation for a successful formulation design. These studies are designed to investigate physicochemical properties of drug alone and in combination with various excipients [1, 2]. This valuable information assists in selection of proper dosage form, choice of most appropriate excipients and also in prediction of in vivo characters of the drug. This leads to reduction in time, cost, resources and challenges involved in the formulation development process. Preformulation studies include determination of fundamental properties of the drug molecule including solubility in different solvents, pH solubility profile, dissociation constant, partition or distribution coefficient, solution and solid state stability and compatibility studies in the presence of excipients. Such a comprehensive preformulation study provides the foundation for development of a robust formulation.

Ganciclovir is a well established drug listed in the USP [3] and information on its physicochemical properties like aqueous solubility, dissociation constants and partition coefficient is available in scientific literature [4, 5]. Still, a detailed account of its solubility in various solvents, pH dependence of solubility and partition coefficient, stability of ganciclovir and its compatibility with various excipients is vital for designing the proposed SLN systems. This would help in deciding the suitable solvent to be used during the process of formulation of nanoparticles and the media for in vitro release testing. Further, it would provide a rational for selecting the most appropriate additives to aid in formulation development. Thus, the broad objective of the preformulation was to evaluate the physicochemical properties and study the drug-excipient compatibility that would be helpful for fabricating the proposed formulations. In the current chapter, results of bulk drug characterization, effect of pH on solubility and partition coefficient, solution and solid state stability and drug-excipient compatibility studies are presented.

4.2. Experimental

4.2.1. Materials

Ganciclovir and excipients were obtained from sources mentioned in section 3.2 of Chapter 3. Ultrapure water (Milli-Q Plus, Millipore®, India) was used for all the studies. Hydrochloric acid, glycine, sodium chloride, dibasic sodium phosphate, potassium

dihydrogen phosphate and sodium hydroxide were of analytical grade and procured from S.D.Fine Chemicals Limited (Gujarat, India). All other reagents and chemicals were of analytical grade.

4.2.2. Instruments/ Equipments

An orbital shaker incubator (MAC instruments, India) was used for solubility studies and partition coefficient studies. Humidity chamber (Thermolab, India) was used for stability studies to maintain room temperature conditions at 25°C ± 2°C/60% ± 5% RH conditions. Frost-free-200-litre refrigerator (LG, India) was used for the studies at refrigerated condition. All pH measurements were performed using a portable pH meter (Hanna Instruments, USA), which was used after calibration with standards of pH 4 and 7. Characterization and compatibility studies were done using Fourier Transform Infrared (FTIR) Spectrophotometer model - IRPrestige-21 (Shimadzu, Japan), loaded with software IRSolutions (version 1.10) for IR data processing and plotting. Thermal analysis was performed using a differential scanning calorimeter (Perkin Elmer, USA), Model: DSC-4000 with integrated thermal analyzer; cooling assembly intracooler and integrating software: Pyris Series – DSC 4000. Drug content determination in drug-excipient compatibility study was carried out using a high performance thin layer chromatography (HPTLC) system (CAMAG, Switzerland), consisting of a Linomat 5 automatic sample applicator, a calibrated syringe of 100 µL capacity, a 10 × 10 cm twin trough glass chamber, a TLC Scanner 3 with a deuterium source and WinCats (version 1.4.1.8154) planar chromatograph software . Chromatography was performed on 10 cm × 10 cm silica gel coated aluminium plates with 0.2 mm thick layer of silica gel 60 F254 (E. Merck, Germany).

4.2.3. Bulk and Physical Form Characterization

Identity of ganciclovir and its bulk characterization were done by standard process by checking UV and infrared (IR) absorption according to its USP monograph [3] and physical form by thermal analysis. A standard solution of ganciclovir (10 µg mL^{-1}) was prepared in methanol and UV spectrum was recorded as described earlier in Chapter 3.

For recording the IR spectrum, the drug sample was suitably mixed with moisture free spectroscopic grade potassium bromide and loaded into the IR holder. It was then placed into the diffuse reflectance attachment of the FTIR and spectrum was recorded in the

range of 400 to 4000 nm^{-1} with a resolution of 4 cm^{-1}. The obtained IR spectrum of ganciclovir was compared with that of the standard.

Physical form of ganciclovir was determined by thermal analysis using DSC. Around 2 mg of finely pulverized pure drug sample was taken and sealed in non-hermetic aluminium pan with lid and placed in the test holder, while an empty sealed aluminium pan was used as the reference. Inert environment was maintained during analysis by purging nitrogen gas at flow rate of 30 mL min^{-1}. Thermogram was acquired at temperature range of 30°C to 300°C with a heating rate of 10°C min^{-1} and the melting temperature was recorded.

4.2.4. Solubility Analysis

Solubility of ganciclovir was assessed in various aqueous and non-aqueous solvents by the shake flask method. Aqueous media included water and buffered and unbuffered solutions of different pH ranging 1-12, while non-aqueous solvents were dimethyl sulphoxide (DMSO), methanol, ethanol, isopropyl alcohol (IPA), dichloromethane (DCM), chloroform and acetonitrile (ACN). Sufficient quantity of drug was added to 2 ml of each media in 10 mL conical flasks and kept for shaking at 37°C, in triplicates, to achieve equilibrium. Excess of drug was maintained in each flask throughout the study. At different points of time (12, 24 and 48 h) the samples were taken and filtered using 0.22 µm syringe filters (Millipore, Mumbai). The filtrates were then diluted suitably and analyzed by spectrofluorimetric method as described in Chapter 3.

Unbuffered solutions in the pH range 1 to 12 were prepared adjusting the pH of water using 0.1N hydrochloric acid or 0.1 N sodium hydroxide solutions and maintaining their ionic strength with 0.5% w/v sodium chloride. Buffered solutions of pH 1 to 12 were prepared by mixing different volumes of two buffer solutions of extreme pH and adjusting to required pH by using 0.1N hydrochloric acid or 0.1N sodium hydroxide solutions [6].

4.2.5. Determination of Apparent Partition Coefficient

The apparent partition coefficient (P_{app}) of ganciclovir was determined by traditional shake flask method using n-octanol as organic phase and water or buffers (pH 1 to 12) as aqueous phase. The organic and aqueous phases were pre saturated with each other by shaking them together for about 12 h at 37.0 ± 0.5ºC and then separating them. 2 mL of aqueous phase containing drug dissolved in it (20 µg mL^{-1}) and 2 mL of organic phase

were added into a 10 mL conical flask and kept for stirring in the orbital shaker incubator at 37.0 ± 0.5ºC, in triplicates. Samples were taken after 24 h, centrifuged (5000 rpm, 10 min) to separate the aqueous layer, diluted suitably and analyzed by the UV-spectrophotometric method as described in Chapter 3. The concentration of drug in aqueous phase at time zero (C_0) and time't' (C_t) were determined and the P_{app} was calculated using the following formula.

$$P_{app} = \frac{(C_0 - C_t)}{C_t}$$

The log of the P_{app} values was taken and the results were expressed as log P_{app} for individual pH.

4.2.6. Stability Studies

4.2.6.1. Solution State Stability

Solution state stability was carried out in different buffered solutions in the pH range of 1 to 12. A known concentration (10 µg mL^{-1}) of ganciclovir standard was prepared in the different pH buffers and the solutions were kept at 25.0 ± 2.0ºC in glass vials, in triplicates. At different time intervals (0, 1, 2, 7, 15, 30 and 60 days), the samples were withdrawn and drug content was analyzed using the stability indicating HPLC method as described in Chapter 3. The amount of drug remaining to be degraded was plotted as a function of time and the degradation rate constant and order of degradation was found out.

4.2.6.2. Solid State Stability

For solid state stability study, the drug sample was stored at various conditions according to ICH stability guidelines [7], i.e., room temperature (RT) condition (25 ± 2 ºC, 60 ± 5% RH) and refrigerated condition (5 ± 2ºC). At different time intervals (0, 1, 2, 3 and 6 months), the samples were withdrawn and drug content was analyzed using the stability indicating HPLC method as described in Chapter 3. On completion of stability period, the samples were also analyzed for any physical or chemical form change by carrying out DSC and FTIR study as described above.

4.2.7. Drug-Excipient Compatibility Study

Compatibility of ganciclovir was studied with various potential excipients for formulation design. Mixtures of drug and different excipients (1:10) were stored at

different conditions according to ICH guidelines as mentioned above and samples were analyzed for assay at various time points till 6 months of study, by using an in house developed and validated stability indicating HPTLC method instead of HPLC method. These excipients are not soluble in commonly used organic solvents used in HPLC method, so may lead to deterioration of the HPLC column. Since HPTLC uses a disposable stationary phase and permits the use of wide range of solvents as mobile phase, it proved to be a suitable approach for determination of ganciclovir in presence of these excipients. The samples were spotted in the form of bands of 4 mm thickness at a constant application rate of 150 nL s^{-1}. Linear ascending development was carried out in a twin trough glass chamber saturated with the optimized mobile phase comprising of butanol-methanol-water-25% v/v ammonia solution (3:2:1:2, v/v/v/v). After the plate was developed and dried, densitometric analysis was performed at wavelength of 254 nm in the scanner set at slit dimension settings of length 4 mm, width 0.1 mm, scanning rate of 20 mm s^{-1} and a data resolution of 100 µm $step^{-1.}$ The retention factor (R_f) was found to be 0.65 ± 0.3. Linear least squares regression analysis showed there was a good linear relationship (R^2 = 0.9992) between peak area and concentration in the range 100–800 ng $spot^{-1}$. The linear regression equation was obtained as peak area = 2.56 × concentration (ng $spot^{-1}$) + 349.87. The intraday and interday precision determined as %RSD ranged between 0.65–3.62% and 1.39-3.27%, respectively. Accuracy, calculated as % recovery, was in the range of 95.65–99.43%. The LOD and the LOQ were 30.42 ng $spot^{-1}$ and 92.2 ng $spot^{-1}$.

Any physical and chemical form change was checked on completion of stability period by carrying out DSC and FTIR analysis. The pure drug solid state stability data was used as the control. The drug content results were plotted as the function of time and order and rate of degradation was determined.

4.3. Results and Discussion

4.3.1. Bulk and Physical Form Characterization

Supplied ganciclovir passed various tests of identification and analysis as per the USP. An absorption maximum of 255 nm was displayed in the UV spectrum for a concentration of 10 µg mL^{-1} in methanol (Figure 4.1).

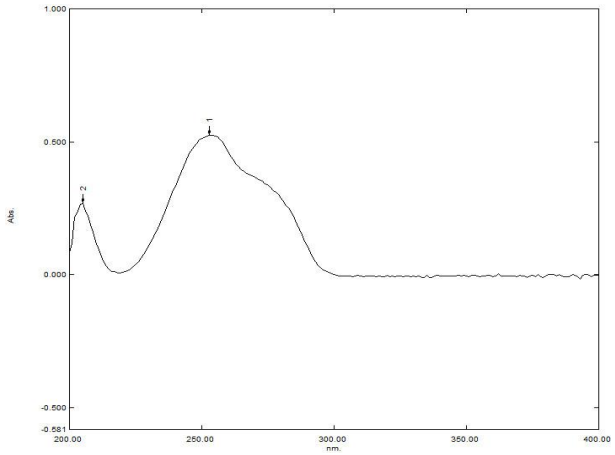

Figure 4.1. UV absorption spectrum of ganciclovir in methanol (USP identification test)

The IR spectrum of the sample (Figure 4.2.) was found to be comparable with that of the crystalline monohydrate form of ganciclovir [8]. The IR spectrum showed characteristic IR bands at 3315.63 (N-H stretching), 3130.47 (aromatic C-H stretching), 2746.63 (aliphatic C-H stretching), 1730.15 and 1693.50 (C=O stretching), 1537.27 (aromatic C=N, C=O stretching), 1485.19 (aliphatic C-H bending), 1307.74 (C-N bending), 1224.80 and 1182.36 (C-O-C asymmetric stretching), 1101.35 and 1070.49 (C-O-C symmetric stretching), 779.24 and 756.10 (aromatic C-H bending).

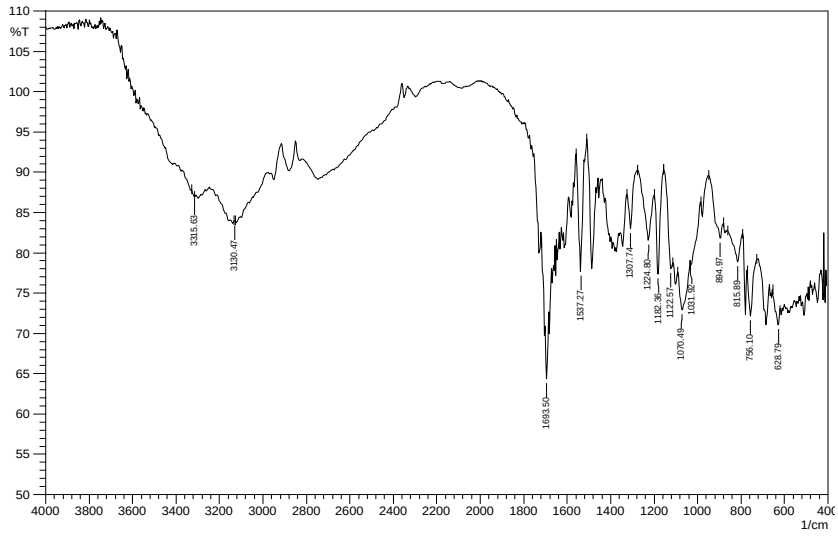

Figure 4.2. FTIR spectrum of ganciclovir

The DSC thermogram of pure drug further confirmed that the drug used in this study exists in the crystalline monohydrate form (Figure 4.3). The thermogram depicted an endothermic loss upto 110°C followed by conversion to anhydrous form which has a

sharp endothermic peak at around 254°C [8, 9] and the drug was found to decompose at its melting temperature, since no peak was obtained on repeated measurement of the same sample.

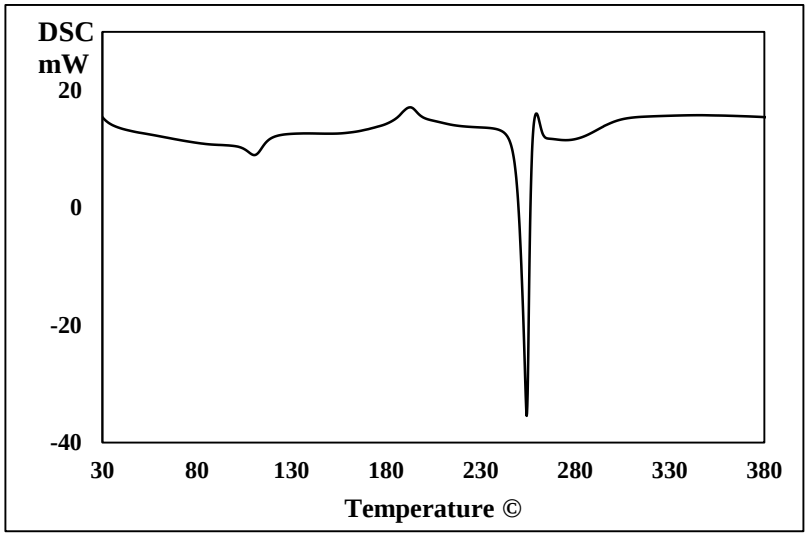

Figure 4.3. DSC thermogram of ganciclovir

4.3.2. Solubility Analysis

The pH solubility profiles of ganciclovir, in buffered and unbuffered media are shown in Figure 4.4 As seen from the profile, there is no significant difference between the solubility values of ganciclovir in buffered and unbuffered solutions.

Solubility was found to be pH dependent. At extreme pH values of below 2 and above 10, ganciclovir depicted higher solubility, which is due to existence of ganciclovir in predominantly ionized form at these pH values. Such observation can also be explained by the two pKa values (pKa_1 = 2.2 and pKa_2 = 9.4) of ganciclovir. At pH values below 2.2 and above 9.4, the ionization increases resulting in higher solubility at these pH.

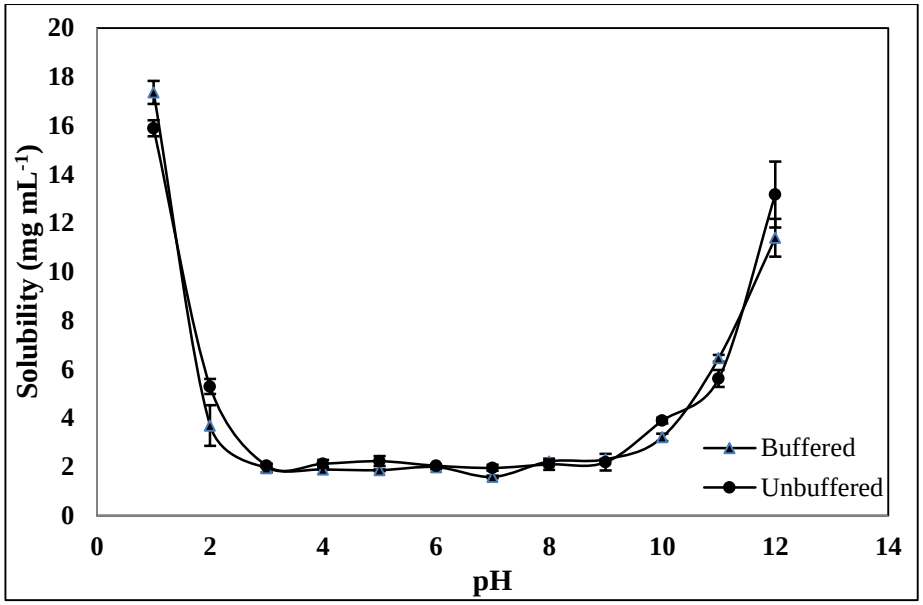

Figure 4.4. pH solubility profile of ganciclovir in buffered and unbuffered media

In solvent solubility studies, solubility of ganciclovir increased with increasing polarity of the solvents (Figure 4.5), the highest solubility being observed in DMSO (dielectric constant = 47.2) and lowest in chloroform (dielectric constant = 4.9). Among the polar solvents, solubility was higher in protic polar solvents (methanol, ethanol, IPA) than the aprotic solvents (DCM, ACN). This can be related to the fact that protic solvents have the tendency to form hydrogen bonds with compounds while the aprotic solvents show non-specific weaker interactions like dispersion forces.

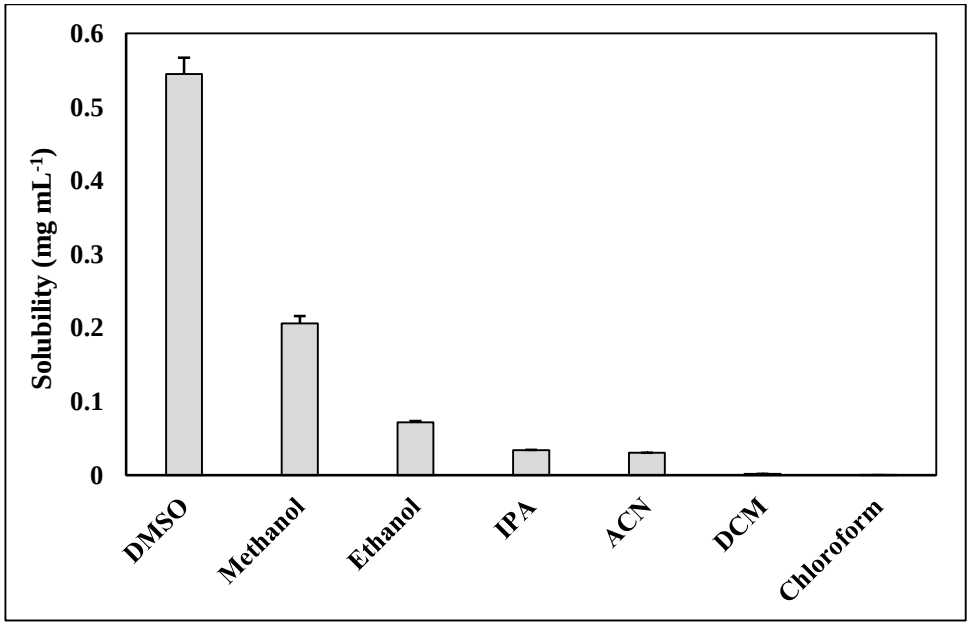

Figure 4.5. Solubility of ganciclovir in different solvents

4.3.3. Determination of Apparent Partition Coefficient

The pH partition profile of ganciclovir between octanol and different aquoues media system is shown in Figure 4.6. From the obtained negative values of log P_{app} , it was evident that, ganciclovir is a highly hydrophilic molecule. The P_{app} values were less at pH values between 3 and 9 as compared to that at extreme pH values. This correlated well with the obtained solubility data, more the ionization of the compound, lesser it partitioned into the organic phase. The experimental n-octanol/water partition coefficient value obtained was 0.025 which was comparable to the reported values of 0.022.

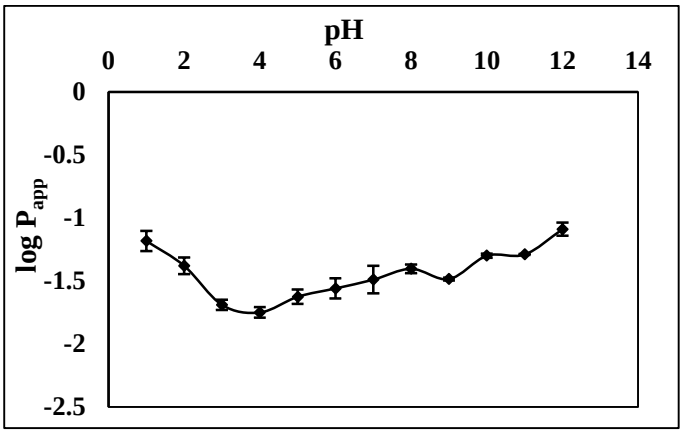

Figure 4.6. pH partition profile of ganciclovir

4.3.4. Stability Studies

4.3.4.1. Solution State Stability

Solution state stability of ganciclovir was found to be pH dependent, with degradation pathways following first order rate kinetics. The log of percentage drug remaining to be degraded was plotted as a function of time for all the pH conditions (Figure 4.7) and the corresponding first order degradation rate constants (K_{deg}) were determined. These constants were then used to determine time for 90% degradation ($t_{90\%}$) at various pH. (Table 4.1). The K_{deg} values were higher at lower pH values and decreased with an increase in pH, while the $t_{90\%}$ values ranged from 6.3 to 112 days. Lower pH was found to be detrimental for ganciclovir possibly due to hydrogen ion catalyzed hydrolysis of the fully ionized moiety (pH < 2.2).

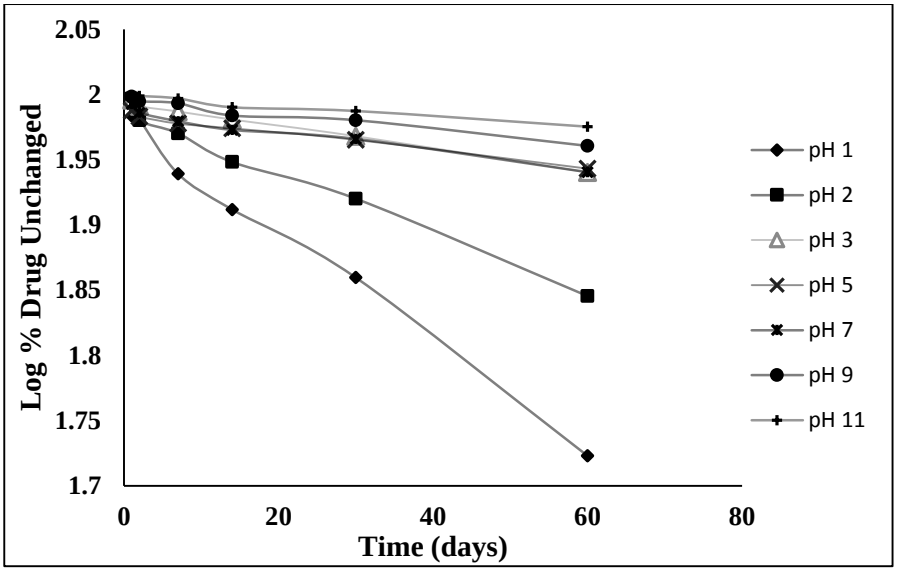

Figure 4.7. Solution state stability profile of ganciclovir in different buffered media

Table 4.1. pH stability data of ganciclovir in solution state

pH	K_{deg} (day^{-1})	$t_{90\%}$ (days)	R^2
1	0.0099	6.30	0.9918
2	0.0055	14.58	0.9876
3	0.0021	44.44	0.9953
5	0.0016	44.14	0.9845
7	0.0018	42.50	0.9656
9	0.0014	70.50	0.9704
11	0.0009	112.00	0.9718

4.3.4.2. Solid State Stability

The degradation rate of ganciclovir in solid state also followed first order kinetics, which is evident by high regression coefficient value. Ganciclovir was stable at refrigerated temperature and at room temperature, with $t_{90\%}$ values of 28.85 and 24.64 months respectively. The physical and chemical form of the drug was also maintained after storage period as the DSC thermogram and FTIR spectrum retained all the peaks seen in the ones recorded for bulk drug at start of the study.

4.3.5. Drug-Excipient Compatibility Study

Table 4.2 gives the degradation rate kinetics of drug alone and in physical mixture with excipients. The binary mixtures was found to be more stable at refrigerated temperature than room temperature, which is supported by K_{deg} values. In thermal analysis, in all the

mixtures, the melting endotherm of drug as well as excipient was well preserved with slight shift in the melting peaks or in the enthalpy of ganciclovir (Figure 4.8). These minor changes could be due to the mixing of drug and excipient, which lowers the purity of each component in the mixture, and thus does not indicate potential incompatibility [10]. The results of thermal analysis of different mixtures are shown in Table 4.3.

Table 4.2. Degradation kinetics of ganciclovir in pure form and in physical mixtures with excipients

Sample	Refrigerated (5 ± 2°C)			Room temperature (25 ± 2°C and 60 ± 5 % RH)		
	K_{deg} ($month^{-1}$)	$t_{90\%}$ (month)	R^2	K_{deg} ($month^{-1}$)	$t_{90\%}$ (month)	R^2
Drug	0.0037	28.85	0.9596	0.0041	24.64	0.9407
Drug + GMS	0.0039	27.27	0.9819	0.0048	21.27	0.9148
Drug + GDS	0.0041	25.14	0.9839	0.0044	24.35	0.9713
Drug + GB	0.0039	26.92	0.9342	0.0048	21.50	0.9531
Drug + PF-68	0.0053	20.29	0.9415	0.0048	21.22	0.9528
Drug + TPGS	0.0048	22.22	0.9597	0.0051	20.48	0.9210
Drug + lecithin	0.0053	19.46	0.9186	0.0051	20.66	0.9856

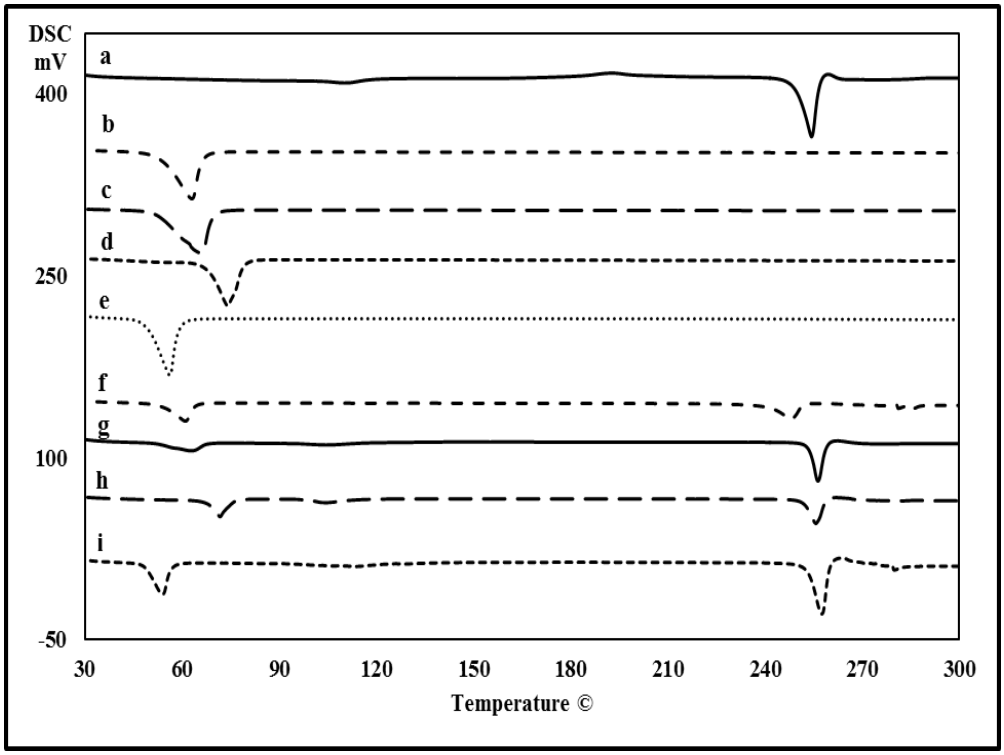

Figure 4.8. DSC thermograms obtained for a) ganciclovir, b) GMS, c) GDS, d) GB, e) PF-68, f) ganciclovir and GMS, g) ganciclovir and GDS, h) ganciclovir and GB and i) ganciclovir and PF-68

Table 4.3. Thermal analysis of ganciclovir with selected excipients

Sample	DSC Peak (°C)		Enthalpy ($J\ g^{-1}$)
	Pure Component	Physical Mixture	
Drug	257.85	-	-176.53
GMS	64.44	63.85	-178.69
		258.82	
GDS	66.75	65.75	-168.16
		259.55	
GB	75.23	74.49	-177.07
		258.6	
PF-68	57.43	57.08	-173.82
		260.74	
TPGS	41.23	40.61	-180.76
		253.07	

These findings were further supported by the IR absorption spectra. In all the drug-excipient combinations studied, the important bands of ganciclovir (listed in section 4.3.1) were identified. Thus, ganciclovir was found to be stable in all the physical mixtures at all the temperatures studied. Individual spectra of IR study are not provided as no effects were observed

4.4. Conclusion

Ganciclovir sample, obtained as gift for this research work, showed satisfactory results of identification tests carried out according to USP. Solubility of ganciclovir was found to be pH dependent, higher at both extreme pH values due to more ionization at these pH. This correlated with the two pKa values of the compound. The solubility of the molecule was much lower in organic solvents. Among the organic solvents, solubility was greater in polar protic solvents followed by polar aprotic and non-polar solvents. Hydrophilicity of ganciclovir was further exemplified the negative value of log P_{app}. Considering these parameters, the poor gastrointestinal permeability of ganciclovir was well validated, which needs to be addressed by designing suitable formulation.

The pH-stability profile of ganciclovir followed first order degradation kinetics with minimal stability at acidic conditions. Solid state stability and excipient-compatibility studies revealed that ganciclovir is stable alone and in the presence of excipients at both refrigerated and ambient temperature. This study gives valuable information regarding deciding formulation excipients and various storage conditions, while the solubility data

generated would help in deciding the solvents and process of nanoparticles preparation. Therefore, preformulation data provided valuable input for designing formulations.

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5. Formulation Design and Characterization

5.1. Introduction

NDDS offer many advantages over conventional delivery systems with enhanced desired therapeutic effects and lowered or no side effects. Recent trend shows that microparticulate and nanoparticulate drug delivery systems have played a significant role in meeting the challenges associated with conventional delivery systems.

Since oral route is the most convenient route of drug administration, nanoparticles often find their applications in oral drug delivery. The advantages offered by nanoparticulate systems in oral drug delivery are improvement in bioavailability of drugs either by increasing the drug solubility, permeability or by overcoming the first-pass effect and P-gp efflux and improved stability of drugs in the GIT [1]. Among the nanoparticulate delivery systems, SLNs have shown good potential in oral drug delivery [2-4]. Composed of biocompatible solid lipids and emulsifiers, SLNs have the ability to incorporate both hydrophilic and lipophilic drugs, and are potential bioavailability enhancer vehicle for various Class II, III and IV drugs. Various absorption mechanisms are proposed for these drug carriers which lead to enhanced oral bioavailability of the drug entrapped into them. These include absorption of the intact nanoparticles through Peyer's patches and M-cells in the intestine or by facilitating lymphatic uptake [5, 6]. Moreover, SLNs generally contain lipophilic or hydrophilic surfactants as stabilizers, some of which have been reported to inhibit P-gp mediated efflux [7].

Oral delivery of ganciclovir poses many challenges like high daily dose, development of resistance and greater intersubject varaibility due to its very low bioavailability. Considering the hydrophilic properties of ganciclovir and potential benefits of nanoparticulate drug carriers, Akhtar et al. developed nanosized niosomal dispersions for oral bioavailability enhancement of ganciclovir [8]. Niosomes comprising of different ratios of cholesterol and spans were prepared and tested in rat model. A five times increment in bioavailability of ganciclovir when administered as niosomal formulation depicted the potential of these nanoscale delivery systems in effective oral drug delivery.

In the present study, solid lipid nanoparticulate delivery systems for ganciclovir have been proposed to overcome the drawbacks of its low and erratic oral absorption. Due to the combined advantages from different carrier systems such as lipo/niosomes and polymeric nanoparticles and feasible scalability, SLNs are very good drug delivery systems to be explored for oral drug delivery. In this project, SLNs were prepared using

different lipids, namely GMS, GDS and GB and studies were undertaken for selection and optimization of critical formulation variables. The selected optimized formulations were further studied for bioavailability

5.2. Experimental

5.2.1. Materials

Ganciclovir and excipients were obtained from sources mentioned in section 3.2 of Chapter 3. Ultrapure water (Milli-Q Plus, Millipore®, USA) was used throughout the study. All solvents were purchased from Merck (Mumbai, India). All buffer salts were of analytical grade and procured from S.D.Fine Chemicals Limited (Mumbai, India).

5.2.2. Instruments/Equipments

A microtip probe sonicator (Microson, Misonix, USA) was used for preparation of emulsion. A digital magnetic stirrer with hot plate (Spinot, Tarsons, India) and vacuum evaporator (Rotavapor, Buchi, Switzerland) were used for solvent evaporation. Temperature controlled centrifuge (Compufuge, Remi, India) was used for separation of nanoparticles and lyophilizer (Freezone, Labconco, India) was used for freeze drying after freezing sample at -20°C in a refrigerator (Frost-free, Vestfrost, India). Zetasizer (Zetasizer nanoZS, Malvern, UK), Transmission Electron Microscope (Jeol 2100F, available at Advanced Instrument Research Facility, JNU, New Delhi) and Scanning Electron Microscope (Zeiss EVO40, available at Advanced Instrument Research Facility, JNU, New Delhi) were used for size and morphological characterization of the nanoparticles.

5.2.3. Preparation of Solid Lipid Nanoparticles

In the present study, SLNs were prepared using w/o/w double emulsion method as it is the most suited method for loading of hydrophilic drugs into the nanoparticulate structure. Nanoparticle formation through this process involves critical steps of formation of stable primary emulsion, formation of secondary emulsion and solvent evaporation from the double emulsion.

A two step emulsification process was followed to prepare SLNs [9, 10]. Internal phase was prepared by dissolving ganciclovir with and without surfactant in water, which was emulsified with the aid of a probe sonicator (1 min, 20W), in the organic phase containing the lipid along with soy lecithin dissolved in DCM and acetone. This primary (w/o)

emulsion was re-emulsified using ultrasonication (3 min, 20W) into an aqueous solution of a surfactant (PF-68 or TPGS), to produce a w/o/w double emulsion. In all the batches, the ratio of aqueous to organic phase for primary w/o emulsion was 1:5 and in the secondary emulsion, the ratio of primary emulsion and outer aqueous phase was 6:10. The organic solvents were then allowed to evaporate first for 3 h at room temperature under magnetic stirring for solidification and hardening of nanoparticles, and then for another 30 min in a rotary evaporator at 30°C. SLNs were isolated by centrifugation at 17500 rpm for 30 min.

The nanoparticles separated by centrifugation were resuspended in water containing sucrose (10% w/w) as cryoprotectant and freeze dried to obtain solid particles. For this, the formulations were frozen overnight in a deep-freezer at -20°C and freeze dried under vacuum (0.1 mbar, -53°C) until free flowing powder was obtained. The product was then transferred to glass container, sealed with parafilm and stored under refrigerated conditions.

5.2.4. Effect of Formulation Parameters

The influence of critical formulation variables on different attributes was investigated by preparing several batches by varying the parameters. The parameters studied were amount of lipid, amount and type of surfactant both in inner and outer phase of the emulsion and stabilizer concentration in primary emulsion. A selected parameter was varied at one time keeping all other parameters constant.

5.2.5. Characterization of Nanoparticles

5.2.5.1. Drug Loading and Entrapment Efficiency

To determine the drug loading (DL) of individual formulation, accurately weighed amount of freeze dried product was transferred to a conical flask and disrupted by addition of suitable amount of DCM and subjecting to ultrasonication (15 min, 25°C). A known quantity of water was then added to the mixture and the contents were stirred overnight to allow ganciclovir to diffuse into the aqueous layer. The aqueous phase was then retrieved from the flask and tested for its ganciclovir content using the HPLC method reported in Chapter 3. DL was then determined by using the formula

$$\text{DL (\% w/w)} = \frac{\text{Amount of drug in the product (mg)}}{\text{Amount of product taken (mg)}} \times 100$$

EE was determined by indirect method [11]. Accurately weighed SLNs were added to known volume of water and shaken on vortex mixer for 30 s. The clear supernatant obtained after centrifugation was taken and the drug content was analyzed in it using the HPLC method described in Chapter 3. The EE was determined by using the formula

$$\text{EE (\%)} = \frac{\text{Total drug added - Drug in supernatant}}{\text{Total drug added}} \times 100$$

5.2.5.2. Particle Size, Size Distribution, Shape and Morphology

The average particle size, size distribution, PDI and zeta potential of each formulation were analyzed by PCS (Zetasizer) using the DLS technique. Freeze dried samples were appropriately diluted with high purity water, filled in disposable polystyrene cells and subjected to particle size analyzer operating at wavelength of 632 nm and light scattering was monitored at a 173º angle at a temperature of 25°C. Values of zeta potential and PDI were directly obtained from the software provided with the instrument.

The morphological characterization of the different nanoparticles was done using SEM and TEM. For SEM analysis, a drop of SLN suspension was dried overnight on an aluminum stub under vacuum. This was then sputter-coated using a thin gold–palladium layer under an argon atmosphere using a gold sputter module in a high-vacuum evaporator. These coated samples were then scanned and photomicrographs were taken at an acceleration voltage of 20 kV. For TEM, SLN suspension was mixed with equal volume of 0.02% w/v phosphotungstic acid and kept for 5 min at room temp for equilibration. A drop of this preparation was then placed on a carbon coated copper grid, excess liquid removed and dried at room temperature. The sample was then micrographed at 200kV on a digital TEM station.

5.2.5.3. In vitro Release Studies

Ganciclovir release from different SLN formulations was evaluated by the dialysis bag diffusion technique [12-14]. Dialysis membrane (Spectrapor, cut off -12500 Da) was soaked in water for 12 h before use for experiment. 2 mL of drug loaded SLN dispersion was filled in the dialysis bag, sealed on both ends and immersed in a beaker containing 100 mL of release medium (pH 6.8 phosphate buffer). The contents of the beaker were stirred at 100 rpm which were maintained at a temperature 37 ± 0.5°C. At time intervals of 5, 10, 15, 20, 30 min followed by 1, 1.5, 2, 4, 6, 8 and 12 h, an aliquot of the sample was withdrawn from the release medium and replaced with the same amount of fresh

medium. All samples were suitably diluted and drug release was estimated using the HPLC method as described in Chapter 3. Cumulative % drug release was calculated and drug release kinetics was studied by subjecting the data to various mathematical models (zero order, first order, Higuchi, Korsemeyer Peppas and Baker Lonsdale). The best fit on the release data was decided based on the value of R^2. The data upto 60% of drug release was used for Peppas model fitting. Time taken for 50% drug release ($T_{50\%}$) was also determined based on best fit model equation.

5.2.5.4. Residual Solvent Analysis

Analysis of the residual solvents was carried out in accordance with USP [15], on an Agilent 6890 gas chromatograph (Agilent Technologies, USA, available at Amol Pharmaceuticals, Jaipur) equipped with a flame ionization detection system. A DB-5 capillary column (30 m × 0.32 mm i.d.; film thickness 0.25 μm) was used. GC conditions used were: oven temperature of 40°C for 20 min, then raised at a rate of 10°C min^{-1} to 240°C, and maintained at 240°C for 20 min. The injector was maintained at 140°C (split mode, ratio 1:5), detector at 250°C and helium was used as the carrier gas (35 cm s^{-1}). Head space samples were prepared in 10 mL vials filled by 10 mL of dimethyl formamide in which 20 mg of drug was dispersed. The head space conditions were: equilibration time 30 min at 100°C; pressurization time 2 min; loop fill time 1 min.

The sequence of injections for analysis was as follows: blank, working standards (six injections for system suitability) and test samples (one vial injection per preparation). Quantitation was based upon external standardization for each residual solvent detected in the sample corrected by sample weight versus the corresponding peak area from an equal volume of the working standard, using the following equation

$$\text{ppm} = \frac{\text{Area of sample}}{\text{Area of standard}} \times \frac{\text{weight of standard}}{\text{weight of sample}} \times \frac{\text{dilution of sample}}{\text{dilution of standard}} \times 10^6$$

5.2.5.5. Thermal Study

Thermal analysis of the SLNs was carried out to assess the physical state of the entrapped drug in the formulations. Measurements were carried out on a DSC instrument as described earlier in Chapter 4. The thermograms obtained for nanoparticulate formulations were compared with those of pure drug, excipients and physical mixture.

5.2.6. Stability of Formulations

The stability of both SLN dispersions and freeze dried particles was evaluated after storage at different conditions, RT (25±5°C) and refrigerated (5±2°C). The nanoparticles were evaluated for particle size, PDI and DL at time intervals of 7, 15 days followed by 1, 3 and 6 months.

5.3. Results and Discussion

On the account of its hydrophilicity, cold homogenization technique and double emulsion technique were the most appropriate methods to prepare SLNs of ganciclovir. The aim of the design and development of formulations was to obtain SLNs with minimum particle size and maximum EE. Initially attempts were made using the cold homogenization technique, but unfavourable results like high particle size and drug leaching resorted to the use of double emulsion solvent evaporation method. Initial batches using this method yielded satisfactory results in terms of particle size and PDI but achieving good drug entrapment was the major challenge. Hence various formulation variables were studied to obtain the desired characters in the SLNs. Different formulation batches, along with their characterization results, prepared by varying formulation parameters are presented in Tables 5.1 to 5.3. Nanoparticles' characters like particle size, EE and in vitro release were found to be dependent on various formulation parameters. The optimized preparations depicted favourable physical qualities like small particle size with uniform distribution and good and stable EE values.

5.3.1. Effect of Amount and Nature of Lipid Matrix

SLNs were formulated using different acylglycerides as solid lipids, GMS, GDS and GB. The type and proportion of each lipid affected various properties of the SLNs. In batches prepared with different quantities of GMS, the particle size and PDI increased from 134.00 to 478.60 nm and 0.27 to 0.47 with increasing quantity of GMS from 25 mg to 200 mg. Similar trend of increasing particle size with increasing lipid amount was observed in SLNs prepared using GDS and GB (Figure 5.1). Batches prepared with GDS level of 200 mg (GAN/SLN/GDS/10) and GB levels of 100 mg and 200 mg (GAN/SLN/GB/9 and GAN/SLN/GB/10) were not evaluated further for drug loading and entrapment due to visual observation of agglomeration during emulsion formation and very high particle size.

Table 5.1. Formula and characters of GMS based ganciclovir loaded SLNs

Batch Code	GAN (mg)	GMS (mg)	Stabilizers (%w/v)				Mean Particle Size (nm) ± SD	Poly Dispersity Index ± SD	Zeta Potential (mV)	Drug Loading (%w/w) ± SD	Entrapment Efficiency (%) ± SD
			Internal (same as external)	Middle (lecithin)	External (PF 68)	External (TPGS)					
GAN/SLN/GMS/1	50	50	0	0.10	0.10	-	148.32 ± 11.40	0.41 ± 0.02	-64.0	8.82 ± 0.69	22.73 ± 4.59
GAN/SLN/GMS/2	50	50	0	0.10	0.25	-	145.53 ± 0.13	0.31 ± 0.05	-62.0	8.62 ± 0.55	21.96 ± 2.71
GAN/SLN/GMS/3	50	50	0	0.10	0.50	-	132.14 ± 6.70	0.27 ± 0.01	-55.8	6.65 ± 0.03	21.11 ± 3.14
GAN/SLN/GMS/4	50	50	0	0.10	1.00	-	100.03 ± 1.00	0.25 ± 0.01	-50.1	5.25 ± 0.06	16.75 ± 1.27
GAN/SLN/GMS/5	50	50	0	0.00	0.25	-	424.30 ± 32.67	0.53 ± 0.06	-34.2	8.89 ± 0.14	14.97 ± 5.15
GAN/SLN/GMS/6	50	50	0	0.05	0.25	-	228.20 ± 0.57	0.29 ± 0.01	-42.6	7.77 ± 0.67	20.13 ± 1.38
GAN/SLN/GMS/7	50	50	0	0.20	0.25	-	124.05 ± 0.92	0.06 ± 0.01	-72.9	5.72 ± 0.27	11.26 ± 2.48
GAN/SLN/GMS/8	50	25	0	0.10	0.25	-	134.00 ± 2.21	0.27 ± 0.08	-62.8	8.33 ± 0.23	19.40 ± 0.85
GAN/SLN/GMS/9	50	100	0	0.10	0.25	-	240.10 ± 0.71	0.39 ± 0.01	-58.9	7.49 ± 0.18	34.54 ± 2.40
GAN/SLN/GMS/10	50	200	0	0.10	0.25	-	478.60 ± 14.99	0.47 ± 0.02	-52.0	6.91 ± 1.26	36.29 ± 2.46
GAN/SLN/GMS/11	50	50	0.10	0.10	0.25	-	165.03 ± 1.51	0.27 ± 0.01	-66.1	11.47 ± 0.20	42.07 ± 1.42
GAN/SLN/GMS/12	50	50	0.25	0.10	0.25	-	143.06 ± 1.36	0.24 ± 0.01	-61.8	11.08 ± 0.34	44.66 ± 2.48
GAN/SLN/GMS/13	50	50	0	0.10	-	0.01	163.94 ± 2.06	0.23 ± 0.01	-65.2	9.45 ± 1.31	20.50 ± 1.89
GAN/SLN/GMS/14	50	50	0	0.10	-	0.02	229.90 ± 27.58	0.21 ± 0.02	-61.5	8.11 ± 1.40	15.48 ± 1.84
GAN/SLN/GMS/15	50	50	0	0.10	-	0.05	329.20 ± 40.73	0.39 ± 0.01	-50.4	7.91 ± 1.03	14.23 ± 1.17
GAN/SLN/GMS/16	50	50	0.01	0.10	-	0.01	157.41 ± 0.58	0.11 ± 0.01	-58.2	10.28 ± 1.32	36.32 ± 0.84
GAN/SLN/GMS/17	50	50	0.02	0.10	-	0.01	216.95 ± 0.41	0.39 ± 0.04	-54.1	10.55 ± 0.83	37.86 ± 0.72

Table 5.2. Formula and characters of GDS based ganciclovir loaded SLNs

Batch Code	GAN (mg)	GDS (mg)	Stabilizers (%w/v)				Mean Particle Size (nm) ± SD	Poly Dispersity Index ± SD	Zeta Potential (mV)	Drug Loading (%w/w) ± SD	Entrapment Efficiency (%) ± SD
			Internal (same as external)	Middle (lecithin)	External (PF 68)	External (TPGS)					
GAN/SLN/GDS/1	50	50	0	0.10	0.10	-	184.68 ± 6.40	0.28 ± 0.06	-51.4	8.94 ± 1.74	32.76 ± 4.28
GAN/SLN/GDS/2	50	50	0	0.10	0.25	-	170.57 ± 1.37	0.25 ± 0.00	-49.0	8.79 ± 1.03	30.54 ± 1.92
GAN/SLN/GDS/3	50	50	0	0.10	0.50	-	148.52 ± 2.14	0.16 ± 0.02	-45.3	8.11 ± 1.23	24.86 ± 2.59
GAN/SLN/GDS/4	50	50	0	0.10	1.00	-	135.16 ± 0.74	0.10 ± 0.02	-40.0	7.52 ± 1.05	22.96 ± 3.94
GAN/SLN/GDS/5	50	50	0	0.00	0.25	-	Primary emulsion not formed				
GAN/SLN/GDS/6	50	50	0	0.05	0.25	-	192.14 ± 3.58	0.39 ± 0.06	-45.0	9.03 ± 0.68	26.47 ± 2.86
GAN/SLN/GDS/7	50	50	0	0.20	0.25	-	167.05 ± 1.17	0.34 ± 0.01	-52.7	7.33 ± 0.74	21.68 ± 2.33
GAN/SLN/GDS/8	50	25	0	0.10	0.25	-	165.34 ± 1.39	0.27 ± 0.02	-59.7	12.1 ± 2.01	22.68 ± 1.65
GAN/SLN/GDS/9	50	100	0	0.10	0.25	-	282.15 ± 10.52	0.28 ± 0.01	-49.2	9.82 ± 1.70	33.06 ± 3.96
GAN/SLN/GDS/10	50	200	0	0.10	0.25	-	Agglomeration seen				
GAN/SLN/GDS/11	50	50	0.10	0.10	0.25	-	177.07 ± 1.12	0.24 ± 0.01	-48.6	11.52 ± 0.91	45.97 ± 1.98
GAN/SLN/GDS/12	50	50	0.25	0.10	0.25	-	167.08 ± 2.15	0.29 ± 0.01	-46.7	10.85 ± 1.10	47.62 ± 0.99
GAN/SLN/GDS/13	50	50	0	0.10	-	0.01	187.19 ± 3.14	0.30 ± 0.03	-56.1	9.80 ± 1.28	29.96 ± 1.61
GAN/SLN/GDS/14	50	50	0	0.10	-	0.02	275.40 ± 4.04	0.32 ± 0.03	-53.1	8.54 ± 1.29	26.02 ± 1.54
GAN/SLN/GDS/15	50	50	0	0.10	-	0.05	434.40 ± 25.65	0.50 ± 0.07	-40.4	6.82 ± 0.82	24.90 ± 1.65
GAN/SLN/GDS/16	50	50	0.01	0.10	-	0.01	161.25 ± 5.64	0.40 ± 0.01	-54.6	9.52 ± 0.50	38.16 ± 0.89
GAN/SLN/GDS/17	50	50	0.02	0.10	-	0.01	169.88 ± 3.34	0.26 ± 0.01	-50.0	8.80 ± 0.96	40.96 ± 2.27

Table 5.3. Formula and characters of GB based ganciclovir loaded SLNs

Batch Code	GAN (mg)	GB (mg)	Stabilizers (%w/v)				Mean Particle Size (nm) ± SD	Poly Dispersity Index ± SD	Zeta Potential (mV)	Drug Loading (%w/w) ± SD	Entrapment Efficiency (%) ± SD
			Internal (same as external)	Middle (lecithin)	External (PF 68)	External (TPGS)					
GAN/SLN/GB/1	50	50	0	0.10	0.10	-	431.03 ± 6.22	0.50 ± 0.01	-62.2	11.07 ± 1.03	24.87 ± 0.58
GAN/SLN/GB/2	50	50	0	0.10	0.25	-	358.00 ± 6.80	0.40 ± 0.04	-54.7	10.89 ± 1.27	24.90 ± 1.29
GAN/SLN/GB/3	50	50	0	0.10	0.50	-	296.80 ± 4.42	0.36 ± 0.03	-50.5	9.55 ± 0.83	22.37 ± 0.52
GAN/SLN/GB/4	50	50	0	0.10	1.00	-	268.36 ± 4.38	0.52 ± 0.01	-46.2	7.91 ± 0.73	18.42 ± 0.39
GAN/SLN/GB/5	50	50	0	0.00	0.50	-	Primary emulsion not formed				
GAN/SLN/GB/6	50	50	0	0.05	0.50	-	393.02 ± 64.06	0.40 ± 0.05	-47.2	10.52 ± 1.63	17.27 ± 2.15
GAN/SLN/GB/7	50	50	0	0.20	0.50	-	282.48 ± 3.29	0.33 ± 0.02	-52.9	8.32 ± 2.18	15.68 ± 1.61
GAN/SLN/GB/8	50	25	0	0.10	0.50	-	225.02 ± 13.86	0.32 ± 0.04	-57.7	12.25 ± 0.59	11.48 ± 2.03
GAN/SLN/GB/9	50	100	0	0.10	0.50	-	618.05 ± 59.65	0.96 ± 0.06	Lumps formed after freeze drying		
GAN/SLN/GB/10	50	200	0	0.10	0.50	-	Agglomeration seen				
GAN/SLN/GB/11	50	50	0.10	0.10	0.50	-	278.34 ± 3.63	0.33 ± 0.04	-49.3	12.01 ± 0.59	32.16 ± 0.68
GAN/SLN/GB/12	50	50	0.25	0.10	0.50	-	253.59 ± 0.99	0.45 ± 0.09	-45.9	12.09 ± 0.18	36.32 ± 0.37
GAN/SLN/GB/13	50	50	0	0.10	-	0.01	374.28 ± 14.29	0.38 ± 0.04	-64.4	10.80 ± 1.96	19.96 ± 2.72
GAN/SLN/GB/14	50	50	0	0.10	-	0.02	401.20 ± 19.38	0.57 ± 0.02	-61.5	9.45 ± 0.28	17.02 ± 2.45
GAN/SLN/GB/15	50	50	0	0.10	-	0.05	560.25 ± 8.39	0.472 ± 0.07	-47.9	7.82 ± 1.28	14.90 ± 0.85
GAN/SLN/GB/16	50	50	0.01	0.10	-	0.01	338.66 ± 6.71	0.22 ± 0.05	-62.5	11.52 ± 1.04	34.16 ± 1.89
GAN/SLN/GB/17	50	50	0.02	0.10	-	0.01	378.86 ± 2.46	0.27 ± 0.03	-59.2	10.40 ± 1.69	33.39 ± 1.72

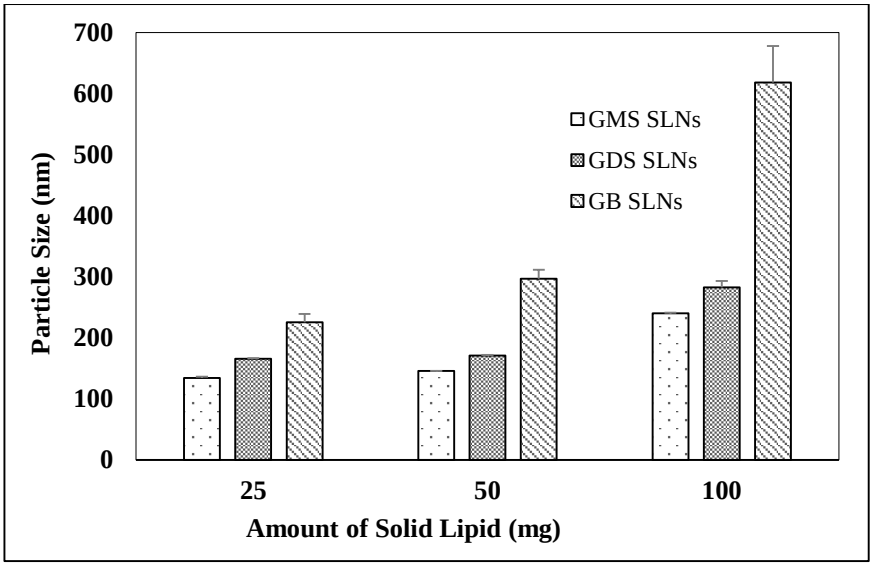


Figure 5.1. Effect of amount and type of lipid matrix on particle size of SLNs

The observation of an increase in particle size of SLNs with increase in lipid content is well in agreement with previous reports of other researchers [16-19]. Such effect was probably caused by the increasing viscosity of dispersed phase (organic phase solution) causing high resistance against the shear forces during the emulsification, resulting in a poorer dispersibility of the lipid solution into the outer aqueous phase. Coarse emulsions were obtained at higher lipid concentrations, which lead to the build of bigger particles during the diffusion process during solvent evaporation. Another reason for such a phenomenon may be the inability of surfactant in the outer phase (which is at fixed concentration) to stabilize the interfacial tension generated by higher amounts of lipid, leading to an increased particle size and agglomeration [9].

Among the different lipids used, the least particle size was observed in batches prepared using GMS and highest was obtained with GB (Figure 5.1.). This can be explained based on the lipophilicity of the lipid used. It has been found that the average particle size of SLN dispersions increase with higher melting lipids [16, 20], which was also established in this study. In the structure of the lipids, it can be seen that GMS has two strong hydrophilic hydroxyl groups and GDS has one hydroxyl group, where as there is no such group in GB. Instead, GB comprises of three long aliphatic chains as substituents on the glycerol moiety, rendering it the most lipophilic of all the lipids used. The more lipophilic the lipid is, the more viscous solution it makes in the organic solvent and as a result the particle size of the nanoparticles formed is more due to increased resistance during emulsion formation.

A significant gain in drug entrapment from 19.4% to 34.54% was seen when GMS amount was increased from 25mg to 100mg (Figure 5.2). Similar pattern was seen with GDS batches. For batches with GB level of 100mg, entrapment could not be determined due to agglomerate formation. Increase in lipid content in the formulations increase the possible sites for drug encapsulation resulting in higher EE values [18, 21] . As compared to GMS and GDS, SLNs prepared with GB showed lower EE, particularly at lower level, due to the lack of hydrophilic moiety in the structure of GB (Figure 5.2). The hydroxyl groups in GMS and GDS could be involved in the better emulsification of the system and encapsulation of ganciclovir into SLNs.

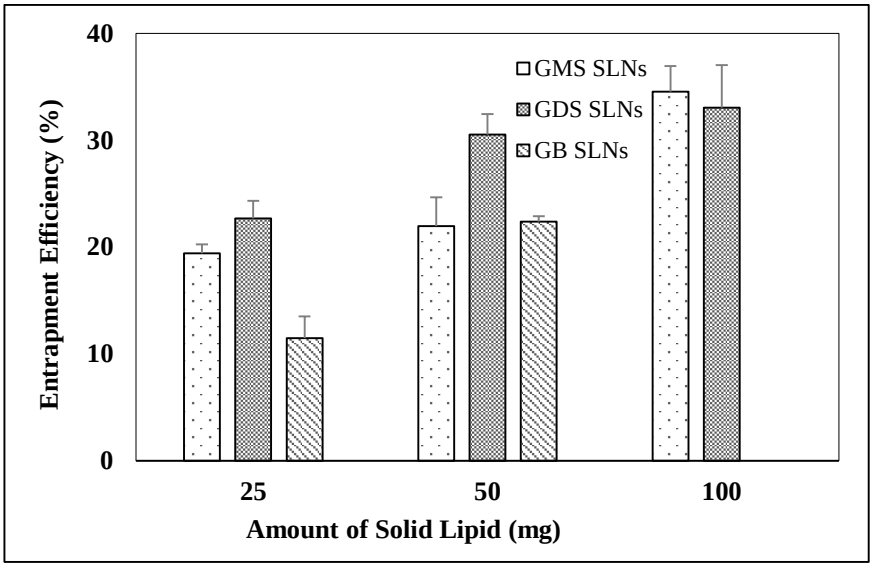


Figure 5.2. Influence of type and amount of lipid on EE of ganciclovir in SLNs

5.3.2. Effect of Surfactants

In preparing nanoparticles using double emulsion technique, the inclusion of surfactants was done, both in primary emulsion and secondary emulsion phase, for achieving a stable product. The particle size and EE of SLNs prepared using GMS, GDS and GB showed dependency on the concentration of the surfactants added during their preparation. At the primary emulsion stage, it was observed that the particle size of nanoparticles decreased with increase in concentration of lecithin from 0.05 to 0.1%, but no appreciable decrease in size was seen when concentration was further increased to 0.2% (Figure 5.3).

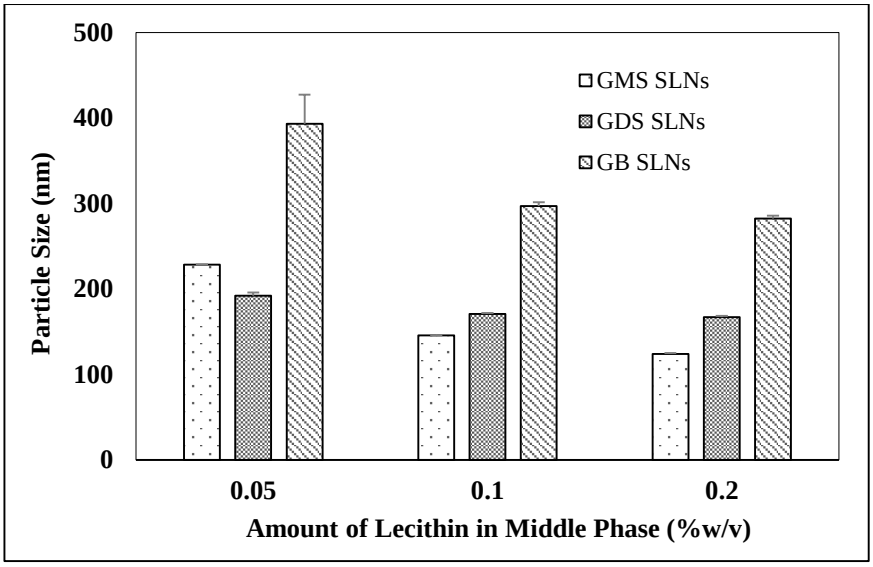


Figure 5.3. Effect of lecithin concentration on particle size of prepared SLNs.

Lecithin is added along with the solid lipid as it is an amphiphilic lipid and has the ability to modify the lipid matrix and help in stabilization of the emulsion and in drug loading into the SLNs [22, 23]. Incorporation of lecithin favours reduction in o/w interfacial tension, supporting increased surface area of the dispersed phase leading to smaller particle sizes [24].

Surfactant in the outer aqueous phase also plays a vital role in determining the characteristics of the nanoparticles by reducing interfacial tension and providing steric and mechanical stabilization of the formed nanoparticles and thus preventing their aggregation [25, 26]. The effect of different concentrations of PF-68 and TPGS on particle size is shown in Figures 5.4 and 5.5, respectively. A trend of decrease in particle size and PDI with increase in concentration of PF-68 was seen in batches prepared using different lipids. In techniques involving solvent evaporation, stabilization of particles is a crucial factor and the concentration of surfactant is the major element that governs the protection of particles. At higher concentrations of surfactant, there is increase in surface active properties and an increased surface area of the dispersed hydrophobic phase. As a result, at constant emulsification power the net shear stress increases causing reduction in particle size [20, 24, 27].

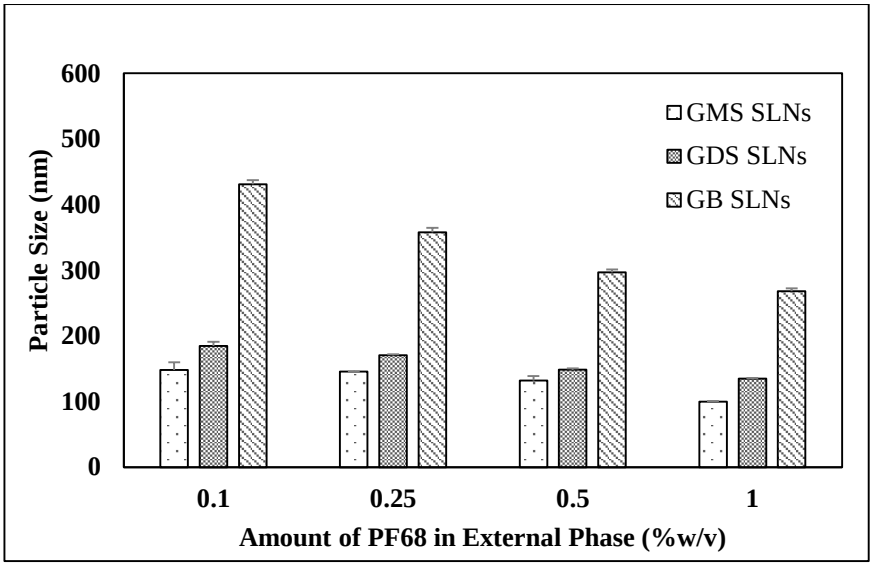


Figure 5.4. Effect of external phase PF-68 amount on particle size of SLNs

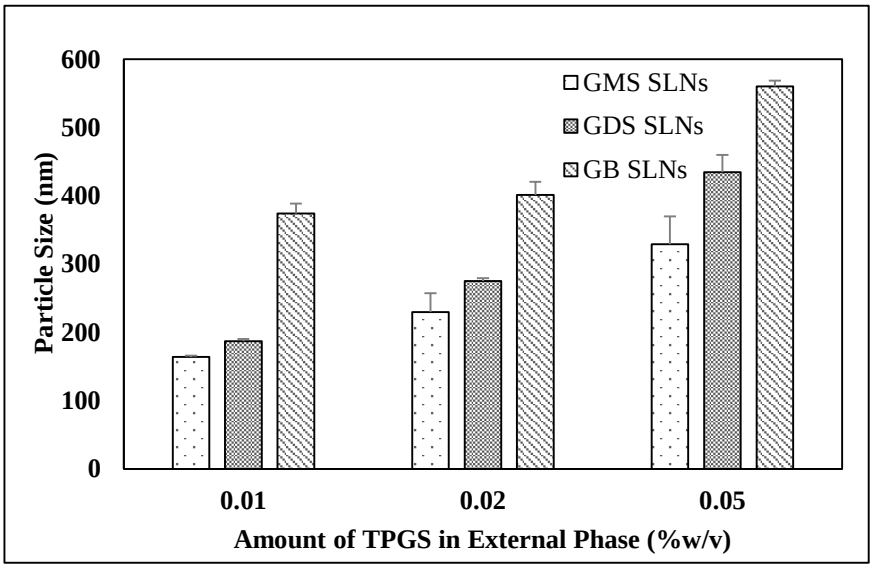


Figure 5.5. Effect of external phase TPGS amount on particle size of SLNs

Due to its unique characters, TPGS is an effective surfactant at concentrations lower than that required with traditional emulsifiers as it has a low critical micellar concentration (CMC) of 0.02% w/w. It has also been found by our group that at concentrations below the CMC, TPGS is effective in enhancing the intestinal permeation of ganciclovir [28]. So, the concentrations of TPGS which were used to prepare nanoparticles were below its CMC. But, contrary to the effect of outer phase PF-68 on particle size, the particle size of nanoparticles prepared using TPGS as stabilizer increased with increase in its concentration (Figure 5.5). This effect would have been due to an increased viscosity of

the outer phase at higher TPGS concentrations. The shear applied could not overcome the increase in viscosity of the dispersion, resulting in higher particle size of the nanoparticles. Thus, further batches with TPGS were prepared at concentration level of 0.01%.

The EE of ganciclovir was not influenced by the amount of outer phase surfactant up to a certain concentration, beyond which a decrease in drug entrapment was observed (Figures 5.6 and 5.7). This effect may be attributed to more leaching of drug from the nanoparticles towards the outer aqueous phase on increase in surfactant concentration. The optimum concentration of PF-68 (0.25% for GMS and GDS and 0.5% for GB based SLNs) and TPGS (0.01% for all) was fixed on this basis.

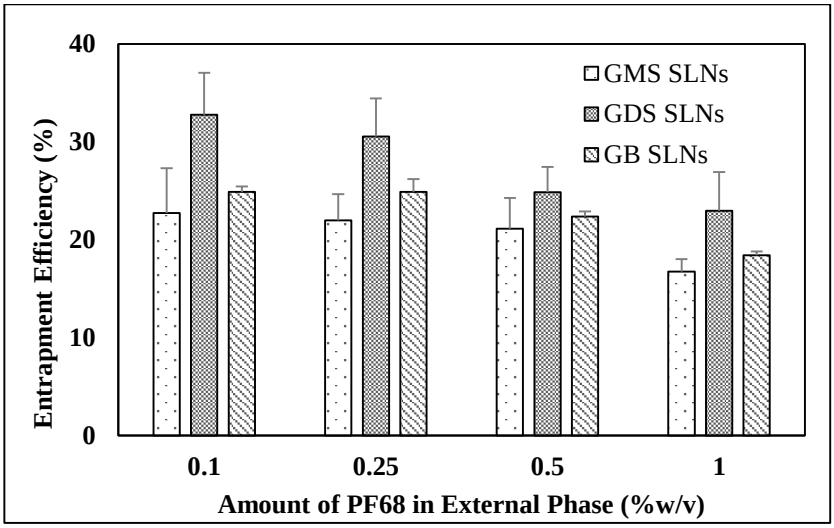


Figure 5.6. Influence of external phase PF-68 concentration on EE of ganciclovir

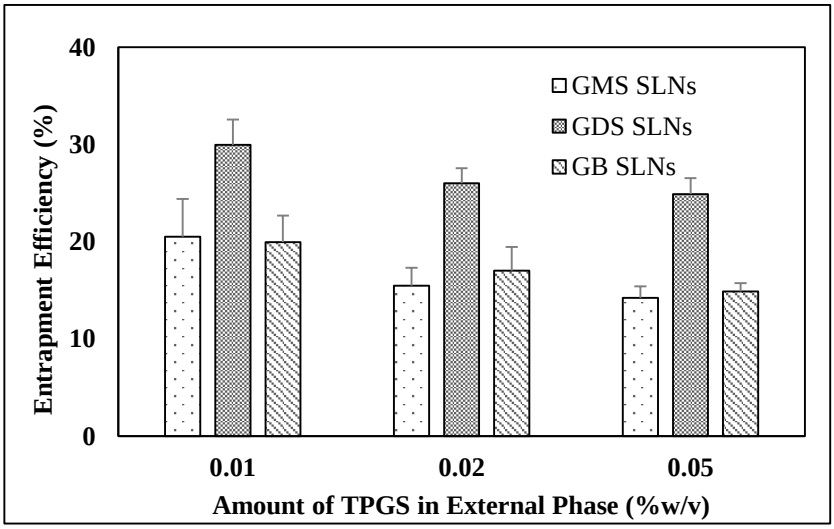


Figure 5.7. Influence of external phase TPGS concentration on EE of ganciclovir

In an attempt to increase the EE of ganciclovir into the SLNs, formulations incorporating surfactant in the inner aqueous phase were prepared. It was seen that inclusion of surfactant in the inner phase did not have any significant effect on the particle size of the SLNs but substantially improved the drug encapsulation (Figures 5.8 and 5.9). Presence of surfactant along with drug lead to increased interaction of the drug with the lipid phase during the formation of primary emulsion, preventing drug partitioning to the external aqueous phase during formation of secondary emulsion and later solvent evaporation [29, 30]. By including PF-68 or TPGS in the internal phase, a remarkable 2 fold increase in EE of ganciclovir was observed in case of GMS nanoparticles and 1.5 times in case of batches with GDS and GB.

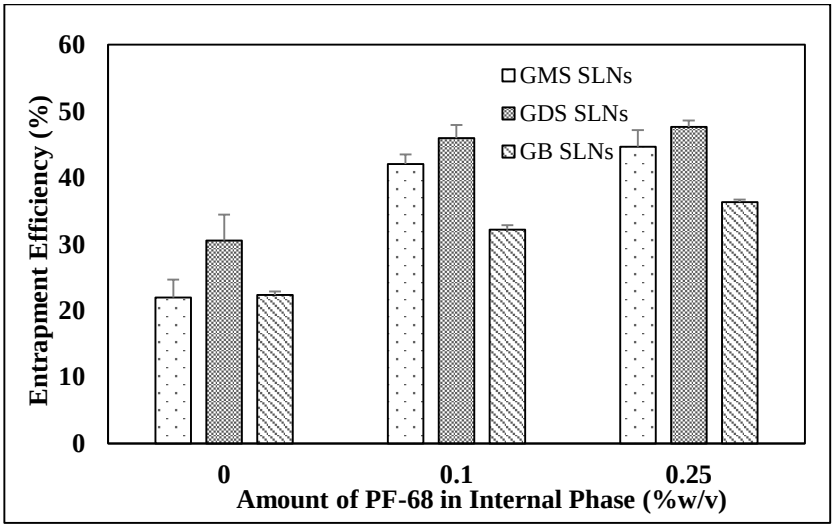


Figure 5.8. Effect of internal phase PF-68 on EE of ganciclovir

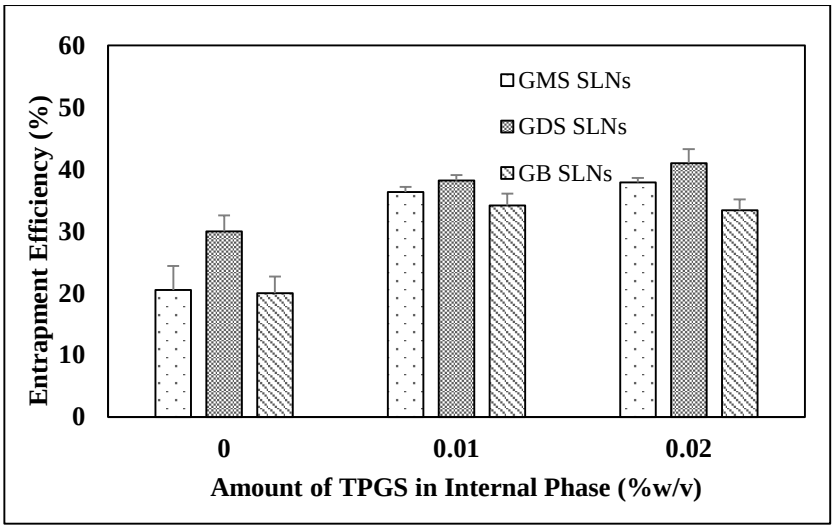


Figure 5.9. Effect of internal phase TPGS on EE of ganciclovir

5.3.3. Effect of Freeze Drying and Redispersibility

Freeze drying of nanoparticles was carried out to obtain a solid and stable product. The effect of freeze drying on particle size and PDI was studied. Redispersibility of the freeze dried formulations was also assessed qualitatively, after reconstituting the product in water and manual shaking. It was observed that formulations prepared using both PF-68 and TPGS as surfactants, were free flowing and exhibited quick redispersibility time (< 1 min) and no significant change in particle size and distribution after freeze drying. The particle size distribution of selected batches of SLNs, before and after freeze drying, is shown in Figures 5.10 and 5.11.

5.3.4. Morphology of Nanoparticles

The shape and surface characteristics of the SLNs were investigated using SEM and TEM. The SEM images (Figure 5.12) indicated that the nanoparticles displayed spherical shape and absence of drug crystals on the surface. TEM images confirmed the nanometer size and the internal globular structure of the SLNs depicting a solid solution matrix model formation, without any aggregation. (Figure 5.13).

5.3.5. In vitro Drug Release

In vitro release profile of ganciclovir from SLN formulations prepared using different lipids and pure drug is shown in Figure 5.14. In case of pure drug, complete diffusion through the dialysis membrane occurred within 0.5 h, while the release of drug encapsulated in SLNs showed a biphasic pattern and was extended upto 4-8 h. All SLN batches displayed an initial burst release of approximately 50-65% in 1 h, which may be due to the weakly bound drug present on the surface of the nanoparticulate matrix. Factors contributing to a fast release are the large surface area, a high diffusion coefficient due to small molecular size, and a short diffusion distance for the drug from outer surface region of the nanoparticle. In later phase, the entrapped drug was released in a slow fashion, extending the release upto 4-8 h, which can be attributed to the slow diffusion of drug from the lipid matrix. The drug release could not be extended beyond 8 h due to the hydrophilicity of drug.

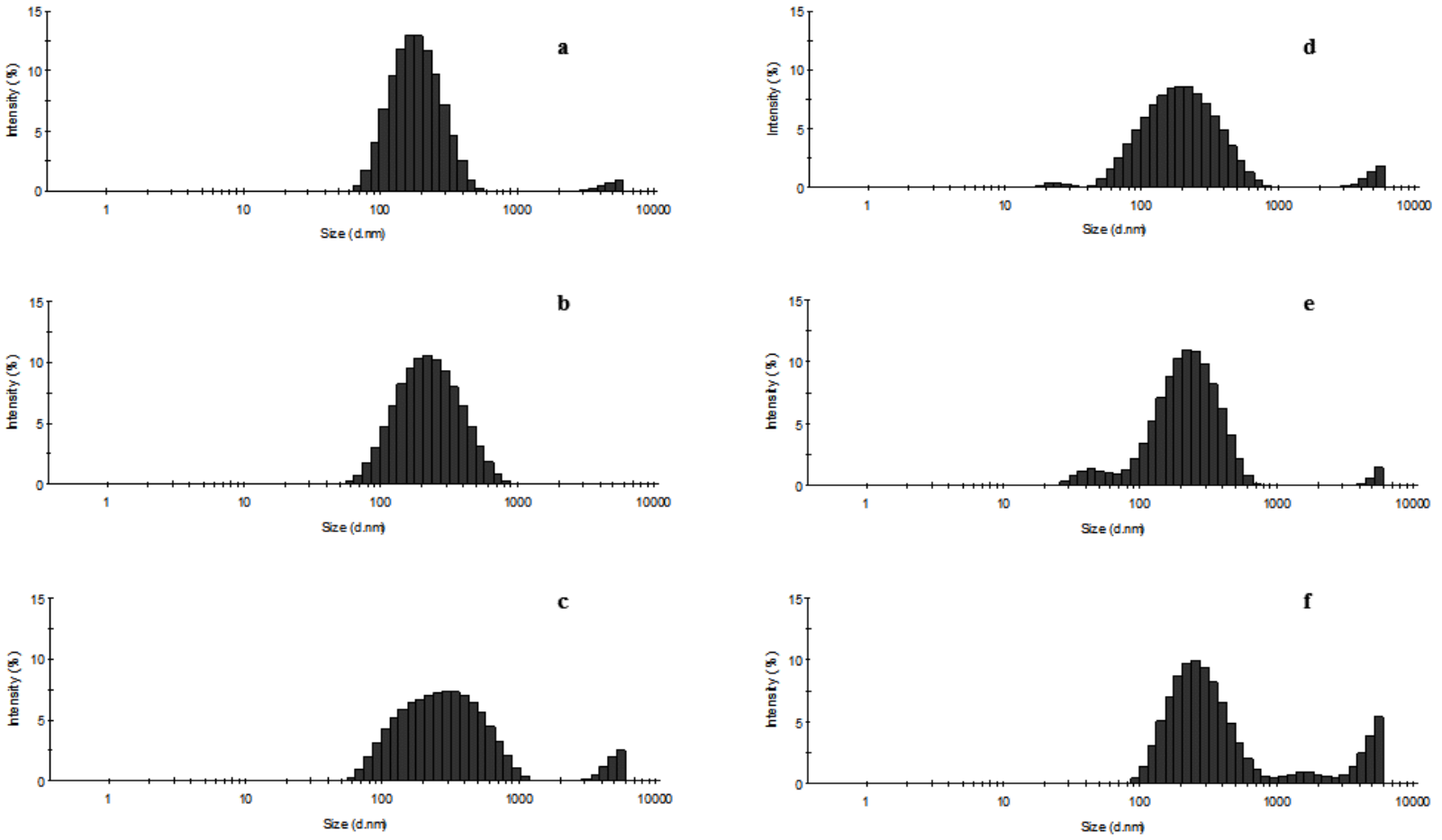


Figure 5.10. Particle size distribution before (a, b and c) and after freeze drying (d, e and f), of batches prepared with PF-68 as surfactant; GAN/SLN/GMS/11 (a and d), GAN/SLN/GDS/11 (b and e) and GAN/SLN/GB/12 (c and f).

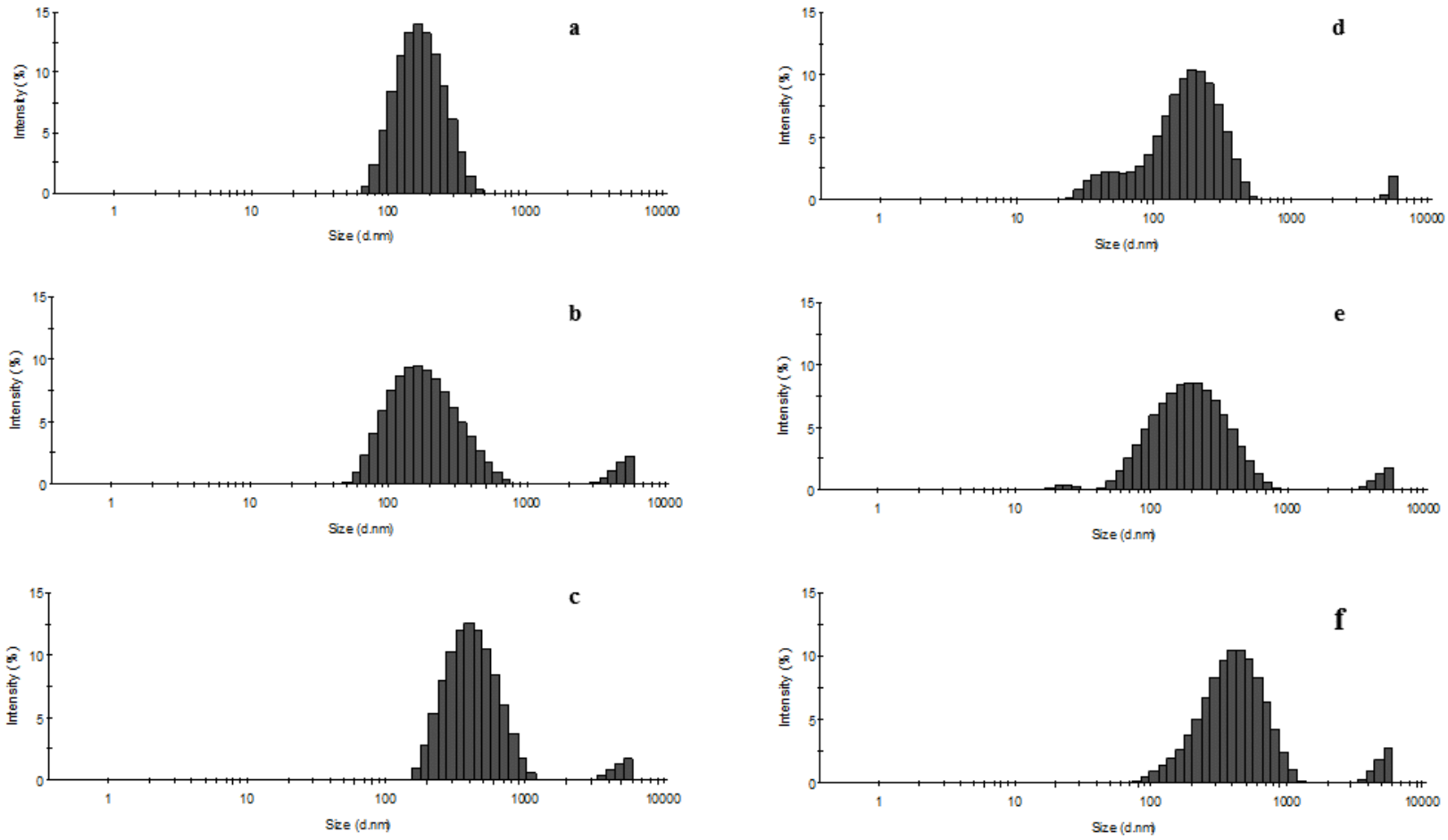
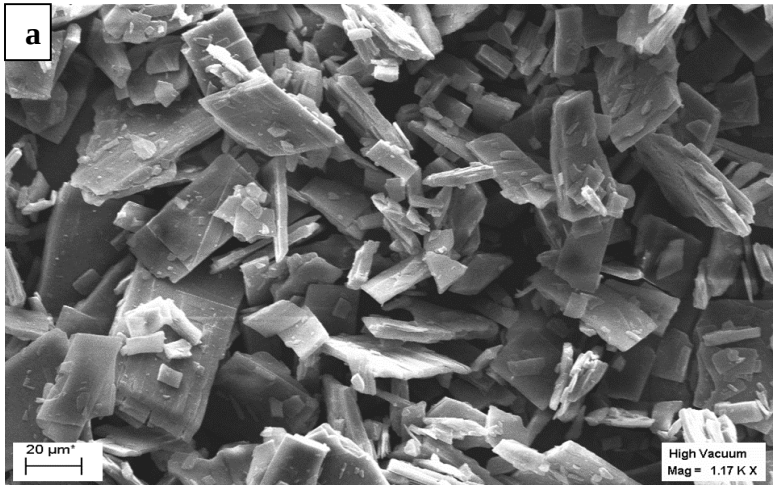
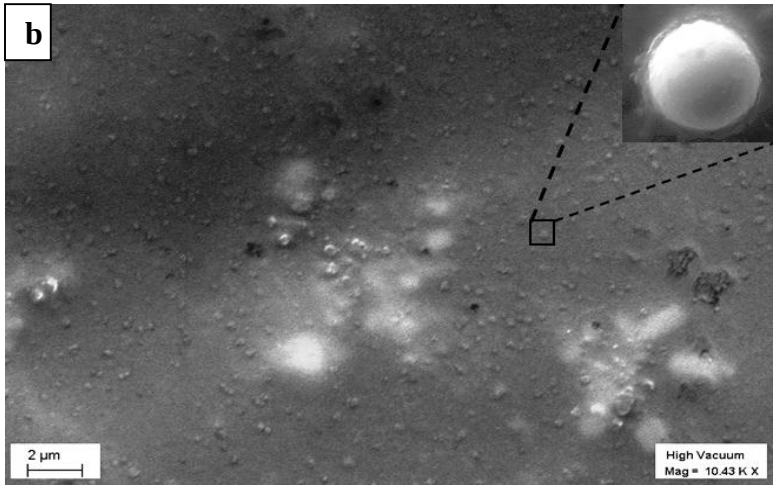
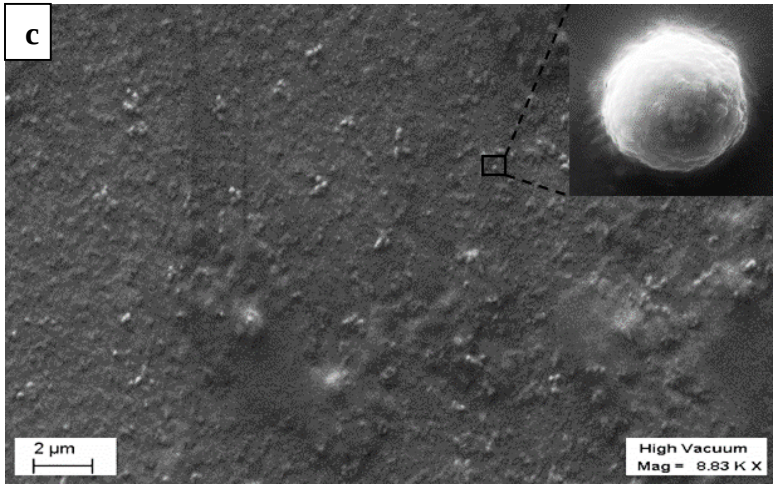
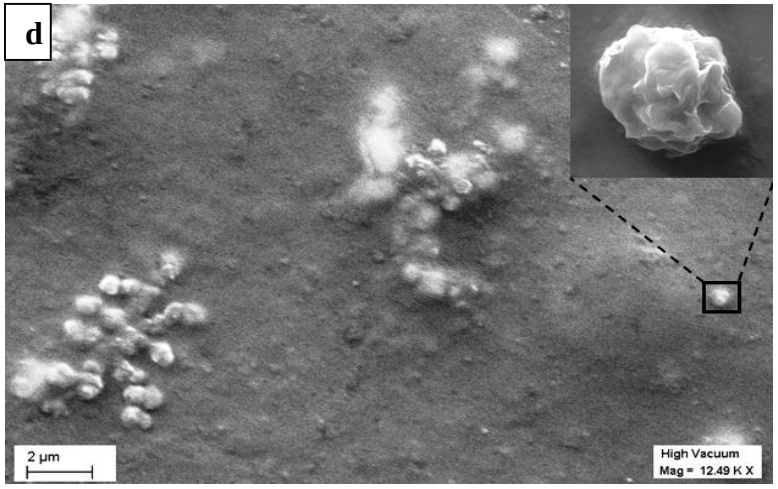


Figure 5.11. Particle size distribution before (a, b and c) and after freeze drying (d, e and f), of batches prepared with TPGS as surfactant; GAN/SLN/GMS/16 (a and d), GAN/SLN/GDS/16 (b and e) and GAN/SLN/GB/16 (c and f).


a
20 µm*
High Vacuum
Mag = 1.17 K X


b
2 µm
High Vacuum
Mag = 10.43 K X


c
2 µm
High Vacuum
Mag = 8.83 K X


d
2 µm
High Vacuum
Mag = 12.49 K X

Continued…

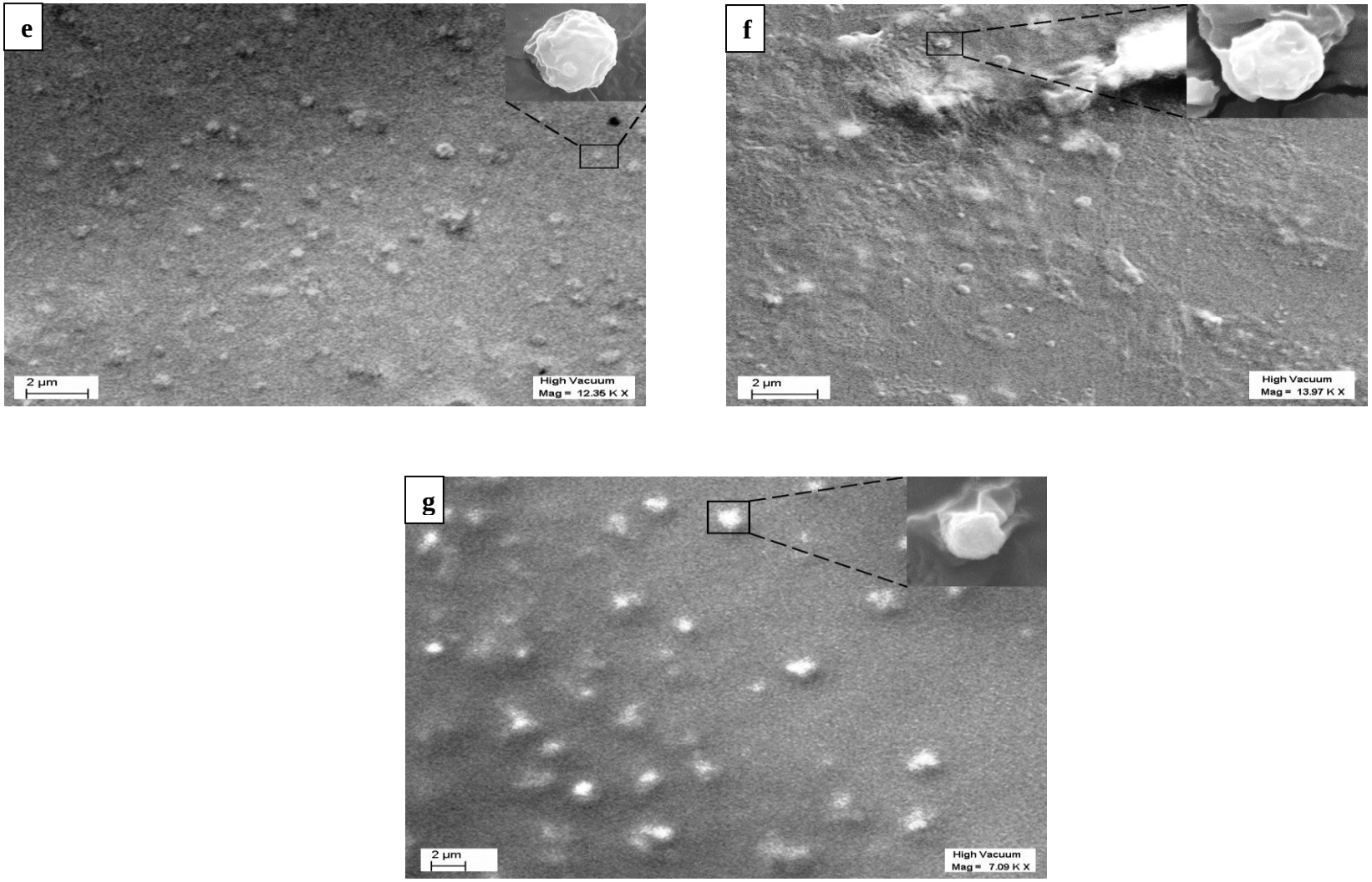


Figure 5.12. Surface morphology of ganciclovir a) and ganciclovir loaded SLNs b) GAN/SLN/GMS/11, c) GAN/SLN/GDS/11, d) GAN/SLN/GB/12, e) GAN/SLN/GMS/18, f) GAN/SLN/GDS/18 and g) GAN/SLN/GB/18) by SEM

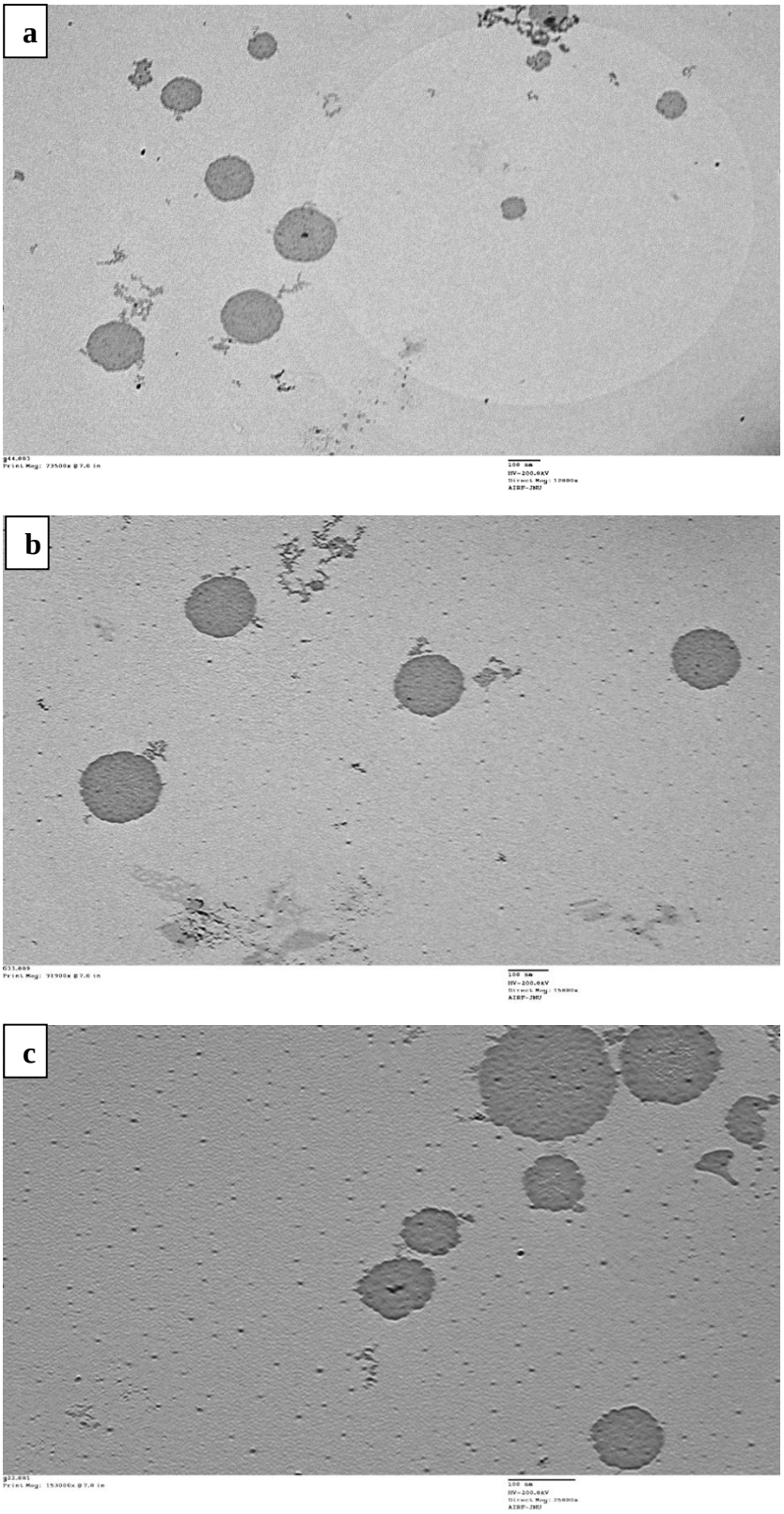


Figure 5.13. TEM images of ganciclovir loaded SLNs, a) GAN/SLN/GMS/11, b) GAN/SLN/GDS/11 and c) GAN/SLN/GB/12

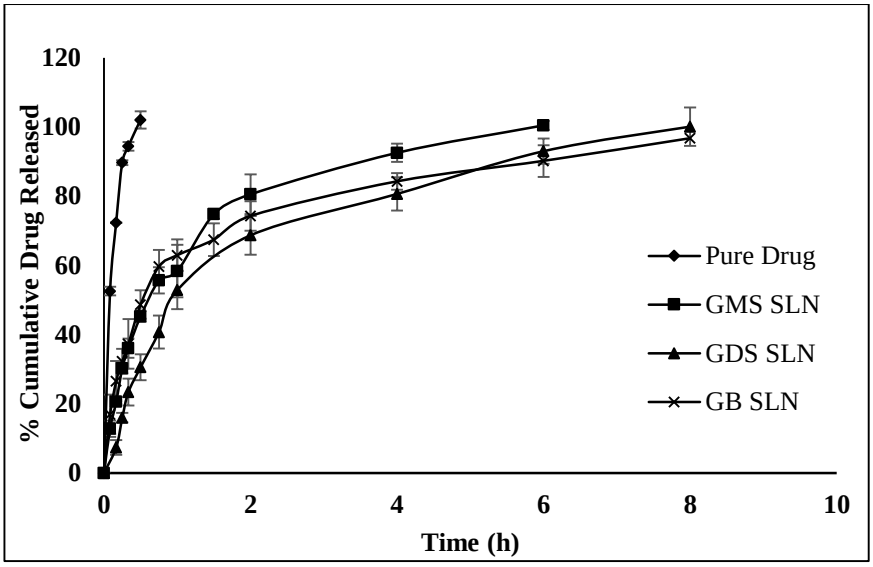


Figure 5.14. In vitro drug release profile of ganciclovir from SLNs prepared using different lipids (Each data represents average of three separate determinations)

Drug release was also affected by the type and amount of lipid matrix used in formulation of SLNs. Among the different lipids used, formulations prepared with GDS and GB displayed the most extended drug release of 8 h (Figure 5.15). The reason may be because of their longer carbon chains or greater lipophilicity as compared to GMS. This finding is in accordance with previous reports by other researchers [31, 32]. Comparative release profiles of formulations prepared with varying the concentrations of lipids used are shown in Figure 5.16. A decrease in drug release was observed with increase in concentration of lipid. Increase in lipid concentration caused increased particle size, leading to lesser surface area and thus causing slower drug release.

A significant retardation in release of ganciclovir was observed when batches were prepared using surfactant in the internal phase as compared to the batches without it (Figure 5.16). The release profiles of all such batches were compared using the model independent approach, by means of dissimilarity (*f1*) and similarity (*f2*) factors [33]. The batches with *f1* values of more than 15% and *f2* values of less than 50% indicated major difference between the release profiles (Table 5.4). Such an effect may be related to the greater entrapment of ganciclovir in the core of nanoparticles as a result of a much stronger interaction of drug with the lipid because of the presence of internal phase surfactant.

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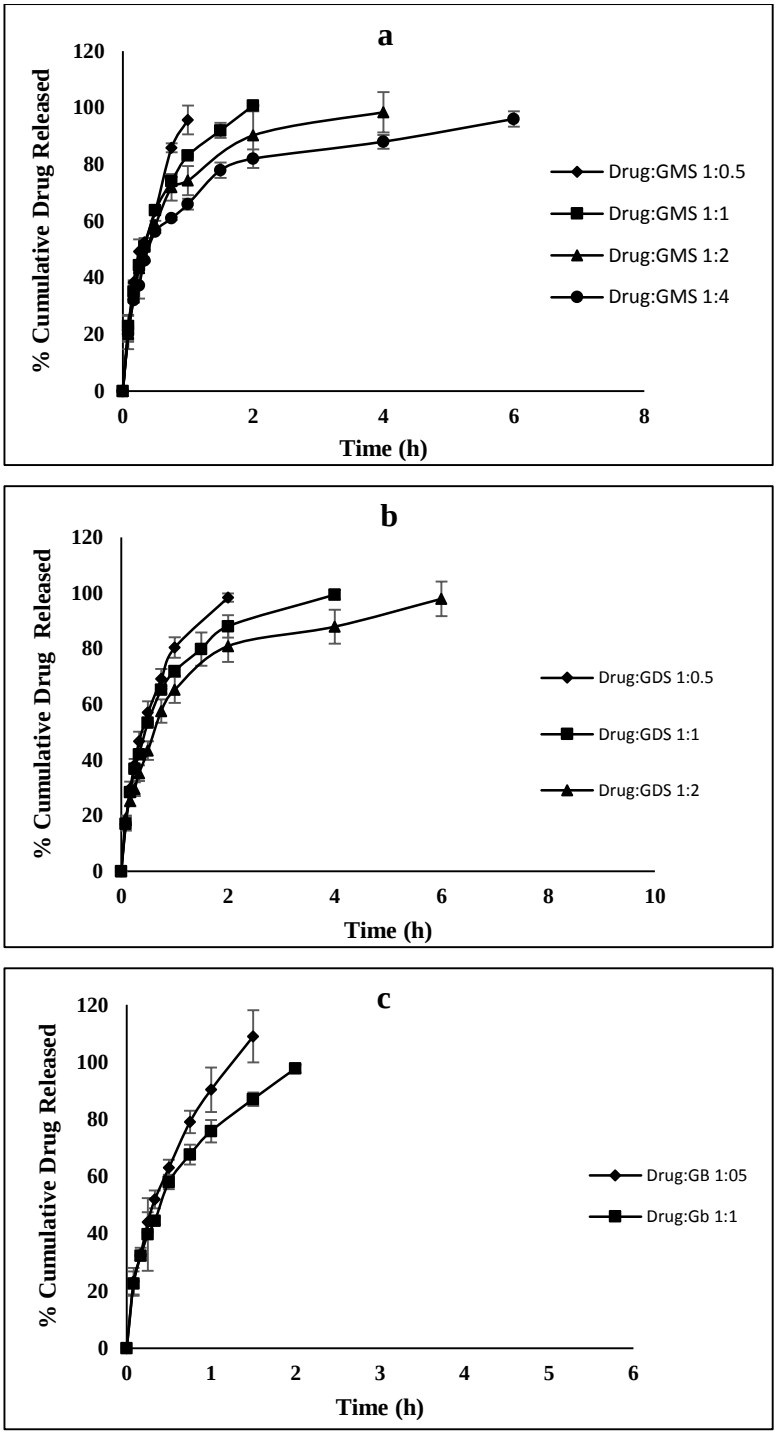

Figure 5.15. In vitro release profiles of ganciclovir from SLNs prepared by increasing amounts of lipids: a) GMS, b) GDS and c) GB. (Each data represents average of three separate determinations)

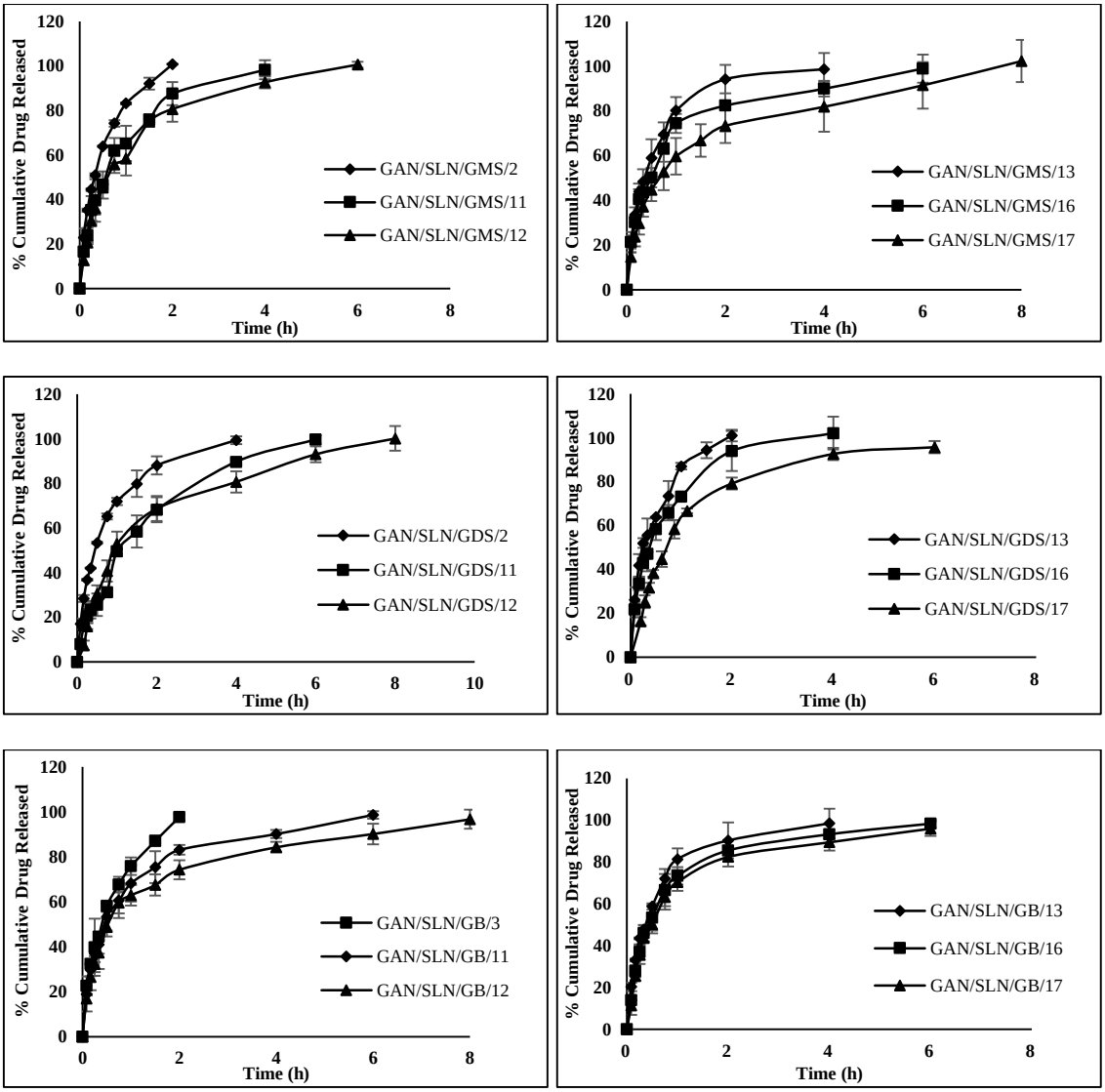

Figure 5.16. In vitro release profiles of ganciclovir from SLNs prepared by increasing amounts of internal phase surfactant. (Each data represents average of three separate determinations)

To elucidate the release kinetics, the dissolution data was fitted into various mathematical models and it was seen that the data was best fitted into the Baker-Lonsdale model. This model appropriately describes the release of drugs from spherical matrices by diffusion mechanism [34]. The release rate constant and $T_{50\%}$ values were calculated based on this best fit model while the mechanism of release was given by the 'n' value obtained on fitting the data into the Korsemeyer-Peppas model. All the kinetic parameters for the drug release from the SLNs is presented in Table 5.4. Values of 'n' in Korsemeyer-Peppas model indicated that the drug release from the nanoparticles follow Fickian

transport (n < 0.5), i.e., release was mainly because of diffusion of drug from the nanoparticulate matrix.

Table 5.4. Model dependent and model independent mathematical parameters of the in vitro release data.

Batch Code	Best fit model parameters (Baker Lonsdale)		$T_{50\%}$ (hr)	'n'value (Peppas model)	*f1*	*f2*
	k	R^2				
GAN/SLN/GMS/2	0.190	0.9903	0.290	0.408		
GAN/SLN/GMS/11	0.105	0.9882	0.525	0.339	20.28[a]	44.91[a]
GAN/SLN/GMS/12	0.083	0.9870	0.661	0.368	26.97[a]	39.00[a]
GAN/SLN/GMS/13	0.125	0.9673	0.440	0.319		
GAN/SLN/GMS/16	0.083	0.9433	0.661	0.291	9.61[b]	59.42[b]
GAN/SLN/GMS/17	0.063	0.9786	0.881	0.323	23.82[b]	42.00[b]
GAN/SLN/GDS/2	0.153	0.9723	0.360	0.466		
GAN/SLN/GDS/11	0.049	0.9549	1.126	0.505	32.80[c]	34.38[c]
GAN/SLN/GDS/12	0.046	0.9529	1.201	0.474	51.45[c]	34.18[c]
GAN/SLN/GDS/13	0.216	0.9942	0.255	0.366		
GAN/SLN/GDS/16	0.125	0.9852	0.440	0.336	12.88[d]	54.66[d]
GAN/SLN/GDS/17	0.074	0.9654	0.741	0.387	32.17[d]	34.16[d]
GAN/SLN/GB/3	0.218	0.9503	0.253	0.420		
GAN/SLN/GB/11	0.083	0.9648	0.661	0.304	11.10[e]	56.90[e]
GAN/SLN/GB/12	0.063	0.9507	0.881	0.290	33.04[e]	34.87[e]
GAN/SLN/GB/13	0.125	0.9512	0.440	0.331		
GAN/SLN/GB/16	0.083	0.9368	0.661	0.304	11.06[f]	57.34[f]
GAN/SLN/GB/17	0.083	0.9554	0.661	0.314	15.52[f]	50.76[f]

Calculated by taking references as [a]GAN/SLN/GMS/2, [b]GAN/SLN/GMS/13, [c]GAN/SLN/GDS/2, [d]GAN/SLN/GDS/13, [e]GAN/SLN/GB/3, [f]GAN/SLN/GB/13

5.3.6. Residual Solvent Analysis

Residual solvent analysis was done to quantify the amount of DCM and acetone remnant in the formulations. The solvents used in the manufacture process, if not completely removed, should be reduced to a concentration which is safe to be administered [35]. According to ICH guidelines, DCM belongs to the category of Class 2 solvents and has the maximum permissible concentration of 600 ppm, while acetone is a Class 3 solvent and a level of 5000 ppm is considered acceptable. The results of residual solvent analysis by gas chromatography yielded concentrations in the range of 205.35-208.83 ppm for DCM and 249.41-283.75 ppm for acetone, much below the permissible limits, suggesting the suitability of the solvent evaporation method utilized for the preparation of SLNs.

5.3.7. Thermal Study

DSC thermograms of the SLNs (Figure 5.17) loaded with ganciclovir reveal the presence of endothermic peaks of solid lipids (≈ 60°C for GMS, ≈ 62°C for GDS and ≈ 76°C for GB), stabilizer (≈ 50°C) and cryoprotectant sucrose (≈ 150°C). While the melting peak of ganciclovir (≈ 250°C) was present in the thermograms of all the physical mixtures (Chapter 4), the absence in the SLNs suggest that it is dispersed in the SLNs in an amorphous state. Such an effect could be attributed to the molecular level dispersion of the drug within the lipid matrix. Similar loss of crystallinity of drug when formulated into SLNs has also been seen in previous studies [36-38].

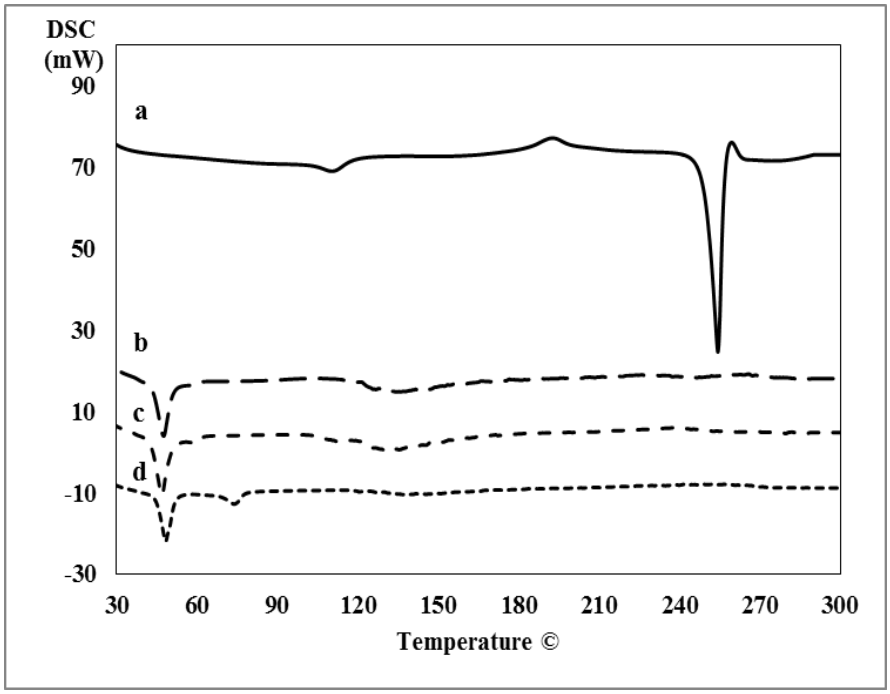

Figure 5.17. DSC thermogram obtained for a) ganciclovir b) GAN/SLN/GMS/11, c) GAN/SLN/GDS/11 and d) GAN/SLN/GB/12

5.3.8. Stability of Formulations

All the nanosuspensions were found to be stable for one week at room temperature and 3 months at refrigerated condition, with no significant change in particle size distribution and EE. Those prepared with GB showed slight aggregation by 3-4 days at room temperature and 15 days at 5°C. However, the aggregation was redispersed with ultrasonic treatment (30 s, 20W).

Freeze dried formulations stored at room temperature displayed good redispersibility, no significant difference in particle size upto 1 month of storage, but an increasing trend of particle size and decreasing trend of DL beyond 1 month was observed. This could be due to leaching out of drug from the SLN matrix due to instability of lipids at room temperature. The formulations at refrigerated temperature were found to be stable with respective to size and DL even after 6 months of storage (Table 5.5).

5.4. Conclusion

Ganciclovir loaded SLNs using triglycerides as solid lipids were successfully prepared using the double emulsion-solvent evaporation technique. Formulation parameters like amount and type of lipid and level of surfactants affected the nanoparticle characters. Optimization of these parameters was done to obtain nanoparticles of minimum size and maximum EE.

The optimized formulations depicted favourable properties like small particle size with narrow size distribution and spherical morphology. The in vitro release was also affected by the formulation parameters and was extended upto 8 h using different lipids. The freeze dried formulations showed good redispersibility and stability at refrigerated conditions. Moreover, the results of residual solvent analysis were within limits and thermal analysis revealed a molecular level dispersion of drug within the SLNs without any chemical or physical interaction between drug and excipients. This suggested the suitability of the preparation technique for formulating stable SLNs. However, EE of the formulations could be reached only upto 45-48%, posing a significant challenge for encapsulating hydrophilic drug into SLN matrix.

Specific designed formulations were selected considering the advantages of small particle size, narrow size distribution, extended release, good in vitro stability and biocompatibility. To establish the suitability of SLNs as potential carriers for better oral delivery of ganciclovir, the optimized formulations were taken further for conducting oral bioavailability and biodistribution studies in rat model.

Table 5.5 Stability of freeze dried SLNs stored at different temperature conditions (1 month and 6 months data)

Batch Code	Initial		1 month				6 months			
			25°C ± 2°C		5°C ± 3°C		25°C ± 2°C		5°C ± 3°C	
	Particle Size (nm)	DL (% w/w)	Particle Size (nm)	DL (% w/w)	Particle Size (nm)	DL (%w/w)	Particle Size (nm)	DL (%w/w)	Particle Size (nm)	DL (%w/w)
GAN/SLN/GMS/11	174.78 ± 1.79	11.47 ± 0.20	200.70 ± 7.29	10.99 ± 0.85	172.54 ± 2.83	11.23 ± 1.03	242.50 ± 5.28	8.77 ± 0.64	180.2 ± 2.18	10.94 ± 0.78
GAN/SLN/GMS/17	171.98 ± 0.93	10.55 ± 0.83	176.20 ± 5.52	9.74 ± 0.32	166.04 ± 3.63	9.94 ± 1.04	238.94 ± 2.78	8.84 ± 0.79	176.5 ± 2.56	10.13 ± 0.84
GAN/SLN/GDS/11	192.14 ± 2.75	11.52 ± 0.91	206.38 ± 3.07	9.84 ± 1.05	215.46 ± 2.29	11.05 ± 0.39	307.20 ± 3.42	9.94 ± 0.84	203.78 ± 3.23	11.04 ± 1.01
GAN/SLN/GDS/17	184.42 ± 2.01	8.80 ± 0.96	200.50 ± 2.38	7.69 ± 0.21	189.94 ± 3.81	7.98 ± 0.51	286.60 ± 8.34	6.01 ± 0.39	178.35 ± 2.21	7.54 ± 0.63
GAN/SLN/GB/12	295.20 ± 6.37	12.09 ± 0.18	347.80 ± 3.97	10.81 ± 0.89	300.42 ± 5.30	12.14 ± 1.36	382.00 ± 3.83	9.69 ± 0.12	307.00 ± 4.32	11.69 ± 0.76
GAN/SLN/GB/17	396.20 ± 6.22	10.40 ± 1.69	447.42 ± 3.95	9.97 ± 0.31	422.00 ± 10.82	10.08 ± 0.67	489.20 ± 4.81	8.18 ± 0.63	438.40 ± 9.21	9.63 ± 0.38

Each data represents the average of three independent determinations

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6. Pharmacokinetic Studies

6.1. Introduction

The goal of a drug delivery system is to provide therapeutic concentration of the drug at the site of action and maintain that concentration for intended period of time. Pharmacokinetics is an integral part of drug development as it enables to study and quantify absorption, distribution and elimination, the events that describe the availability of drug in the body for exerting its therapeutic effect. Preclinical pharmacokinetic study is performed to provide an insight into the in vivo fate of the drug and its efficacy. The suitability of the in vitro characters of the delivery systems also needs to be further substantiated using the in vivo studies. Pharmacokinetic studies in animal models is done at early stage of product development as a proof of concept and for extrapolation of pharmacokinetics in humans.

As discussed earlier, nanoparticulate systems have been found to be advantageous over conventional drug delivery systems in terms of both spatial and temporal delivery. Selective targeting, extended circulation and protection of drug molecule are some of the key benefits of nanoparticulate drug delivery systems. To estimate this altered in vivo behaviour of nanoparticulate delivery systems, preliminary pharmacokinetic studies are performed in animal model. The plasma drug concentration profile and biodistribution characters in various organs has been studied for different nanoparticulate drug delivery systems and marked modifications have been found with respect to pure drugs in several studies [1, 2]. In the present study SLNs of ganciclovir have been developed with an objective to enhance its oral bioavailability. The plasma and tissue concentrations after oral dosing of the developed SLNs were determined in rat model and compared with that for pure drug administration to study the expected performance of the designed formulations. Different pharmacokinetic parameters were determined to study the possible effect of the developed formulations on pharmacokinetic and biodistribution characters. The study was aimed at providing rationale for designing the proposed delivery system for increasing therapeutic effectiveness of ganciclovir.

6.2. Experimental

6.2.1. Materials and Instruments/Equipments

Drug and all chemicals were obtained from sources mentioned in previous chapters. Diethyl ether, purchased from Merck (Mumbai, India) was used as inhalation anesthetic. All surgical instruments like scissors, forcep, syringes and gavage needles were used

after sterilization. A Kinematica™ Polytron™ PT 1600E Benchtop Homogenizer (Thermo Fisher Scientific Inc., USA) was used for preparing tissue homogenates. A light microscope (Olympus BX41, Japan) was used to take images of intestinal sections for histopatholgy study.

6.2.2. Animals

Male Wistar rats were obtained from Hisar Agricultural University, Haryana, India. Rats were housed in standard laboratory conditions and experiments were conducted in adherence to the approved protocols of IAEC (Protocol numbers IAEC/RES/13/12, IAEC/RES/13/12/REV/15/8, IAEC/RES/13/12/REV-2/17/13 and IAEC/RES/18/15).

6.2.3. In vivo Pharmacokinetic and Biodistribution Study

Pharmacokinetic studies of single dose administration of pure drug by i.v. and oral and selected SLN formulations by oral route were carried out in rat model. The drug concentration was determined in plasma and tissues namely, brain, heart, spleen, kidney, liver and lungs to study various pharmacokinetic parameters and determine the biodistribution profile of the drug and formulations. All the procedures were followed as per the standard protocol.

The animals were divided into groups of 3 randomly and each study was performed in triplicate. Animals were fasted overnight (12-15 h) prior to study and water was given ad libitum. They were marked, weighed and dosed according to their body weight. The pure drug was administered i.v. as solution prepared in sterile water for injection. A dose of 2 mg kg^{-1} was injected via the tail vein route by restraining the upper body of the rat in a holder. Orally, the drug and SLNs dispersions, in a dose of 50 mg kg^{-1}, were administered by gavage method using an oral feeding needle [3]. The SLNs were reconstituted and used immediately for the study.

Blood samples (0.5 mL at each sampling point) were collected from the retro-orbital sinus at 5, 10, 15, 30, 45 min, followed by 1, 2, 3, 4, 6, 8 and 12 h following administration for i.v. group and at 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 12 and 24 h for oral groups The blood was collected into centrifuge tubes containing 10% EDTA as anticoagulant. Plasma was separated and transferred into clean tubes after centrifugation of blood samples at 12,000 rpm for 15 min and frozen at −20°C until analysis. After oral dosing, at sampling time points of 0.5, 1, 2, 4, 8 and 12 h, immediately after blood collection, tissues (brain, heart, kidney, spleen, liver and lungs) were collected and processed for

tissue homogenates as described in Chapter 3. Drug concentration analysis in all the plasma and tissue samples was carried out within 15 days of sample collection, using the extraction technique and the developed bioanalytical method described in Chapter 3.

6.2.4. Pharmacometric Data Analysis

The pharmacokinetic analysis of the concentration time data was performed using the non-compartmental analysis of WinNonlin software version 2.1 (Pharsight, Mountian View, CA). Data were expressed as mean ± SD. The apparent bioavailability (F) of orally administered ganciclovir and relative oral bioavailability (F_{rel}) of SLNs over pure drug was calculated by

$$F\ (\%) = \frac{AUC_{oral}}{AUC_{IV}} \times \frac{Dose_{IV}}{Dose_{oral}} \times 100$$

$$F_{rel} = \frac{AUC_{SLN}}{AUC_{pure\ drug}} \times \frac{Dose_{pure\ drug}}{Dose_{SLN}}$$

For comparison of pharmacokinetic parameters one way ANOVA followed by post hoc Bonferroni's test was employed (GraphPad Prism version 3.0) and $P<0.05$ was considered as the level of significance.

6.2.5. Histopathological Evaluation for Local Toxicity

The histopathological evaluation was carried out by an experienced histopathologist. The intestines of the control group (pure drug) and the test (SLNs treated) group were removed 4 h after oral gavage administration of drug solution, washed using saline and immersed in a 10% aqueous solution of formalin. A transverse section was prepared, stained using hematoxylin–eosin, and examined under light microscopy.

6.3. Results and Discussion

In vivo pharmacokinetic and biodistribution studies were carried out for SLNs prepared with different lipids. Formulations for pharmacokinetic studies were selected based on highest EE and particle size below 250 nm. Batches prepared with GMS, GDS and GB were tested. The batch codes, as mentioned in chapter 5, were GAN/SLN/GMS/11, GAN/SLN/GDS/11, GAN/SLN/GDS/16 and GAN/SLN/GB/12. In this chapter, these batches are coded as GMS-PF68, GDS-PF68, GDS-TPGS and GB-PF68, respectively, for ease of reference.

6.3.1. Plasma Pharmacokinetic Study

i.v. and oral pharmacokinetics of pure drug were studied in order to determine the absolute bioavailability of ganciclovir in rat model. The plasma drug concentration of ganciclovir following single i.v. (2 mg kg^{-1}) and oral (50 mg kg^{-1}) dose is given in figure 6.1 and pharmacokinetic data is listed in table 6.1. i.v. plasma profile showed a rapid decline in concentration till 2 h followed by slow elimination, with detectable plasma levels of upto 12 h, while oral profile showed rapid but low absorption with attainment of C_{max} at 0.5 h followed by a decline with detectable concentration upto 12 h.

Calculated data for AUC in i.v. administration was found to be 2591.03 ng h mL^{-1} with 2 mg kg^{-1} dose and that for oral administration of pure drug was found to be 2671.64 ng h mL^{-1} with 50 mg kg^{-1} dose. The oral bioavailability of ganciclovir calculated from this data was found to be 4.12% (F=0.0412). This value was in agreement with the reported bioavailability of ganciclovir.

Oral pharmacokinetic studies of SLN formulations also showed rapid absorption but with much higher plasma drug concentration depicting enhanced extent of absorption (Figure 6.1). The pharmacokinetic parameters generated on non-compartmental analysis of data are presented in table 6.1.

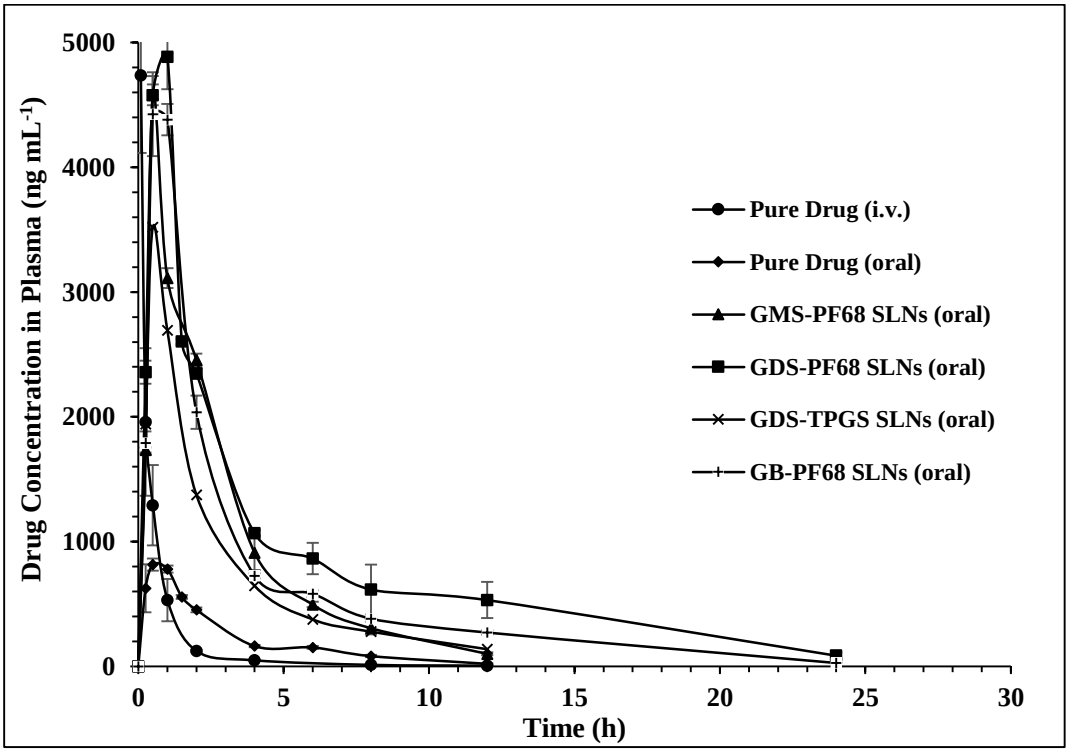

Figure 6.1. In vivo plasma profile of ganciclovir following i.v. administration of pure drug and oral administration of pure drug and SLNs.

Table 6.1. Summary of pharmacokinetic parameters of ganciclovir in plasma after i.v. and oral dosing of pure drug and SLNs in rats

Parameters	**i.v. Pure Drug (Dose 2 mg kg^{-1})**	**Oral Pure Drug (Dose 50 mg kg^{-1})**	**Oral GMS-PF68 SLNs (Dose 50 mg kg^{-1})**	**Oral GDS-PF68 SLNs (Dose 50 mg kg^{-1})**	**Oral GDS-TPGS SLNs (Dose 50 mg kg^{-1})**	**Oral GB-PF68 SLNs (Dose 50 mg kg^{-1})**
t_{max} (h)	-	0.5	0.5	1.0	0.5	0.5
C_{max} (ng mL^{-1})	-	815.32 ± 128.43	4581.92 ± 1457.45**	4886.42 ± 814.21**	3521.09 ± 351.50**	4426.28 ± 688.88**
AUC (ng h mL^{-1})	2591.03 ± 245.38	2671.64 ± 217.37	12468.51 ± 1696.16***	20149.80 ± 772.37***	9861.63 ± 782.37**	14715.98 ± 641.28***
V_d (mL kg^{-1})	0.0025 ± 0.0002	0.0023 ± 0.0010	0.0028 ± 0.0002	0.0062 ± 0.0004***	0.0046 ± 0.0004**	0.0047 ± 0.0002**
Cl (mL h^{-1} kg^{-1})	0.0008	0.0008	0.0008	0.0008	0.0008	0.0008
$t_{1/2}$ (h)	2.22 ± 0.23	2.06 ± 0.93	2.54 ± 0.20	5.56 ± 0.33***	4.11 ± 0.23**	4.21 ± 0.18**
MRT (h)	0.96 ± 0.13	3.23 ± 0.62	3.16 ± 0.37	6.47 ± 0.83**	4.19 ± 0.03*	4.57 ± 0.08*
F (%)	-	4.12 ± 1.4	19.33 ± 2.62***	31.11 ± 1.19***	15.22 ± 2.45**	22.72 ± 1.00***

All the values are expressed as mean ± SD (n=3). *P<0.05, **P<0.01 and *** P<0.001 as compared with the oral pure drug group; one way ANOVA followed by Bonferroni's test for multiple comparison.

Oral administration of GMS-PF68 SLNs showed significant increase in C_{max} and AUC to 4581.92 ± 1457.45 ng mL^{-1} and 12468.51 ± 1696.16 ng h mL^{-1}, respectively, indicating improved absorption of ganciclovir as compared to the pure drug administration. Similarly, oral administration of GDS SLNs formulated with PF-68 with stabilizer resulted in 6 times increase in C_{max} and 7.5 times increase in AUC and of ganciclovir as compared to pure drug. However, the increase in C_{max} and AUC was only 4.3 and 3.7 times, respectively, when GDS SLNs were formulated with TPGS as stabilizer. On administration of GB-PF68 SLNs the C_{max}, AUC and relative bioavailability were increased by about 5.5 times when compared to pure drug. The bioavailability was calculated for every individual formulation against the pharmacokinetic data of i.v. administration of pure drug (Table 6.1). It was seen that the relative bioavailability of GMS-PF68, GDS-PF68, GDS-TPGS and GB-PF68 was enhanced by about 5, 8, 4 and 5.5 times, respectively, as compared to pure drug. This enhanced bioavailability may be attributed to the increased absorption of ganciclovir due to transport of the nanocarrier system through M cells of Peyer's patches, which in turn bypasses the effect of P-gp efflux. Additionally, SLNs have also been seen to be absorbed by chylomicron formation via the lymphatic pathway [2, 4-6].

It was also observed that the V_d, $t_{1/2}$ and mean residence time (MRT) of ganciclovir were increased in case of SLNs as compared to pure drug administration. The change in V_d is suggestive of an altered distribution of ganciclovir, while an increased $t_{1/2}$ and MRT in comparison to pure drug may be due to extended residence in blood and prolonged release property of the formulations. If a sustained release formulation is administered and the $t_{1/2}$ is found to be longer than that after an i.v. dose of the compound, it is the apparent $t_{1/2}$ which is measured. The rate of decline of the drug plasma concentration is not only due to elimination, but other factors such as absorption rate or distribution rate influence the plasma concentration decay. $t_{1/2}$ and MRT of ganciclovir were more prolonged in case of GDS and GB SLNs, which is consistent with the more extended release property of these formulations as compared to GMS SLNs, as discussed in Chapter 5. The difference in pharmacokinetic parameters and distribution profile of different batches SLNs are expected due to difference in physicochemical properties of the three different carriers which alter the properties of the SLNs.

6.3.2. Tissue Biodistribution Study

The biodistribution of ganciclovir was studied after administration as pure drug and SLNs, in various highly perfused organs: brain, heart, spleen, kidney, liver and lungs. The drug biodistribution profiles in all tissues except brain showed a biphasic pattern for both pure drug and SLNs, with achievement of t_{max} in 0.5 h and rapid elimination followed by slow elimination with detectable levels till 12 h post dosing (Figure 6.2). The pharmacokinetic parameters in the tissues are given in Table 6.2.

An altered distribution profile of ganciclovir was seen in various organs when administered as SLNs. Ganciclovir, when given as SLNs, was found to distribute more in brain, heart and lungs, while less uptake was seen in liver, spleen and kidney as compared to pure drug administration. These findings also explain the enhanced values of V_d.

Biodistribution studies of pure drug after oral administration showed slower and poor permeation of ganciclovir to the brain with C_{max} of 94.76 ng g^{-1} achieved in 2 h post dosing and AUC of 771.04 ng h g^{-1}. The C_{max} and AUC were found to increase on administration of SLNs. After oral administration of GMS-PF68 SLNs, the C_{max} was increased to 104.43 ng g^{-1} and AUC obtained was 968.66 ng h g^{-1}. GDS-PF68 SLNs yielded the maximum increase in C_{max} and AUC values to 136.15 ng g^{-1} and 1401.87 ng h g^{-1}, respectively, while only a slight increase as compared to pure drug was seen in case of GDS-TPGS SLNs. Studies with GB-PF68 SLN formulations also resulted in a significant increase in C_{max} to 123.73 ng g^{-1} and AUC to 1255.05 ng h g^{-1} in brain. The enhanced permeation into brain by SLNs is seen due to the enhanced ability of the lipophilic nanocarrier coated with hydrophilic surfactant to cross the BBB and is consistent with previous reports by other research groups [7-10].

Ganciclovir concentration in heart after pure drug administration was found to be more than that found in brain and C_{max} of 383.59 ng g^{-1} was achieved in 0.5 h. Significant increase in C_{max} by 3-4 times and AUC values by 2-fold were observed on oral administration of all SLN formulations (Table 6.2). Similar trend was observed in biodistribution of ganciclovir in lungs. The C_{max} in case of pure drug was 2264.2 ng g^{-1}, which was significantly increased by about 1.5 times in GMS-PF68 SLNs, and 2 times in case of GDS-PF68, GDS-TPGS and GB-PF68 SLNs. The AUC values were also increased significantly as compared to the pure drug. GDS and GB SLNs also showed

increase in $t_{1/2}$ in lungs indicating their long circulating character. This increased uptake of SLNs in brain, heart and lungs may result in possible increased efficacy of ganciclovir in cases of CMV encephalitis, endocarditis and pneumonitis, respectively, which are the severe manifestations of the end stage CMV disease.

Biodistribution studies in RES organs, liver and spleen, were also performed and it was found that drug concentration levels in these organs after administration as SLNs were not as high as that reached after pure drug administration. Pure drug produced a C_{max} of 2293.94 ng g^{-1} in liver, which was nearly similar in case of GMS-PF68 SLNs. However, a significant reduction in C_{max} (2293.94 to 1305.69 and 860.26 ng g^{-1}) was observed in GDS-PF68 and GDS-TPGS SLNs. The C_{max} was further reduced to 820.09 ng g^{-1} in case of GB-PF68 SLNs. A significant reduction in AUC values in liver was also seen in all the SLN formulations administered, when compared with pure drug administration. Biodistribution studies in spleen revealed a significant reduction in both C_{max} and AUC values of ganciclovir attained after oral administration of SLNs, as compared to those obtained with pure drug (Table 6.2). Absence of enhanced distribution to liver and spleen may be indicative of the escape of nanoparticles from the phagocytic uptake of the RES due to the stealth property of the surfactants PF-68 and TPGS [11, 12].

Among all the organs studied, pure drug ganciclovir was found to distribute the most in kidney, with C_{max} of 4683 ng g^{-1} and AUC of 31202.34 ng h g^{-1}. A decreased uptake of ganciclovir in kidney was observed with significant ($P<0.001$) reduction in AUC values, in case of SLNs. Since renal excretion is the primary route of elimination of ganciclovir, a reduced distribution towards kidney suggests decreased excretion, thus supporting the increased $t_{1/2}$ and MRT of ganciclovir in plasma on administration as SLNs. It is known that the surface properties of nanoparticles contribute to their in vitro and in vivo performance, including their ability to traverse biological membranes. Hence, a modified distribution pattern of ganciclovir was observed when administered as SLNs.

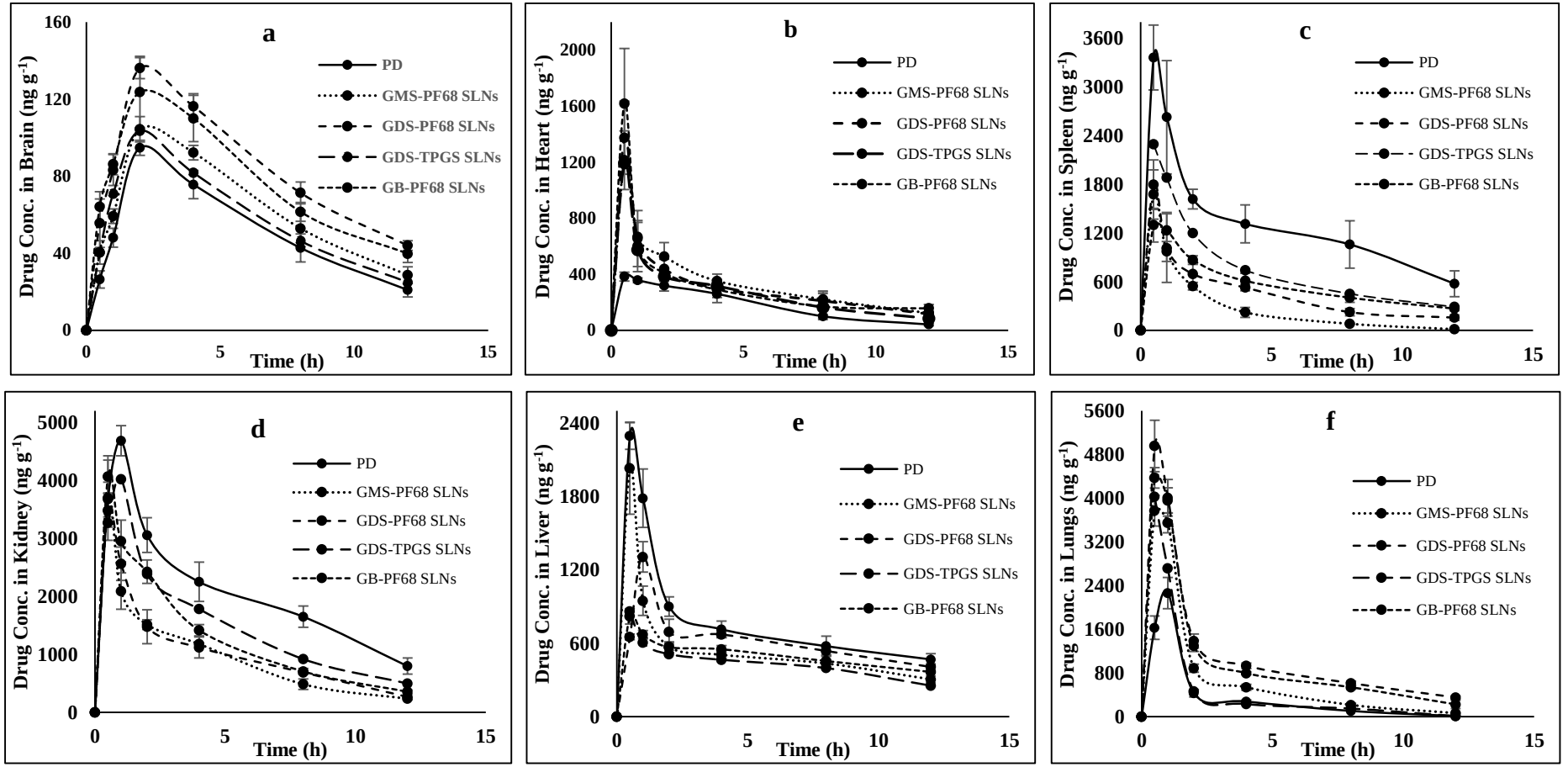

Figure 6.2. Biodistribution profile of ganciclovir following single oral dose of pure drug (PD) and SLNs in tissues: a) brain, b) heart, c) spleen, d) kidney, e) liver and f) lungs.

Table 6.2. Summary of pharmacokinetic parameters of ganciclovir in different tissues after oral dosing of pure drug and SLNs in rats

Tissue	Pharmacokinetic Parameters	Treatment				
		PD	**GMS-PF68 SLNs**	**GDS-PF68 SLNs**	**GDS-TPGS SLNs**	**GB-PF68 SLNs**
Brain	t_{max} (h)	2.0	2.0	2.0	2.0	2.0
	C_{max} (ng g^{-1})	94.76 ± 3.94	104.43 ± 6.59	136.15 ± 18.61**	103.55 ± 27.49	123.73 ± 5.51*
	AUC (ng h g^{-1})	771.04 ± 41.77	968.66 ± 98.39*	1401.87 ± 72.01***	877.24 ± 125.38	1255.05 ± 54.05***
	$t_{1/2}$ (h)	4.57 ± 0.39	4.79 ± 0.83	6.01 ± 0.79	4.67 ± 0.18	5.51 ± 0.46
	MRT (h)	7.41 ± 0.60	7.90 ± 1.10	9.43 ± 1.18	7.51 ± 0.47	8.79 ± 0.65
Heart	t_{max} (h)	0.5	0.5	0.5	0.5	0.5
	C_{max} (ng g^{-1})	383.59 ± 32.42	1214.74 ± 210.22*	1374.92 ± 260.75**	1196.22 ± 78.52*	1618.832 ± 392.63**
	AUC (ng h g^{-1})	2409.39 ± 42.10	4941.45 ± 552.49***	4858.02 ± 234.64**	3907.05 ± 521.48*	4994.30 ± 832.68***
	$t_{1/2}$ (h)	3.21 ± 0.96	4.79 ± 0.91	6.20 ± 2.23	4.24 ± 0.29	5.63 ± 0.90
	MRT (h)	5.05 ± 0.79	6.66 ± 1.35	7.98 ± 2.66	5.75 ± 0.59	7.41 ± 0.77
Spleen	t_{max} (h)	0.5	0.5	0.5	0.5	0.5
	C_{max} (ng g^{-1})	3363.25 ± 399.91	1675.09 ± 303.75***	1374.92 ± 305.49***	2293.50 ± 237.58**	1296.91 ± 206.22***
	AUC (ng h g^{-1})	20322.29 ± 4303.27	3449.96 ± 214.46***	6527.05 ± 128.02***	11385.64 ± 682.38***	9251.91 ± 446.01***
	$t_{1/2}$ (h)	5.90 ± 0.29	1.94 ± 0.79***	4.43 ± 0.81	5.86 ± 1.48	6.16 ± 0.94
	MRT (h)	8.40 ± 0.58	2.64 ± 0.07***	6.09 ± 1.36	7.51 ± 1.26	8.74 ± 1.27

Continued……

Table 6.2. Continued..

Tissue	Pharmaco-kinetic Parameters	Treatment				
		PD	**GMS-PF68 SLNs**	**GDS-PF68 SLNs**	**GDS-TPGS SLNs**	**GB-PF68 SLNs**
Kidney	t_{max} (h)	1.0	0.5	0.5	1.0	0.5
	C_{max} (ng g^{-1})	4683.00 ± 260.60	3694.03 ± 729.74	3480.77 ± 289.64*	4021.11 ± 253.53	4067.46 ± 283.36
	AUC (ng h g^{-1})	31202.34 ± 1102.62	12817.89 ± 378.39***	13925.06 ± 1500.35***	21351.47 ± 483.64***	17687.54 ± 822.39***
	$t_{1/2}$ (h)	5.37 ± 1.39	3.55 ± 0.26*	3.67 ± 0.32	4.38 ± 0.62	3.96 ± 0.06
	MRT (h)	7.69 ± 1.75	4.90 ± 0.18*	5.28 ± 0.33*	6.13 ± 1.26	5.34 ± 0.17*
Liver	t_{max} (h)	0.5	0.5	1.0	0.5	0.5
	C_{max} (ng g^{-1})	2293.94 ± 108.90	2030.58 ± 375.74	1305.69 ± 124.56**	860.26 ± 48.56***	820.09 ± 62.46***
	AUC (ng h g^{-1})	18059.64 ± 2370.52	11469.44 ± 1663.14**	13751.43 ± 829.82*	8747.72 ± 435.72**	13786.12 ± 1443.85*
	$t_{1/2}$ (h)	13.16 ± 1.83	11.32 ± 2.40	10.93 ± 0.48	9.82 ± 1.38	14.78 ± 3.16
	MRT (h)	17.51 ± 2.53	15.04 ± 2.95	15.75 ± 0.90	13.81 ± 2.48	19.63 ± 4.55
Lungs	t_{max} (h)	1.0	0.5	0.5	0.5	0.5
	C_{max} (ng g^{-1})	2264.2 ± 285.19	3771.12 ± 271.62**	4955.98 ± 472.44***	4027.49 ± 276.36	4372.87 ± 186.07***
	AUC (ng h g^{-1})	4457.79 ± 296.90	8736.08 ± 440.40***	16285.84 ± 391.27***	6069.76 ± 365.25	13602.39 ± 1477.65***
	$t_{1/2}$ (h)	1.73 ± 0.34	2.66 ± 0.25	5.44 ± 0.53***	1.51 ± 0.23	4.43 ± 1.09**
	MRT (h)	2.41 ± 0.14	2.88 ± 0.12	6.31 ± 0.66***	2.05 ± 0.16	4.99 ± 1.11**

All the values are expressed as mean ± SD (n=3). *P<0.05, **P<0.01 and *** P<0.001 as compared with the pure drug group; one way ANOVA followed by Bonferroni's test for multiple comparison.

6.3.3. Histopathological Evaluation for Local Toxicity

Photomicrographs of the intestinal mucosa of rat exposed to pure drug ganciclovir and SLNs are shown in Figure 6.3. As indicated, the epithelium of each group was undamaged, and the villus structure was intact. There was no significant difference in arrangement and structure of nuclei and cells between ganciclovir pure drug and ganciclovir loaded SLNs, indicating that SLNs have no significant immediate local toxicity in the intestinal tract.

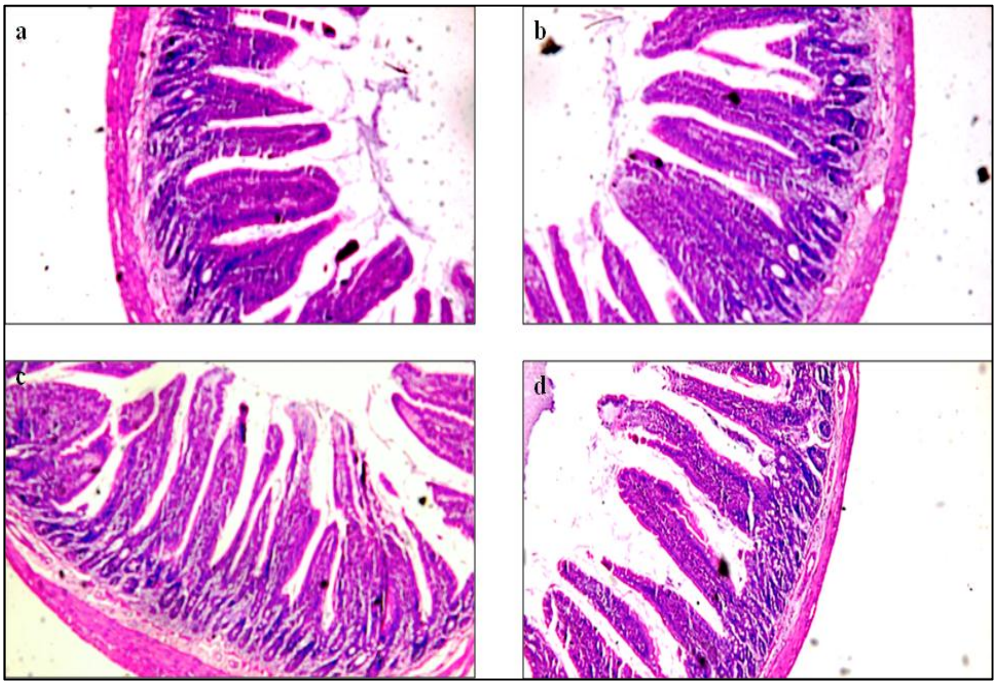

Figure 6.3. Photomicrographs of rat intestine at 4 h after oral gavage administration of a) pure drug, b) GMS SLNs, c) GDS SLNs and d) GB SLNs at dose of 50 mg kg^{-1}. (original magnification ×10).

6.4. Conclusion

Pharmacokinetic study revealed that ganciclovir had a low oral bioavailability of approximately 4% when administered as pure drug solution, with pharmacokinetic parameters in good agreement with that obtained after i.v. dosing. The SLNs demonstrated an enhanced bioavailability of ganciclovir, with significantly altered pharmacokinetic and biodistribution profile on oral administration as compared to pure drug. SLNs showed prolonged residence time in plasma and a higher V_d and $t_{1/2}$ than pure drug. Biodistribution studies illustrated that ganciclovir is distributed more towards brain, heart and lungs when administered as SLNs and less towards liver, spleen and

kidneys as evident from the C_{max} and AUC values. The histological evaluation depicted no change in architecture of cells showing absence of local toxicity of SLNs on the intestine.

Thus, the pharmacokinetic studies confirmed that SLNs are suitable carriers for effective oral delivery of drugs which may address to drug-specific limitations like poor bioavailability. The biodistribution profile can also be modulated to achieve an enhanced therapeutic efficacy.

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7. Conclusions and Future Perspectives

7.1. Conclusions

In the present research work, solid lipid nanoparticulate drug delivery systems were designed for better oral delivery of ganciclovir, a highly water soluble but poorly bioavailable antiviral agent. Formulations were prepared using different solid lipids (GMS, GDS and GB) and surfactants (PF-68 and TPGS) as excipients employing a double emulsion method.

For quantitative analysis of the drug, spectrometric and liquid chromatographic methods were developed and validated separately. The UV spectrophotometric, spectrofluorimetric and RP-HPLC analytical methods were found to be simple, selective, accurate, precise and robust. The methods were successfully used for the preformulation studies and in vitro evaluation of developed formulations. The developed RP-HPLC bioanalytical method was sensitive, accurate, precise, stability indicating and showed no interference of biomatrices in the analysis. It was successfully employed for estimation of ganciclovir in biological samples like plasma and tissue homogenates collected during in vivo pharmacokinetic and biodistribution studies.

Preformulation studies indicated that ganciclovir has a pH-dependent solubility, with greater solubility at extreme pH conditions. The poor solubility of ganciclovir in organic solvents and very low apparent partition coefficient at all pH values confirmed its highly hydrophilic nature. In liquid state, ganciclovir was found to be stable, with maximum stability at higher pH conditions. Solid state stability and drug-excipient compatibility studies revealed that ganciclovir was stable alone and in presence of excipients used throughout the study period. The absence of interaction with the excipients indicated their suitability of use in formulations.

Ganciclovir loaded SLNs were prepared using a double emulsion solvent evaporation technique and the effect of various formulation variables was studied. It was observed that nanoparticle characters like average particle size and distribution, drug content, entrapment efficiency and release pattern were dependent on these formulation variables.

The optimized formulations depicted the desired characters of low particle size, in the range of 140-170 nm in case of GMS and GDS SLNs and 250-340 nm in case of GB SLNs and entrapment efficiencies in the range of 35-48%. In vitro drug release was extended upto 8 h and the release profile was explained by the Baker-Lonsdale model for spherical particles. Morphological examination by SEM and TEM displayed

homogenous solid, spherical and non-porous particles. The formulations depicted good redispersibility after lyophilization and presence of residual solvents in the formulations within the prescribed limits suggested suitability of the preparation technique. Freeze-dried formulations were found to be stable in terms of particle size and drug loading even after 6 months of storage at refrigerated conditions.

Results of the in vivo pharmacokinetics and biodistribution studies in rat yielded a multi fold increase in oral bioavailability of ganciclovir and an altered distribution character when administered as SLNs. The absolute bioavailability of ganciclovir was increased from approximately 4% to 16-32% in case of SLNs. The increased half-life and MRT of SLNs indicated prolonged circulation of the nanoparticles in the blood and body. Pharmacokinetic parameters and distribution profile were found to be different for different batches of SLNs due to difference in physicochemical properties of the three different carriers, thereby altering the properties of the SLNs. An increased uptake of SLNs was seen in brain, heart and lungs which may prove beneficial in severe complications of the infection like encephalitis, endocarditis and pneumonitis. Liver, spleen and kidneys showed reduced uptake of nanoparticles probably due to the stealth effect of the hydrophilic coating of surfactants. Modified distribution can be used for targeting specific organ with low dose and no or less side effects. Histology of the rat intestine exposed to SLNs did not reveal any abnormality in structure of cells. Visual monitoring of animals post dosing also did not show any undesirable effect.

Collectively, these results indicate that SLNs are promising delivery systems to be developed to enhance the oral bioavailability of ganciclovir, so that the dose of the current therapy can be reduced, the inconvenience of i.v. administration can be avoided and overall the patient compliance be improved.

7.2. Future Perspectives

The major challenge which still remains with the formulation of SLNs incorporating hydrophilic drug is achieving good EE. Further work is needed to improve the EE and decrease the amount of lipid and thus the formulation cost. SLNs coated with enteric polymers could be designed for further enhancing the oral bioavailability. Extensive in vitro cell uptake and in vivo studies could be done to establish the exact mechanism of enhanced absorption of nanoparticles. More detailed and multiple dosing pharmacokinetic studies including toxicology studies can be done to determine possible

side effects. Effect of particle size of formulations on the distribution profile can also be studied. Scale up of optimized batches could be done to check the potential scope for commercialization of these formulations. Further clinical studies in human subjects need to be done to establish the expected benefits of the delivery system. Similar studies can also be done on other hydrophilic drugs of different categories.

Appendix

List of Publications and Presentations

Publications

1. Garima Balwani, Emil Joseph, Satish Reddi, Vibhu, Ranendra N Saha., 2013. Rapid, Simple and Sensitive Spectrofluorimetric Method for the Estimation of Ganciclovir in Bulk and Pharmaceutical Formulations. *Journal of Spectroscopy*. Volume 2013, Article ID 972806
2. G. Balwani, E. Joseph, S. Reddi, V. Nagpal, R. N. Saha. Studies on Effect of TPGS on Absorption of Ganciclovir in Rats. 2013 *AAPS Annual Meeting and Exposition*, Abstract M1091.
3. Garima Balwani, Emil Joseph, Pooja Makhija and Ranendra Narayan Saha. Ganciclovir-Loaded Solid Lipid Nanoparticles Prepared with TPGS as stabilizer – Formulation, Characterization and In Vitro Drug Release. *Pharmaceutica Analytica Acta*, Volume 6 (1): 102.
4. Garima Balwani and Ranendra N Saha., A Stability Indicating HPTLC Method for Quantitative Estimation of Ganciclovir in Bulk, Pharmaceutical Formulations and Compatibility Studies. *Communicated.*

Paper Presentations

1. Balwani G., Makhija P., Joseph E., Saha R N. Design and Optimization of Solid Lipid Nanoparticles for Oral Delivery of Ganciclovir. 14th International Symposium of the Controlled Release Society-Indian Chapter (CRS-IC), Mumbai, 2015.
2. Garima Balwani, Pooja Makhija, Ranendra N. Saha. Development of UV Spectrophotometric Method for Ganciclovir and its Application in Preformulation Studies and Estimation in Marketed and In-house Developed Formulations. 65th Indian Pharmaceutical Congress (IPC), Delhi NCR, 2013
3. G. Balwani, R. N. Saha. Development and Validation of RP-HPLC Method for Estimation of Ganciclovir in Bulk and Formulations. 7th AAPS-NUS PharmSci@Asia Symposium, Singapore, 2012
4. G. Balwani, R. N. Saha. Development and Validation of Spectrofluorimetric Method for Estimation of Ganciclovir in Bulk and Formulations. 7th AAPS-NUS PharmSci@Asia Symposium, Singapore, 2012

5. Balwani G., Gupta A., Saha R N. Studies on Effect of Excipients on Release of Stavudine from Controlled Release Tablets Prepared by Melt Granulation. 12th CRSIC International Symposium, Mumbai, 2012
6. Karthikeyan V, Sekar Vasantha Kumar, Garima Balwani, R N Saha. Formulation and Evaluation of Immediate Release Tablets of Risperidone Using Superdisintegrant-Croscarmellose Sodium. 62nd Indian Pharmaceutical Congress, Manipal, India, 2010

Book Chapter

1. Saha R.N., Balwani Garima and Vibhu. Transmucosal Drug Delivery: Potential Role of Nanoparticles, in NanoBiomedicine: Volume 6 on Drug Nanocarriers, Studium Press LLC, USA (*In Press*)

Biography of Prof. Ranendra N. Saha

Dr. Ranendra Narayan Saha is Shri B K Birla & Shrimati Sarala Birla Chair Professor (Senior Professor of Pharmacy) and Director of BITS Pilani-Dubai Campus. In 2011, he has been awarded Shri B. K. Birla and Shrimati Sarala Birla Chair Professorship at BITS Pilani for contributions in teaching and research. He has vast experience in the field of Pharmacy especially in Pharmaceutics, Novel Drug Delivery Systems and Pharmacokinetics. He received "Pharmacy Professional of the Year 2013" award given by Indian Association of Pharmaceutical Scientists and Technologists. He is also recipient of "The Best Pharmacy Teacher Award" for the year 2005, awarded by Association of Pharmaceuticals Teachers if India (APTI), in recognition of his contribution in teaching and research in the field of Pharmacy. He has more than 33 years of teaching, research and administrative experience. In his research career, he has successfully completed several projects funded by industries and government funding agencies. He is on advisory board and selection committee member of a number of Universities in India and abroad. He has published more than 90 papers in international, national journals and presented more than 100 papers in Conferences/ Seminars/ Symposiums. He has also delivered several invited lectures at Seminars and Conferences, Universities in India and abroad.

Biography of Garima Balwani

Ms. Garima Balwani has completed her Bachelor of Pharmacy (B. Pharm.) from Delhi Institute of Pharmaceutical Sciences and Research, New Delhi in the year 2008 and Master of Pharmacy (M. Pharm., Pharmaceutics) from BITS Pilani, Pilani Campus in 2010. She has been working as a research scholar in Department of Pharmacy at BITS Pilani, Pilani Campus from 2010 to 2015. She is the recipient of the INSPIRE fellowship from Department of Science and Technology (DST), Government of India, awarded to top rank holders in the postgraduate degree to pursue doctoral research. She has published research articles in well renowned journals and presented papers in national and international conferences.