APPLICATION OF CASSETTE DOSING TO INCREASE THE THROUGHPUT OF PHARMACOKINETIC SCREENING

THESIS

Submitted in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

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2002

Those moments shared with Dr. Madhu Dikshit, Dr. D. P. Sahu, Dr. P. K. Srivastava, Dr. V. L. Sharma, Dr. V.K. Sharma, Dr. A. K. Dwivedi, Dr. R. Pal, Dr. Amit Mishra and Dr. Saman Habib will always be treasured.

The unflagged friendship of my well wishers and friends, especially Chitra, Sajeev, N. Sridhar, Pravin, ARS, AAVI, Subbu, Venku, Preeti, Basha, Becket, Munvar, STVS, Jagan, Phillip, Satya, Vijay, Kiran Babu, Murthy, Pavan, Pushkar, Rajesh, Vishal and Amrita will be a nostalgia for me.

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CERTIFICATE

This is to certify that the thesis entitled **APPLICATION OF CASSETTE DOSING TO INCREASE THE THROUGHPUT OF PHARMACOKINETIC SCREENING** and submitted by **Rajanikanth M** ID. No. **2000PHXF006** for award of Ph. D Degree of the Institute, embodies original work done by him under my supervision.

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Date: 7.10.2002

ABBREVIATIONS USED

I	- 1-(4-propionyl phenoxy)-3-[N^4-3',4'-dimethoxyphenyl)-piperazinyl]-N^1-propan-2-ol
II	- 1-(phenoxy)-3-[N^4-phenyl piperazinyl]-N^1-propane
III	- 1-(2-isopropyl phenoxy)-3-[N^4-(2'-methylphenyl)-piperazinyl-N^1-propan-2-ol
AAG	- α-acidglycoprotein
ADME	- absorption-distribution-metabolism-excretion
ANOVA	- Analysis of Variance
APCI	- Atmospheric Pressure Chemical Ionization
API	- Atmospheric Pressure Ionization
AUC	- area under the curve
AUMC	- area under the first moment curve
BSA	- bovine serum albumin
C	- concentration
C_{max}	- Maximum concentration achieved upon oral administration
C_0	- concentration at time 0 min
C_t	- concentration at time 't'
CDRI	- Central Drug Research Institute
CL	- clearance
CN	- Cyano
CS	- Calibration Standard
CTA	- Clinical Trial Application
CV	- Coefficient of Variation
CYP	- cytochrome
CYP3A	- sub family of CYP
DMPK	- Drug Metabolism and Pharmacokinetics
DMSO	- dimethyl sulfoxide
ESI	- Electrospray Ionization
ESI/MS	- Electrospray ionization-Mass Spectrometer (y)
EST	- Estrogen sulphotransferase
F	- bioavailability (fraction absorbed)

FDA	- Food and Drug Administration
Flu	- Fluorescence
g	- gram
g	-centrifugal force
GC	- Gas Chromatography
G.I.T	- gastrointestinal tract
GST	- Glutathione-S-Transferase
h	- hour
HPLC	- high performance liquid chromatography
HTPK	- High Throughput Pharmacokinetics
HTS	- High Throughput Screening
I. C. H.	- International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
I.D.	- inner diameter
IND	- investigational new drug
IPA	- Isopropyl alcohol
I.S	- internal standard
i.v.	- intravenous
k_a	- absorption rate constant
k_{dis}	- disappearance rate constant
k_e	- elimination rate constant
k_m	- metabolic rate constant
LC	- Liquid Chromatography (same as HPLC)
LC/MS	- Liquid Chromatography-Mass Spectrometer
LC/MS/MS	- Liquid Chromatography-Tandem Mass Spectrometer
LLOQ	- lowest limit of quantitation
LOD	- Limit of Detection
LOQ	- Limit of Quantitation
MAA	- Marketing Authorization Application
MAT	- mean absorption time
MPS	- Mobile Phase Standard
MRM	- Multiple Reaction Monitoring
MRT	- mean residence time
MS	-Mass Spectrometer (y)

MS/MS	-Tandem Mass Spectrometer (y)
MSS	- Mixed Stock Solution
min	- minute
mg	- milligram
ml	- milliliter
mM	- millimolar
N/n	- number
NADP	- nicotinamide adenine diphosphate
β-NADPH	- reduced nicotinamide adenine diphosphate
NCE	- new chemical entity
NDA	- new drug application
NRS	- Normal Rat Serum
ng	- nanogram
nm	- nanometer
O.D.	- Outer diameter
OPA	- ortho phosphoric acid
PDA	-Photo Diode Array
PST	- Phenol sulphotransferase
PEG	- polyethylene glycol
pH	- negative logarithm of hydrogen ion concentration
PK	- pharmacokinetics
pK_a	- negative logarithm of dissociation rate constant
p.o.	- per oral
Q. C	- Quality Control
RBC	- red blood cells
RPLC	- reversed phase liquid chromatography
RSD	- relative standard deviation
S9	- supernatant of 9000 x g centrifuged liver homogenate
SIR	- Selected Ion Recording
SOP	- Standard Operating Procedures
s.d	- standard deviation
sec	- second
TOF	- Time of Flight

t	- time
$t_{1/2}$	- half life
t_{max}	- time of maximum concentration
UDP	- Uracil di phosphate
UGT	- UDP-dependent glucuronosyl transferase
USP	- United States Pharmacopoeia
UV	- ultraviolet
V_d	- volume of distribution
V_{ss}	- steady state volume of distribution
v/v	- volume by volume
WSS	- Working Standard Solution

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Chapter 1

INTRODUCTION

1.1 New Drug Discovery and Development

1.1.1 Introduction

In the face of constant threat in endangerment of the species against the wrath of disease, man has been surviving by searching for drugs against the diseases and the search goes on and on till the diseases which manifest in one form or the other are wiped out. The events following the administration of the drug can be divided into two phases: a) a pharmacokinetic phase, in which adjustable elements of dose, dosage form, frequency and route of administration are related to drug level-time relationships and b) a pharmacodynamic phase in which the concentration of the drug at the site(s) of the action is related to the magnitude of the effect produced (1). Magnitude of both desired response and toxicity are function(s) of drug concentration at the site(s) of action. Any chemical entity can qualify as a drug when it posses the desired attributes of bioavailability, potency, chemical tractability and selectivity (2). In highlighting the importance of pharmacokinetics in drug development and therapy, Kaplan (3) has rightly said, "what body does to the drug is equally important to what drug does to the body". Awareness of the benefits of understanding pharmacokinetics and concentration-response relationship has lead to extensive application of such information by the pharmaceutical industry to drug design, selection and development.

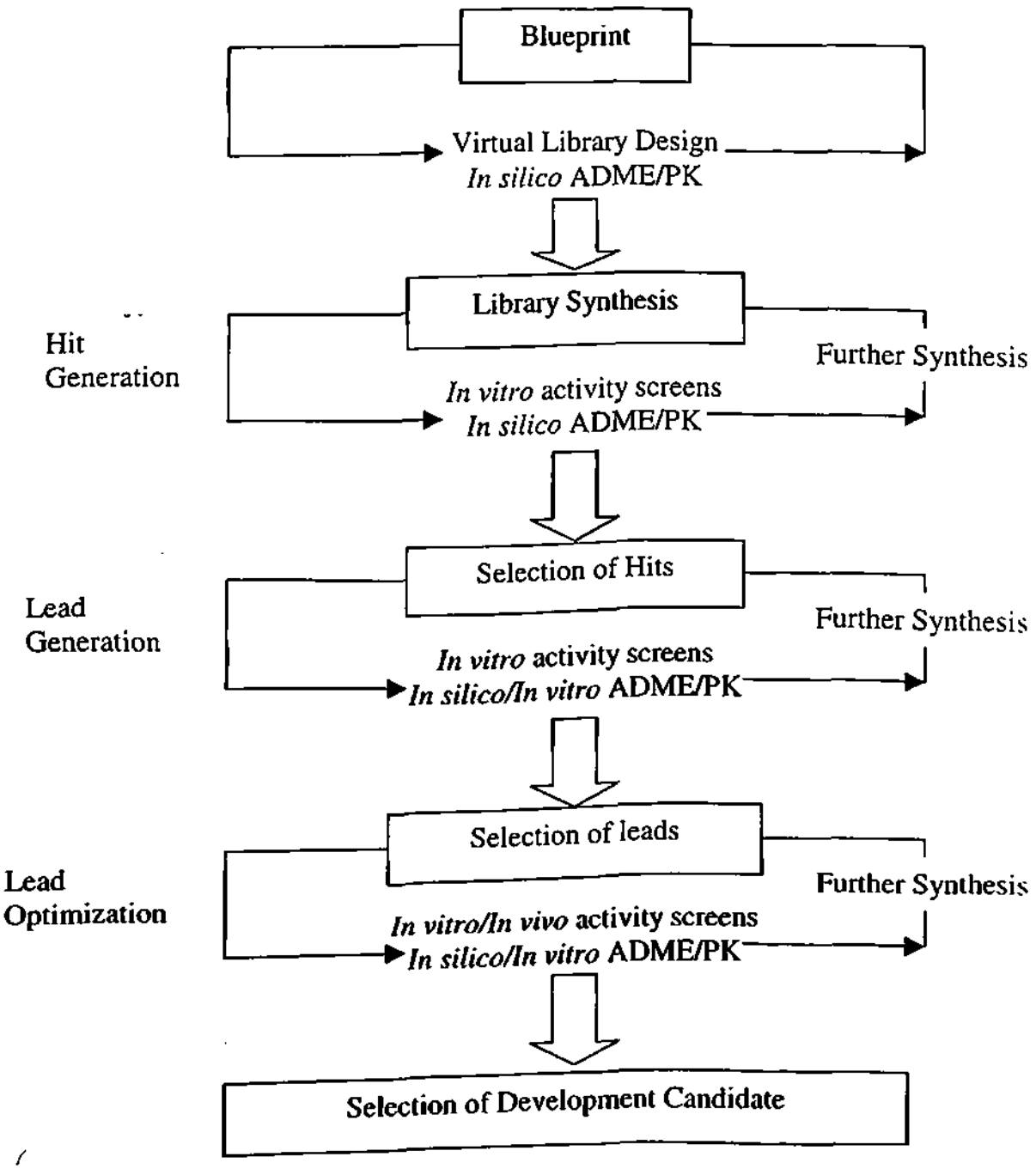
Drug research encompasses several diverse disciplines united by a common goal, namely the development of novel therapeutic agents. The search for new drugs can be divided functionally into two stages: discovery and development (4).

1.1.2 New Drug Discovery

The process of drug discovery can be divided into a number of distinct phases: 'hit generation', 'lead generation', 'lead optimization'. The goal of the 'hit generation' step is to screen large compound libraries to find molecules with a specific biological response. This consists of setting up a working hypothesis of the target enzyme or receptor for a particular disease, establishing suitable models (or surrogate markers) to test biological activities, and screening the new drug molecules for *in vitro* biological activities. Ideally a hit should have reproducible activity in a relevant bioassay, a confirmed chemically tractable structure, high purity and a potential for novelty (5). At the lead generation step, medicinal chemists use a variety of empirical and semiempirical structure-activity relationships to modify the chemical structure of a compound to improve the *in vitro* activity of the hits. However, good *in vitro* activity

cannot be extrapolated to good *in vivo* activity unless a drug has good bioavailability and a desirable duration of action. The lead optimization phase typically includes ADME screening paradigms to improve upon the degree of potency, which has already been achieved. **Figure 1** shows the phases in drug discovery and the iterative nature of the process with biology and ADME information being fed back to the medicinal chemists to guide each new round of synthesis (6).

Figure 1: Role of ADME/PK in phases of drug discovery.



1.1.2.1 Role of pharmacokinetics and metabolism in drug discovery:

Rational drug discovery requires an early appraisal of all factors impacting on the likely success of a drug candidate in the preclinical, clinical and commercial phases of the drug development (7). The use of pharmacokinetic and ADME studies in optimization of lead molecules is the marrying of a traditional drug development activity with a research one (8). The study of absorption, distribution, metabolism, excretion and pharmacokinetics (ADME/PK) has developed into a relatively mature discipline in drug discovery through the application of well-established *in vitro* and *in vivo* methodologies (7). For ADME/PK this strategic shift has been occurring for sometime, driven by the concerns that poor pharmacokinetics was the major cause of compound failure in drug development programs. Considering pharmacokinetics to be the weakest link in the drug development chain was a major influence in expanding ADME/PK from its traditional role as a preclinical safety support function towards the earlier stages of drug discovery (9). A growing awareness of the key roles that pharmacokinetics and the drug metabolism play as determinants of *in vivo* drug action has led many drug companies to include examination of pharmacokinetics and drug metabolism properties as part of their screening processes in the selection of drug candidates. Consequently, industrial drug pharmacokinetics & metabolism scientists have emerged from their traditional supportive role in drug development to provide valuable support in the drug discovery efforts. Having become established as a routine part of lead optimization, four factors are primarily responsible for determining the nature and extent of ADME/PK studies. These are the phases of the discovery program, the number of compounds available for study, tools available and, most importantly, the specific requirements of the drug candidate defined within the product profile (7). The common ADME/PK issues encountered during lead optimization are concerned essentially with systemic exposure and the potential for drug-drug interactions (10). Problems with systemic exposure typically become apparent through the failure of molecules with *in vitro* potency to show subsequent efficacy *in vivo*. The various problems with systemic exposure are related to absorption (solubility, permeability and gut stability), distribution (plasma-protein binding, tissue binding) clearance (first-pass metabolism, renal, hepatic, biliary, etc.), while issues related to drug-drug interactions are such as enzyme induction and inhibition (10). Hence it is now more common for initial ADME/PK studies to be conducted prior to *in vivo* efficacy testing to evaluate systemic exposure and make best use of animal models.

whose use is often constrained for practical and ethical reasons. The capacity to relate systemic drug concentrations to a pharmacodynamic effect can ensure that rational decisions are made in the context of the target product profile. For instance, a project might strive excessively to meet targets for elimination half-life of a product that would be consistent with one-a-day dosing, when in reality the duration of action is not directly governed by the plasma concentration (**11, 12**). While aiming for a long half-life is undoubtedly an advisable general principle of lead optimization, it is important to ensure that the time and effort expended are justified on the basis of likely improvements in the pharmacodynamic profile.

After establishing that ADME/PK findings are relevant to compound progression, it is necessary to investigate the underlying causes of any problems that might be encountered. A good understanding of these problems is the cornerstone of a rational ADME/PK lead optimization strategy because it ensures that the appropriate tests are conducted and that the synthetic program is directed accordingly (**13**).

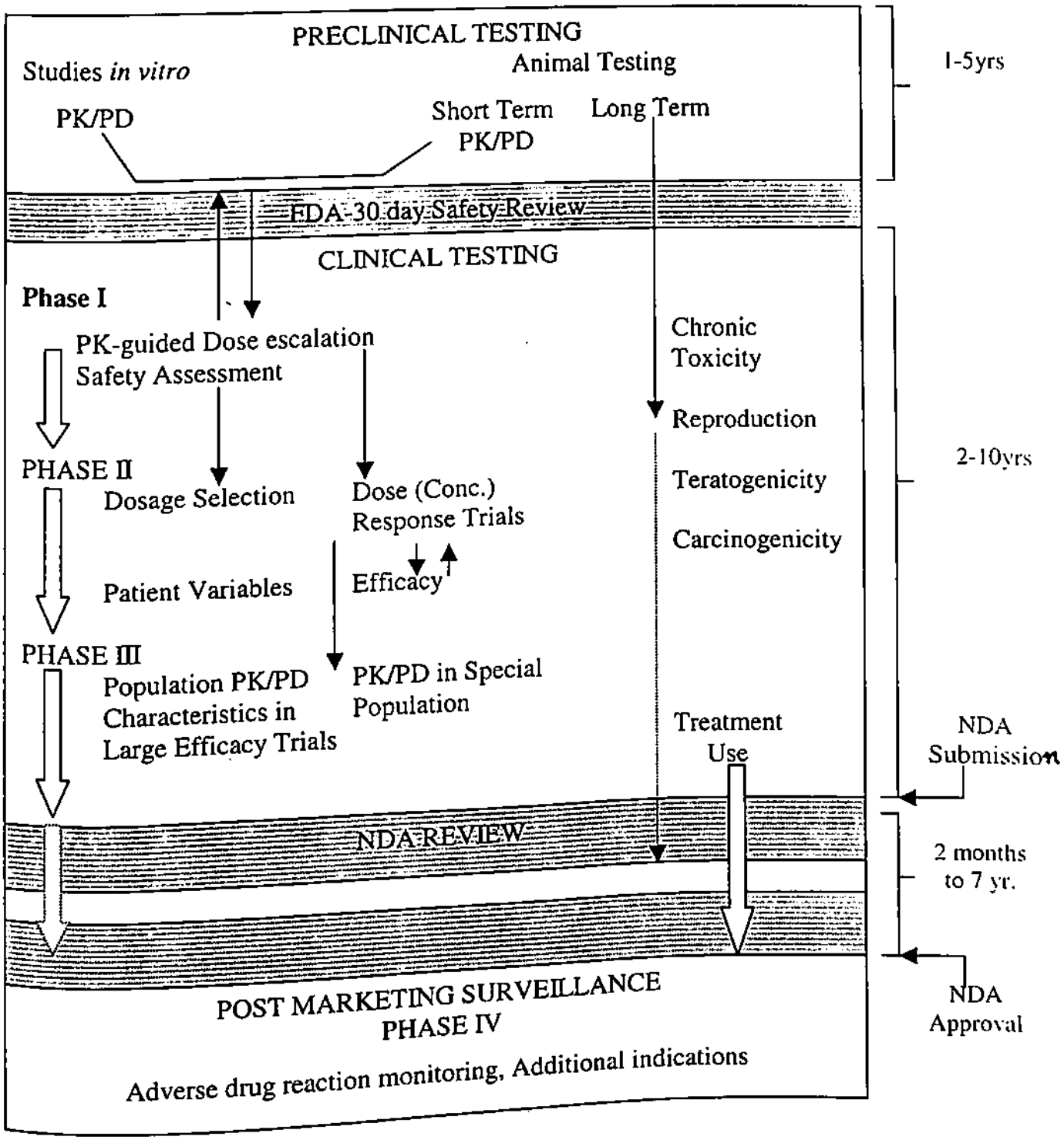
1.1.3 New Drug Development

Drug development may be defined as a series of specialized events performed to satisfy criteria, internal (i.e., competitive industry benchmarks) and external (i.e., regulatory compliance), to yield a novel drug (**4**). The process involves preclinical research and development, and clinical trials commonly divided into four phases (**14**), as illustrated in the **Figure 2** (**1, 15**).

1.1.3.1 Preclinical studies

The preclinical stage of the drug development focuses on the activities that are necessary for filing an IND (Investigational New Drug)/CTA (Clinical Trial Application). The completed IND/CTA contains information that details the drug's composition and the synthetic processes utilized for its production. The IND/CTA also contains animal toxicity data, protocols for early clinical phase trials, and an outline of specific details and plans for evaluation. Process research, formulation, metabolism and toxicity are the major areas of responsibility in this development stage.

Figure 2: The phases of drug development (PK, pharmacokinetics; PD, pharmacodynamics). (Figure modified from reference 1 and 15).



Preliminary data from early animal toxicology and pharmacokinetic studies are obtained to determine the optimal doses and dosage form for the initial phase clinical trials. The preclinical pharmacokinetics studies involve the development of appropriate bioanalytical methods for the quantititation of the drug and metabolite concentrations in the evaluation of the pharmacokinetics. Typically it involves a series of studies focusing on the absorption, distribution, metabolism and excretion (ADME)

of the NCE. These early studies also provide insight into the extent of safety monitoring necessary during the phase I. The data from these studies support the IND/CTA submissions and the clinical development for all indications. All the issues that are expected to attract the attention of the regulatory agencies are identified at this time and are addressed in the clinical plan (4).

1.1.3.2 Clinical Trials

The clinical development stage is comprised of three distinct phases (I-III), and culminates in the filing of New Drug Application (NDA)/Marketing Authorization Application (MAA). Each phase involves process scale-up, pharmacokinetics, drug delivery, and drug safety activities (4).

1.1.3.2.1 Phase I studies

The objectives of Phase I clinical studies are to define the initial parameters of toxicity and tolerance and their relation to dosage. It also includes the study of relevant pharmacokinetics of drug in healthy subjects and optimization of drug delivery system. Pilot pharmacokinetics (single and multiple doses), bioavailability and single dose ADME studies are usually carried out in this phase (**14, 16**).

1.1.3.2.2 Phase II studies

Phase II trials are carried out to evaluate the efficacy in a limited number of patients and to define both the therapeutic dose range and dosing regimen for a particular disease. Studies on intra- and inter-subject variability, dose proportionality, multiple dosages ADME are also carried out (**14, 16**).

1.1.3.2.3 Phase III studies

Phase III studies are performed in considerably large number of patients than phase II and are frequently carried out on a multi-center basis. The principal aims are to demonstrate long-term safety and tolerance and to compare the new drug with the existing standard treatment. Possible drug-drug interactions, effects of age, sex and other subject variables on both pharmacokinetics and pharmacodynamic variables are also studied at this stage (**14, 16**).

1.1.3.2.4 Phase IV studies (Post-marketing Surveillance)

These studies are conducted after a product license has been obtained following NDA/MAA review. They are generally large-scale, long-term trials designed to investigate the incidence of relatively rare adverse reactions. Most phase IV studies are used to extend the range of the approved indications (**14, 16**).

1.1.3.3 Role of Pharmacokinetics in phases of Drug Development

Pharmacokinetics (PK) is the quantitative study of the time course of drug absorption, distribution, metabolism and elimination. This includes the relationship of ADME to the intensity and time course of therapeutic and toxicological effects of drugs or chemicals (**17**). The term ADME is typically used in reference to nonclinical studies. In reality, ADME is generic, being applicable without any restriction to pharmacokinetics/metabolism investigations in humans as well as animals. Fundamentally ADME is fully critical in all phases of a fully integrated drug development program.

The initial charge of early development programs is to file the IND application such that approval may be secured to investigate a new chemical entity (NCE) in human beings. Animal toxicology studies comprise the foundation of the IND. In this context, ADME studies provide supportive information to augment the interpretation of toxicological findings. Of primary importance among them are drug exposure, expressed in terms of AUC (area under the drug plasma concentration – time curve) and C_{max} (maximum drug concentration in plasma), or an alternative parameter which are then related to dose levels and toxicological outcomes (**18**). Based on the toxicokinetic data at no-observed toxic effect dose, an acceptable exposure limit in humans can be defined. To assist in putting the toxicokinetic data into a broader perspective, the basic pharmacokinetic behavior of the NCE is assessed in the toxicology species. Some typical studies that may be conducted at this stage of development are listed in **Table1**.

Table 1: Studies to be conducted for IND.

Toxicokinetics
Pharmacokinetics/absolute bioavailability in toxicology species (male/female)
Protein binding
Erythrocyte/plasma distribution
Whole body autoradiography/tissue distribution
Mass balance in toxicology species
Metabolic profile in toxicology species
Pharmacodynamics
Allometric scaling

In later development, the focus of ADME work is shifted to human studies. These are intended to more fully define the disposition of the drug in humans, particularly in the target therapeutic population. Ultimately these data are integrated to a NDA or Market Authorization Application (MAA) to secure final approval to market the drug (**18**). Possible studies, which may be performed during clinical development, are listed in **Table 2.**

Table 2: ADME studies which support NDA/MAA

NON CLINICAL
Toxicokinetics in chronic and reproductive/teratology studies
Multiple dose pharmacokinetics in toxicology species
Biliary excretion/enterohepatic recirculation
Metabolic identification in toxicology species
Multiple dose whole-body autoradiography/tissue distribution
Placental transfer
Milk secretion
Effects on metabolic systems (induction)
CLINICAL
Single/multiple dose pharmacokinetics in safety/tolerance studies
Dose proportionality
Food effect
Repeated measures
Mass balance/metabolic profile and identification with radiotracer
Pharmacokinetics in subpopulations (gender, age, genetics, liver and renal failure)
Drug interactions
Milk penetration
Pharmacodynamics
Population pharmacokinetics/pharmacodynamics in patients
Pivotal bioequivalence

ADME data gathered during the full course of development are incorporated into drug labeling, which intended to optimize therapeutic utilization of the drug in the target population (**18**).

1.2 The trend towards HTS

The provisions of the Harris-Kefauver Amendments to the Food, Drug, and Cosmetic Act in 1962 resulted in the requirement of sufficient pharmacological and toxicological research in animals before a drug can be tested in human beings. This has greatly increased the time and cost of required marketing a new drug (**15**). This amendment has resulted in revolutionary changes in the drug discovery programs adopted by the industry. As the time taken for a compound to reach Phase II human trials from the initiation of a typical project is usually more than 5 years, it is evident that anything that can be done to speed up the discovery process could help selected compounds to reach the market sooner and thus give an earlier return on company's investment. But NCEs fail at multiple points along the discovery and development track. Some might even make it to the market, only to be withdrawn following an initial successful launch, with the cost in lost revenue to the companies who have invested their time and money in their development being potentially crippling. The recent withdrawal of the anticholesterol drug Baycol™ (Bayer AG, Leverkusen, Germany) is the latest example of a compound with excellent potential in its therapeutic class, but which will now not realize any value for the discovering company (**19**). Often the withdrawal is for safety reasons, such as the unforeseen occurrence of an adverse reaction or a cross-reaction that was only likely to become apparent with large-scale use, or abuse, of the compound in man (**19**). Many IND application fail during the preclinical and clinical development and recently it was estimated that 46% of NCEs entering the clinical development were dropped due to unacceptable efficacy and 40% due to safety reasons (**20**). Hence, one of the keys to developing a successful drug is in minimizing the unpredictable features of the molecule. This includes trying to predict the way in which the molecule affects the body, and vice versa, including the interaction with any other drugs and assorted xenobiotics that might be present from time to time during its therapeutic use. Consequently, the development of a drug is a long and complex process with a high failure rate before it reaches the market (**21**).

In recent years there has been a significant shift in the focus of drug discovery activities from a detailed knowledge gathering science to high volume throughput

events. This has resulted in accelerated drug development strategies, which focus on producing drug candidates or NCEs and accomplishing goals in less time than with traditional development approaches. The key elements of accelerated developmental strategies involve the early identification of the most promising drug candidates. The premise of this approach focuses on maximizing the return on investment via cost effective application of resources. The return on investment is captured by bringing a profitable drug to market faster and by utilizing the resources more efficiently (4). Two accelerated development strategies have emerged to maximize the return on investment, which are:

1. Quantitative process approaches:

These are aimed at achieving high throughput of analysis. The focus is on sample volume with the primary objective of accommodating increases in sample generation. This approach is typically accomplished with the addition of more resources and/or improved methods for analysis, and is highly effective when a "go" decision is made for lead candidate development. The activities associated with faster analysis are generally independent of sample-generating approaches. The incorporation of an automated task into an existing method for analysis is the example of a quantitative process approach (4).

2. Qualitative process approaches:

These are supposed to eliminate candidates that have unsuitable characteristics, which form the weak link in the drug development pipeline. Here, analysis that focuses on pharmaceutical properties are performed during the earlier stages of drug development. This approach usually requires the development of a new application that is highly integrated with the sample-generating responsibilities that lead to faster decisions to "stop" development activities. Predictive *in vivo* and *in vitro* models of metabolic stability are examples of a qualitative process approach (4).

Accelerated development exploits the relationship between quantitative and qualitative process approaches. Often, it is the balance between the two approaches that creates new opportunities for development success as well as significant challenges for analysis. Typically, one approach is developed in response to the other, followed by refinement and integration.

1.2.1 Need for HTPK

Even though a detailed knowledge-gathering science still remains crucial for the drug discovery process, but it has been overwhelmed by the vast array of the screening events that are aimed at identifying the potential drug candidates. Hence drug discovery is becoming highly dependent on technologies, which enhance the ability to quickly generate, test and validate a discovery. Today in the race to develop the next selling drug, the key tools for rapidly generating potent and selective compounds are combinatorial chemistry and functional genomics (22). The recent developments in robotics and combinatorial chemistry synthesis in pharmaceutical research and development have driven the production of a very large number of compounds with potential activity for pharmacological activity. High-density synthesizers can synthesize millions of promising compounds per year (22). Even in the absence of combinatorial paradigms, medicinal chemists are turning to automated and parallel compound synthesis (23). These technological advances have now given discovery scientists the ability to deliver large numbers of lead compounds for final optimization. In parallel with this, the capacity for screening these molecules against targets of interest is rapidly growing beyond 100,000 per week (24). Turning a chemical lead into a marketable medicine requires a balance of potency, safety and pharmacokinetics. The pace generated by the chemists in the drug discovery cycle is further progressed by the discovery project teams by focussing on automation with 96-well microplate technology as a means of accelerating *in vitro* pharmacological testing. These rapid advances have given birth to a plethora of jargon phraseology such as "high-throughput screening (HTS)", "ultra HTS", "compound decks" and "bioprospecting" or "fishing" for leads (25). The net effect of these "upstream" events have generated pressure on throughput further downstream in the optimization processes that seek to align potency with good pharmacokinetics generating a "bottleneck" in the drug discovery cycle. The traditional pace and analysis of pharmacokinetics and metabolism is, very slow and careful, describing in detail the intricate processes governing the distribution and disposition of drugs. The traditional analytical support involves the use of manual sample preparation (liquid/liquid and/or solid phase extraction) combined with HPLC (radiochemical, PDA, UV or fluorescence detection) or Gas Chromatography (GC) estimation. These processes are full of limitations. Manual sample preparation is labor intensive and is not cost effective. Conventional LC techniques are usually not very selective and specific. They often require extensive method development. The GC

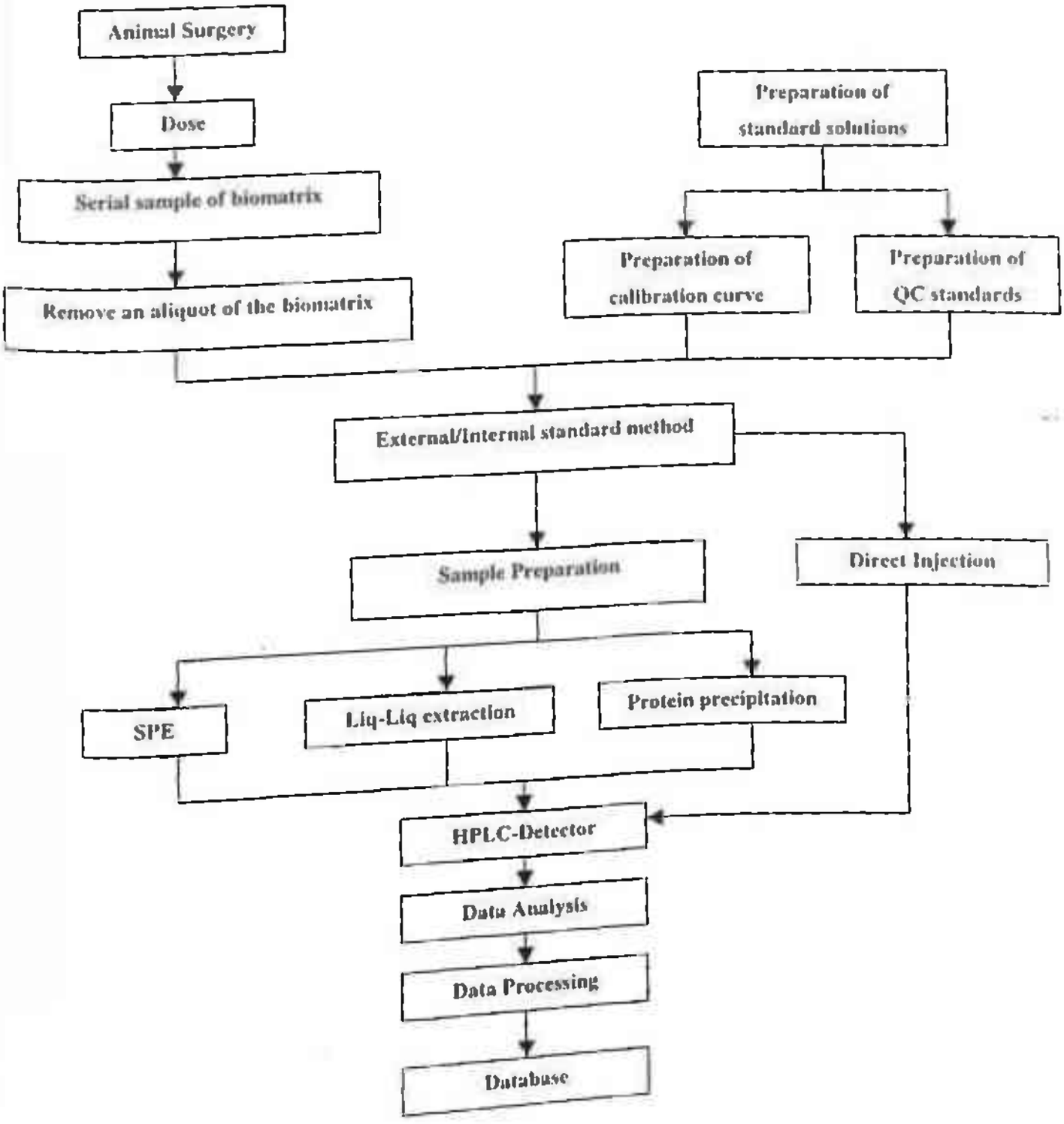
techniques are complex and generally require several extraction steps combined with derivatization and thus time consuming

Moreover a typical preclinical pharmacokinetic study involves:

1. Initial surgery required in preparing animals for dosing, actual dosing and collection of blood samples.
2. Calibration of an analytical method.
3. The preparation of samples prior to analysis.
4. The actual quantitative analysis.
5. Data reduction and reporting.

The complete process is summarized in **Figure 3**:

Figure 3: Typical process involved in a pharmacokinetic study.



Although it is imperative that all samples, standards and quality controls are treated identically, processing each sample sequentially is time-consuming. This

suggests the introduction of new technologies at the sample generation and preparation stage to improve the throughput.

In addition to the fundamental changes in discovery technology, cost and value are the important dynamics, which operate within the processes of developing drugs that have a major impact on the need for deriving information on kinetics at an early stage. The overall cost of bringing a new medicine to the market place is very high- estimates vary widely between £100 and £300 million (26). However such is the rate of the dropout of the compounds, the industry currently spends more on compounds that fail in the development than it does on those that succeed to the market place (27-28). Any means of uncovering this early and 'failing fast, but failing cheap' will be highly valuable in reducing the fallout rate at the more costly stage of the development cycle. The corollary to this is that any early information gained on a compound that adds confidence to the likelihood of successful development should similarly reduce the ratio of failure to success. Thus, there is a need to bring ADME/PK studies, which are traditionally very low throughput processes into the realms of high throughput to aid selection and to add value to NCEs.

1.3 High-throughput approaches to pharmacokinetics (HTPK):

The solution to this problem by the introduction of novel concepts such as 'N-in-One' dosing or Cassette Dosing, Sample Pooling approaches, use of automation for sample preparation and bioanalysis and use of new experimental approaches for the evaluation of many substances in parallel, which have shown to be a useful ways to increase the speed with which compounds are studied (29).

Earlier, the pharmacokinetic analysts quantitated the compounds with UV or diode-array detectors following the HPLC. Methods of development were focused on discovering a way to make analyte, and perhaps a key metabolite or two, elute from the column in 'empty regions' of the chromatogram. This difficulty of resolving even one or two analytes from a biomatrix caused a delay in the development of the newer concepts for increasing the throughput of PK studies. The advent of liquid chromatography (LC) coupled with atmospheric pressure ionization (API) mass spectrometry (MS) and tandem mass spectrometry (MS/MS), has resulted in analytical methods, which are renowned for its sensitivity and selectivity. LC/MS and/or LC/MS/MS typically require minimal sample preparation and facilitate rapid analysis. A key technique of LC/MS is the use of multiple reaction monitoring (MRM) which

involves the isolation of the precursor ion of the analyte in the first quadrupole of the instrument, fragmenting it in the collision chamber, and then isolating a selected product ion of the precursor in the third quadrupole. This allows for the quantitation of multiple compounds simultaneously (30,31). These advances in the field of bioanalysis have given birth to the possibilities of novel concepts of increasing the throughput of PK screening.

1.3.1Concepts of HTPK

1.3.1.1 Cassette Dosing or N-in-One dosing:

Cassette Dosing is a procedure for higher-throughput screening in drug discovery to rapidly assess pharmacokinetics of large numbers of NCEs. It involves simultaneous administration of multiple compounds to a single animal. Blood samples are collected, and the plasma/serum samples obtained are simultaneously analyzed by means of an assay method that permits concurrent assay of many compounds in a single sample (4,32 - 34). Consequently, the pharmacokinetics of multiple compounds can be assessed and ranked rapidly with small number of experimental animals and shortened assay times. Cassette Dosing is generally used to screen NCEs in two general ways: for systemic clearance (i.v. dosing) and for oral plasma/serum drug levels (p.o. dosing). Compared with conventional pharmacokinetics studies, cassette dosing has the advantages of:

a) Rapid assessment of pharmacokinetic parameters of a group of compounds.

b) Reduced animal requirement.

c) Reduction in the requirement of labor and more over it is not labor intensive.

d) Elimination of intra-species variability in assessing the PK of a group of compounds.

e) Decrease in the overall costs of bringing a new drug into market.

The design and concept of cassette is based on a set of intuitive assumptions:

a) The size of the cassette (n) is limited only by the sensitivity of the assay and the solubility of the compounds. Large cassettes are difficult to assay using HPLC well. In the construction of the cassette, compounds with similar retention times are to be avoided or the analytical method should be modified so as to obtain good resolution. The physico-chemical properties of the compounds play a major role in forming a solution of the mixture.

b) The pharmacological nature of the compounds also defines the composition of the cassette. Compounds with antagonistic pharmacological characters are avoided in

designing cassette. Usually compounds of the same series with different intensities of the same pharmacological activity are selected in designing a cassette.

c) There is a possibility of encountering drug - drug interactions. The probability of the interactions increases in large cassettes. Controlling the size of cassette can control the frequency of the occurrence of interactions to a good extent. In, most cases, these interactions are encountered when the clearance of one drug is reduced by the co-administration of a second drug. However, most interactions are not that severe and also enough alternate pathways of clearance exist so that inhibiting one of them does not have big effect in the clearance **(24, 31-35)**

d) Drug-drug interactions in cassette dosing usually lead to false positives. A false positive is a result in which a compound appears to have acceptable pharmacokinetic characteristics when dosed in a mixture but would be identified as unacceptable if dosed singly. Mutually competitive inhibitors of elimination pathways tend to decrease clearance thereby increase plasma levels and AUC. This may be acceptable in a screening mode, which will be corrected in the later single-compound pharmacokinetics determination. Most of the interactions will lower the clearance hence; good compounds will not be eliminated from further consideration **(32,36-37)**.

e) Interactions are dose dependent, so lowering the dose of the compounds as far as possible will tend to weaken the interaction, reducing the frequency of occurrence of a compound potent enough to affect clearance. Thus, one may guard against competitive inhibition of a shared metabolic enzyme by keeping dose small **(31-38)**.

f) Drug-drug interactions can sometimes lead to false negatives. A false negative is a result in which a compound appears to be unacceptable when dosed as cassette but would be identified acceptable if dosed singly. These are more serious than false positives, as such compounds will be discarded without further testing. The possibilities of false negatives exist in case of protein binding displacement of one compound by the other, presence of drug metabolizing enzyme activator. The possibilities of the occurrence of false negatives can be detected by applying the concept of N-in-One to basic *ex vivo* studies such as *in vitro* metabolism studies, estimation of protein binding, *in situ* absorption determination, followed by single-compound studies. These *ex vivo* studies should be included as one of the determinants in defining the composition of the cassette.

g) Lastly, even if the absolute values are wrong, the correct rank order will be observed **(31-38)**. The use of 'Right Box' Analysis as a measure for success of a

screening method can increase the predictive power of Cassette Dosing. It involves placement of the test compound into a correct category or the Right Box (22).

1.3.1.2 Sample Pooling

The other approach of increasing the throughput of the PK, which involves reduction in the number of samples to be analyzed, is Sample Pooling. The concept of sample pooling involves individualized dosing (one compound per dose per animal), One-in-One dosing, followed by pooling of the samples of all the compounds for the respective time points. This result in one combined sample per time point for simultaneous quantitation of drug concentrations thereby enhancing the throughput of PK (39). Compared with conventional pharmacokinetics studies, sample pooling has the advantages of:

a) Rapid assessment of pharmacokinetic parameters of a group of compounds.
b) Reduced assay workload due to reduction in the bioanalysis time.
c) Elimination of drug-drug interactions in assessing the PK of a group of compounds.
d) Decrease in the overall costs of bringing a new drug into market.

The application of the concept of Sample Pooling depends upon certain factors:

a) The concept requires an assay method, which has the capability for concurrent estimation of multiple compounds in a single sample.
b) The number of compounds, which can be estimated, depends on the sensitivity of the assay method. As the number of compound(s) (n) increase, so does a factor of 'n' dilute the individual compounds.
c) Compounds, which can undergo chemical interactions, cannot be included in the premises of the concept.

1.4 Future Perspectives

Advances in MS currently offer many intriguing possibilities for further development of HTPK. Triple quadrupole MS systems continue to improve in sensitivity with the development of enhanced vacuum pumping systems and ion optics. The requirement for method development is negated by improvements in in selectivity and detection limits such that instrument optimization for ultimate performance is no longer required. In addition, such instrumentation allows PK investigation at more pharmacologically relevant doses. MS manufacturers are introducing new software tools to either automatically obtain optimized MS/MS characteristics from an infused

sample or simply from a loop injection that, when operated in a batch-mode for multiple-analyte screening, will dramatically reduce analytical method development.

There could also be a drug discovery use for the hybrid quadrupole-TOF technology, currently expensive and with niche role in proteomic analysis. This combines the power of quadrupole with the ability to obtain accurate mass MS/MS data on samples of interest by using a TOF analyzer (40). This generates simultaneous parent and metabolite information from a single chromatographic run, thereby facilitating more data to be obtained from a single biological sample.

A new paradigm in drug discovery has emerged in which the entire sample collection is rapidly screened through the introduction of automation technologies for sample preparation. More recent technologies such as microtitre plate, robotic instrumentation and MS, have all advanced sufficiently to provide a platform on which to build a more fully automated approach to perform analysis of biological matrix samples, thereby promoting HTPK. However, as yet, the promise is not fully realized, as no solution works 'out-of-the-box', and efforts to fully validate such methodology continue in an attempt to increase throughput whilst retaining the quality of analysis. A key differentiator in further development of automation technologies is likely to be software. A majority of hardware platforms, particularly for the manipulation of microtitre plates, are already highly competent. However, fully unattended operation will only become a reality when intelligent, decision-making software will become available. Such developments are likely to follow when the focus of the instrument manufacturers shifts from purely supporting the HTS-end of the operation to encompassing the wider drug discovery arena (39).

The drug discovery process is heading towards systems involving fully robotized process from sample collection through to data reporting, linking together all necessary processes such a s sample preparation and MS. This will ultimately result in a report file, containing concentration data along with all appropriately logged experimental details, which will feed straight into database fields, and create a truly 'turn-key' approach to HTPK.

1.5 Research Envisaged

The literature survey gives an insight into the existing problem and its importance in the field of drug discovery and development, which is a very rapidly growing and the number of compounds generated is continuously increasing. This has resulted in increased pressure on the downstream activity and efficacy screening process to increase their output to meet the demands generated by the chemists. With pharmacology gearing up itself with high throughput *in vitro* activity screening techniques matching the output generated by the medicinal chemists, the low-throughput activity of discovery pharmacokinetics is directly opposed to the need to address the high attrition rate of compounds in drug development caused by poor PK profiles. This slow rate of pharmacokinetics has led to the formation of a bottleneck in the drug discovery cycle. This calls for the need to bring the traditionally low-throughput PK screening into the realms of high-throughput screening.

The solution to the problem lies in exploring the novel concepts such as Cassette Dosing and Sample Pooling. Cassette Dosing involves dosing mixtures of NCEs to a single animal and simultaneously quantify all the NCEs in the samples generated resulting in PK data for all the compounds at once. Sample Pooling involves dosing of the NCEs individually to different animals and then pooling common time point samples before processing and then the samples are analyzed to generate the PK data of all the compounds simultaneously. Both the methods capitalize on liquid chromatography/ tandem mass spectrometry (LC/MS/MS) as a sensitive and specific analytical method to simultaneously quantify all the NCEs. The limited availability of LC/MS/MS prompted an investigation to alternatively apply the concepts used to achieve HTPK screening for lesser number of compounds using traditional analytical techniques. Since Cassette Dosing provides an added advantage of decreasing the number of animals involved in the study, this concept was taken up for the investigation. It was also planned to investigate the extent to which traditional analytical techniques will serve the purpose and also study the role of advance analytical techniques, which offer advantages.

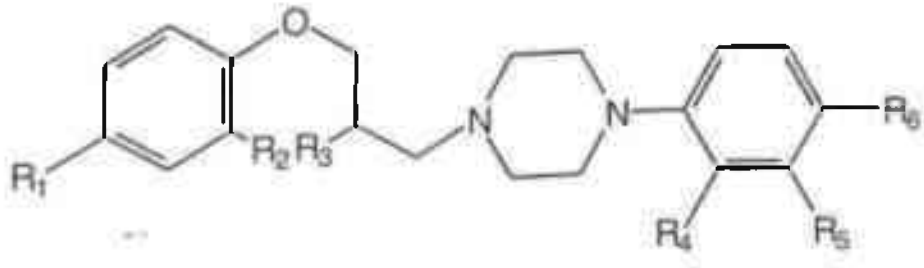
The concept of Cassette Dosing was applied to screen three in house CDRI molecules of the class of aryloxy-substituted arylpiperazines for illustrative purpose. The cassette consisted of 1-(4-propionyl phenoxy)-3-[N^4-3',4'-dimethoxyphenyl]-piperazinyl]-N^1-propan-2-ol, 1-(phenoxy)-3-[N^4-phenyl piperazinyl]-N^1-propane, 1-(2-isopropyl phenoxy)-3-[N^4-(2'-methylphenyl)-piperazinyl-N^1-propan-2-ol which were

potent antihypertensives, synthesized in the institute as a part of drug discovery program. Prior to the start of the studies, the criterion for the selection of the compounds with most favourable PK parameters was defined. Since the compounds were antihypertensives, the desired criteria for the selection was set as low clearance, high AUC and long residence time

The basic concept of Cassette Dosing was utilized for the generation of PK parameters. This was followed by Discrete dosing of the compounds at the same dose level so as to generate a baseline data for comparison of the feasibility of the concept of Cassette. Apart from this, the possibility of its application to various *in situ* and *in vitro* studies usually carried out to predict before performing *in vivo* studies was also explored in the present investigation.

During the various phase(s) of the work, good laboratory practice (GLP) guidelines were followed. This was essential since GLP sets " A standard by which experiments are designed, performed, monitored, audited, recorded, analyzed and reported so that there is public assurance that the data is credible". The various acceptable parameters and our data confirmation to the acceptable limits are discussed under individual chapters. The various equipments and the chemicals used in the experiments are listed under individual chapter(s). The protocols involving animal experiments were approved by the Local Ethical Committee for Animal Experimentation prior to the start of the study. Animals were cared for in accordance with principles of The Guide for the Care Use of Laboratory Animals (Department of Health, Education and Welfare, no. [NIH] 85-23). In the text that follows, the compounds have been designated as I, II and III respectively. The structure(s) of I, II and III and Internal Standard [(I. S.) used in HPLC/MS assay] are given in **Figure 4**.

Figure 4: Chemical structures of I, II III and I. S.



Compound	Substitution					
	R_1	R_2	R_3	R_4	R_5	R_6
I	$COCH_2CH_3$	H	OH	H	OCH_3	OCH_3
II	H	H	H	H	H	H
III	H	$CH(CH_3)_2$	OH	CH_3	H	H
I.S.	$COCH_2CH_3$	H	OH	H	H	C_6H_5

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CHAPTER 2

BIOANALYTICAL ASSAY METHOD DEVELOPMENT AND VALIDATION

2.1 Introduction

Methods of measuring drugs in biological media are increasingly important to many societal groups. Problems related to medicine, toxicology, pharmacology, forensic science, environmental research, new drug development, drug abuse, clinical pharmacokinetics and drug researches are highly dependent on biopharmaceutic methodology (1,2,3). Analytical methods employed for the quantitative determination of drugs and their metabolites in biological samples are the key determinants in generating reproducible and reliable data which in turn play a significant role in evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data (4).

2.1.1 Bioanalytical Method Development

Once decided to generate the pharmacokinetic parameters, the accurate measurement of concentration of drug/metabolite becomes mandatory. Therefore, the measurement techniques/assay must be highly specific and very sensitive for the parent drug/metabolite(s) in question. For quantitative bioanalysis of pharmaceutical compounds aiming at providing the best estimate of the concentration of an analyte in a complex biomatrix, a wide range of biochemical and analytical techniques are available. The rational selection and development of an assay method require special knowledge about the drug to be analyzed. A sound knowledge of physico-chemical properties like, lipophilicity, pKa, volatility (in case of GC), U.V. absorption, fluorometric and electrochemical characteristics, molecular weight and fragmentation pattern (in case of MS) and chemical stability under storage conditions and throughout a particular assay procedure is an essential *priori* to assay development (5).

The choice of a suitable end step (actual measurement or detection step) is cardinal to the development of an assay procedure and also largely determines the requirements for sample preparation. Solely, U.V. spectrophotometry and spectrofluorometry are not applied in pharmacokinetic studies, due to the lack of specificity. However, these detection techniques are very useful when used in combination with a separation procedure like liquid chromatography. The combination of a chromatographic and detection systems greatly contribute to the selectivity of a particular method, as the chromatographic conditions provide large flexibility, in such a way that there is no interference in the drug/metabolite(s) detection from other drugs, endogenous sample constituents etc (5). The development

of atmospheric pressure ion sources, such as ESI and APCI, has added liquid chromatography-mass spectrometry to the list of the bioanalytical assay methods. In particular, the combination of LC with tandem MS, LC-MS-MS, offers high specificity, speed, good precision, accuracy, wide dynamic range, high sensitivity and applicability to almost every type of compound. These properties have made LC-MS-MS a generally accepted tool in bioanalytical support of clinical pharmacology studies (6).

If not already defined as part of the lead developability assessment program, a bioanalytical chemistry method needs to be developed and characterized for the quantification of the drug candidate in physiological fluid matrices. It is therefore essential to employ well-characterized and fully validated analytical methods to yield reliable results, which can be satisfactorily interpreted. Analytical methods and techniques are constantly undergoing changes and improvements and in many instances they are at the cutting edge of the technology. These analytical technologies are emphasized with unique characteristics, which can vary from analyte to analyte. Moreover the appropriateness of the technique may also be influenced by the ultimate objective of the study. Specific validation criteria are needed for methods intended for analysis of each of the analyte (7).

2.1.2 ANALYTICAL METHOD VALIDATION

Bioanalytical method validation includes all the procedures to demonstrate that a particular bioanalytical method for the quantitative determination of the concentration of the analyte (or a series of analytes) in a particular biological matrix is reliable for the intended application (7). Many of the principles, procedures and requirements of bioanalytical method validation are common to all types of methodologies. For pharmaceutical methods, guidelines from the United States Pharmacopoeia (USP) International Conference on Harmonization (ICH) and Food and Drug Administration (FDA) provide a framework for performing such validations (8). In general, methods for regulatory submission must include studies on specificity linearity, accuracy, precision, range, detection limit, quantitative limit and robustness.

Doing a method validation can be tedious, but the consequences of not doing it right are wasted time, money and resources (8). In early stages of drug development, it is usually not necessary to perform all of the various validation

studies. Many researchers focus on specificity, linearity, accuracy and precision studies for drugs in preclinical through Phase II (preliminary efficacy) stages. The remaining studies pertaining validation are performed when the drug reaches the Phase II (efficacy) stage of development and has a higher probability of becoming a marketed product (8).

The process of validating a method cannot be separated from the actual development of the method conditions, because the developer will not know whether the method conditions are acceptable until validation studies are performed. The development and validation studies may therefore be an iterative process. Results of the validation studies may indicate a necessity for change in the procedure. During each method validation study, key method parameters are determined and then used for subsequent studies.

The following principles of bioanalytical method validation provide steps for the development of a new method (7):

- Parameters essential to ensure the acceptability of performance of bioanalytical method validation are accuracy, precision, selectivity, sensitivity, reproducibility and stability.
- A specific detailed description of the bioanalytical method should be written. This may be in the form of a protocol, study plan, report and /or Standard Operating Procedures (SOP).
- Each step in the bioanalytical method should be investigated to determine the extent to which environmental, matrix material or procedural variables, from time of collection of materials up to the analysis and including the time of analysis, may affect the estimation of the analyte in the matrix. In the case of LC-MS-MS based procedures, it is essential that appropriate steps be taken to ensure the lack of matrix effect(s) throughout the application of the method, especially if the nature of the matrix changes from that used during initial method validation.
- Whenever possible, the same biological matrix as that intended in the samples should be used for validation purposes. (For tissues of limited availability, appropriate proxy matrices may suffice.)
- The accuracy, precision, reproducibility, response function and selectivity of the method with respect to endogenous substances should be established with

reference to the biological matrix. With regard to selectivity, there should be evidence that the substance being quantified is the intended analyte.

The concentration range over which the analyte will be determined must be defined in the bioanalytical method, based on the evaluation of actual standard samples over the range, including their statistical variation. This defines the standard curve. It is necessary to use sufficient number of standards to define adequately the relationship between concentration and response. The relationship between response and concentration must be demonstrated to be continuous and reproducible. The number of standards to be used will be a function of the dynamic range and the nature of the concentration-response relationship. In many cases, five to eight concentrations (excluding blank) may define the standard curve.

For bioanalytical method validation the accuracy and precision can be accomplished by analysis of replicate sets of the analyte samples of known concentrations-quality control sample(s) (QC)-from an equivalent biological matrix. Three concentrations representing the entire range of the standard curve: one within 3x of the Lowest Limit of Quantitation (LLOQ) (low QC sample), one near the center (middle QC), and one near the upper boundary of the standard curve (high QC). The specific *a priori* acceptance criteria for the mean value of accuracy should be within ± 15% of the theoretical value except at LLOQ, where it should not deviate by more than ± 20%. The precision around the mean value should not exceed 15% of coefficient variation (%CV), except for LLOQ where it should not exceed 20%. Other methods of accuracy and precision, which meet these limits, may be equally acceptable.

The stability of the analyte in biological matrix at intended storage temperature (s) should be established. In addition the influence of freeze/thaw cycles should be studied. The stability of the analyte at ambient temperature should be evaluated over a period of time that encompasses duration of typical sample preparation, sample handling and analytical run time

The specificity of the assay methodology should be established using independent sources of the same matrix. This requirement may not be necessary for hyphenated mass spectrometry-based methods.

Recovery should be reproducible at each concentration

- Acceptance/Rejection criteria for spiked, matrix-based calibration standards and validation QC samples should be based on nominal (theoretical) concentration of the analyte(s).

To achieve rapid screening of preclinical PK parameters by the application of the concept of Cassette Dosing, sensitive HPLC assay method(s) for the simultaneous estimation of I, II and III in biological matrices such as serum and rat liver S9 fraction were developed and validated. Depending upon the need of selectivity and sensitivity, methods using different instruments such as HPLC/Flu, HPLC/MS were developed. Since the aim was to achieve the goal as economically as possible, traditional detectors were utilized wherever possible.

2.2 EXPERIMENTAL

2.2.1 HPLC/Fluorescence conditions (System A)

An HPLC. Shimadzu, Japan (LC-10ATvp) with CBM-10A (Communication Bus Module). FCV10ALvp (quaternary valve) and DGU-14 (Degasser) was used to pump the mobile phase [65:35%v/v acetonitrile:phosphate buffer(25mM, adjusted to pH 4 with orthophosphoric acid)] at a flow rate of 1.5ml/min. Chromatographic separations were performed on a C18 reversed phase column (Spheri-5, 5µm, 220x4.6 mm I.D) preceded by a guard column (30x4.6mm I.D) (Perkin Elmer, Norwalk,CT,USA) of the same material. Mobile phase solvents were filtered and degassed before use. Samples were injected by SIL-10ADvp auto injector fitted with 50µl loop. The compounds were found to have native fluorescence in the mobile phase (excitation = 250nm, emission = 350nm). After the elution the compounds were monitored using a model RF-10Axl, spectrofluorimeter detector set at 250nm(Ex)/350(Em). The chromatography was carried out at ambient temperature. Chromatographic peaks were integrated using Class LC10 workstation (Shimadzu, Japan).

2.2.2 LC-MS conditions (System B)

A Jasco PU980, intelligent pump was used to deliver a premixed mobile phase composed of methanol - acetonitrile - potassium hydroxide (1mM, pH adjusted to 4) (45:45:10%v/v) at a flow rate of 1mL/min. The mobile phase was degassed in an ultrasonic bath (Bransonic Cleaning Co., USA) prior to the analysis. Chromatographic separations were achieved on a Ultremex 5 CN 30 x 4.6 mm 5µ, (SI No. 03A-0050-EO). The samples were injected through a manual injector (Rheodyne model No. 7125,Cotati, USA) fitted with a 20µL loop. Automated data acquisition was triggered using contact closure signals of the manual injector. The total effluent from the column was split so that one-tenth was injected into the electrospray LC/MS interface. ESI-MS analysis was performed using a Micromass Quattro II Triple Quadrupole Mass Spectrometer equipped with an electrospray source. Data acquisition and analyses were performed using MassLynx version 3.3 software. Nitrogen was used as both the nebulizing gas ($10Lh^{-1}$) and as curtain gas ($250Lh^{-1}$). The ESI capillary was set at 3.5kV while the cone was at 61V. Data was acquired in Selected Ion Recording mode (SIR). A source temperature of 80°C was used. The dwell time was 0.8s,the inter

channel delay was set at 0.03 s and the span was 0.2 Da. The analytes were analyzed by quantifying for SIR (positive ESI) masses of 429.4, 369.3, 297.3 and 369.3 for I, II, III and I.S respectively.

2.2.3 Mobile phase conditions

2.2.3.1 System A

Mobile phase comprising 65:35%v/v acetonitrile:phosphate buffer(25mM, adjusted to pH 4 with orthophosphoric acid) at a flow rate of 1.5ml/min was employed.

Phosphate buffer: Phosphate buffer was prepared by dissolving 6.82 gm potassium dihydrogen orthophosphate in 2 L triple distilled water and adjusting the pH to 3.5 with 40% orthophosphoric acid. The buffer was filtered through a 0.22µm cellulose membrane under reduced pressure. Mobile phase was prepared by mixing acetonitrile and buffer in 65:35%v/v ratio and degassed in an ultrasonic bath (Astracon Ultrasonic Bath, Misonix Inc., NY, USA) for 15 minutes just before chromatography.

2.2.3.2 System B:

Mobile phase for this system comprised of 45:45:10%v/v methanol:acetonitrile:potassium hydroxide (1mM, adjusted to pH 4 with glacial acetic acid). The mobile phase was pumped at a flow rate of 1 ml/min.

Potassium hydroxide solution: 0.56 g of Potassium hydroxide was dissolved in 10 mL of triple distilled water resulting in 1M solution. 1 mL of 1M potassium hydroxide solution was added to 1 L of triple distilled water to make 1mM solution of potassium hydroxide. The pH of the solution was adjusted to pH 4 with glacial acetic acid. The pH-adjusted potassium hydroxide was filtered through a 0.22µm cellulose membrane under reduced pressure.

2.2.4 Development and validation of assay method for simultaneous estimation of I, II and III in serum using HPLC and Flu detection (System A):

A sensitive, selective and reproducible HPLC assay method using isocratic elution and fluorescence detection was developed to simultaneously estimate compounds I, II and III for the generation of preclinical *in vivo* and/or *in vitro* data, which will aid in rapidly screening for the compound with most suitable PK characteristics. The method was carefully optimized for chromatographic separation, sample preparation and post column detection. Various mobile phases with different

composition of the buffer were tested to elute the compounds on different stationary phases. The chromatograms were studied for peak shape, sensitivity and selectivity. The sample preparation was optimized after a thorough check for the sample clean up using various solvents for 0.5mL of normal rat serum. An appropriate model to define the linearity of calibration standards was selected after performing linear regression using different weighing schemes on Microsoft Excel (ver 5.0).

(a) Stock and standard solutions

Individual stock solutions of I, II, and III (200µg/ml) were prepared by dissolving 10mg in 50ml acetonitrile. Mixed stock solution (MSS) of all the three compounds (16µg/ml) was prepared by transferring 800µl in 10ml volumetric flasks and the volume made up with acetonitrile. Further dilutions were prepared by appropriate dilution in the range of 2000-62.5ng/ml for the determination of recovery. The detailed scheme for preparing the analytical standards is shown in **Table 1.**

Table 1: Preparation of Mobile Phase standards (MPS)

Sample Code	Concentration (ng/ml)	Volume of solution to be diluted	Volume of mobile phase to be used (ml)
MPS 6	2000	1 ml of MSS	7
MPS 5	1000	1ml of MPS 6	1
MPS 4	500	1 ml of MPS 5	1
MPS 3	250	1 ml of MPS 4	1
MPS 2	125	1 ml of MPS 3	1
MPS 1	62.5	1 ml of MPS 2	1

(b) Calibration curve

Calibration and quality control (QC) samples of all the analytes from 12.5ng/ml-400ng/ml in serum were prepared by adding various volumes of mixed stock solutions in appropriate volume of pooled drug free rat serum so that the volume ratio of the organic phase added was less than 2.5%. Calibration and the QC standards were stored at -30°C until analysis. Prior to HPLC analysis these analytical standards and the QC samples were processed according to the method outlined below. The detailed

scheme for preparing the calibration standards and QCs are shown in the **Tables 2a and 2b.**

Table 2a: Preparation of Calibration standards (CS)

Sample Code	Concentration (ng/mL)	Volume to be added	Volume of NRS (mL)
CS6	400	50 µl ofMSS	2
CS 5	200	1 ml of CS6	1
CS 4	100	1 ml of CS5	1
CS 3	50	1 ml of CS4	1
CS 2	25	1 ml of CS3	1
CS 1	12.5	1 ml of CS2	1

Table 2b: Preparation of QCs

Sample Code	Concentration (ng/mL)	Volume to be added	Volume of NRS (mL)
QC Hi	400	250µl of MSS	10
QC Med	80	2 ml of QC Hi	8
QC Lo	16	2 ml of QC Med	8

(c) Sample preparation

To blank or spiked serum (0.5ml), 1ml of acetonitrile was added. The tubes were vortex mixed for 15 sec and then centrifuged at 1000rpm at 10°C for 5min. 750µl of the supernatant was transferred to a clean conical tube and evaporated to dryness under reduced pressure in speed vacuum concentrator (Savant Instrument, Farmingdale, NY, USA) below 40°C. The residue was acidified with 200µl of 0.5N hydrochloric acid and washed with 2x2ml n-hexane. The acid layer was basified with 50µl of 2M KOH and extracted with 4ml of 2% isopropylalcohol in ether. The organic layer was transferred into another tube by snap freezing the aqueous layer in liquid nitrogen and evaporated to dryness in speed vacuum concentrator. This whole process of extraction required 4 - 5 h for processing 30 samples. The residue was reconstituted in 0.1ml of acetonitrile and injected into HPLC. The calibration curve was obtained by linear regression (y=mx) of the peak heights of I, II, III versus concentration with Microsoft Excel version 5.0 on the IBM PC computer.

(d) Stability in autoinjector

Replicates of the spiked samples at different concentrations of 16 ng/mL, 80 ng/mL, and 400 ng/mL were processed and reconstituted at the same time. The reconstituted samples were placed in the autoinjector and one set was injected immediately and the other after 12 h. The %RSD for all the three concentration levels was calculated.

e) Method validation Program

The validation program for the HPLC method included within and between precision and accuracy studies on three different days. These studies were carried out in triplicates at three different concentrations: low16 ng/ml, medium 80 ng/ml, and high 400 ng/ml concentration levels.

i) Specificity

The specificity was defined as non-interference in the regions of compounds of interest with the endogenous substances, drug metabolites or other compounds of the cassette in the determination of the concentration.

ii) Limit of detection (LOD) and quantitation (LOQ)

The detection limit of the HPLC assay method (LOD) of I, II, III is the drug quantity in the serum after the sample clean-up corresponding to three times the baseline noise (S/N>3). The limit of quantitation (LOQ) was defined as the concentration quantity of the sample, which was quantified, with less than 20% deviation in precision.

iii) Accuracy and precision

The accuracy of each sample preparation was determined by injection of calibration samples and three QC samples on three different days (n = 27; three each of low, medium and high concentration). The precision was determined by one-way ANOVA as within and between % RSD. The accuracy was expressed as % bias:

$$\% \text{ Bias} = \frac{(\text{Observed concentration} - \text{Nominal concentration}) \times 100}{\text{Nominal concentration}}$$

2.2.5 Development and validation of assay method for simultaneous estimation of I, II and III in serum using HPLC/MS (System B)

An alternative method for the estimation of I, II and III with increased selectivity and sensitivity was developed using HPLC/MS. HPLC-Fluorescence method could not provide selectivity due to co-eluting metabolites of the compounds II and III, when rat liver *in vitro* metabolism experiment was performed and moreover when administered orally, the levels obtained were low along with the interference in the analyte regions by the metabolites. Hence HPLC/MS was adopted for the estimation of the analytes in SIR mode, which provided a very high selectivity along with improved sensitivity. A sensitive, selective, reproducible and robust assay method for the simultaneous estimation of all the three compounds was developed in positive ion mode. Various mobile phases with different type, compositions of buffers were tested to elute the compounds on different stationary phases. The chromatograms were studied for peak shape, sensitivity and selectivity. The sample preparation was optimized after a thorough check for the sample clean up using various solvents for 0.1mL of normal rat serum. Internal standard (I. S) was employed for quantifying the analytes in the method. An appropriate model to define the linearity of calibration standards was selected after performing linear regression using different weighting schemes on Microsoft Excel (ver5.0).

(a) Stock and standard solutions

Stock solutions (SS) of I, II and III (1mg/ml) and I.S (200 µg/ml) were prepared separately by dissolving 10 mg of I, II, III and 20 mg of I. S in 10 ml and 100 ml acetonitrile respectively. Subsequent working standards (WSSA) of I, II and III (5, 1, 0.25 µg/ml) and working IS (WISA) (0.5 µg/ml) were made in acetonitrile from stock solutions by appropriate dilution. Further dilutions were prepared from working standard in mobile phase in the range of 2.5– 100 ng/ml for plotting the standard curve and determination of recovery of I, II and III. The detailed scheme of preparation of the mobile phase standards is given in the Tables 3a, 3b.

Table 3a: Preparation of working stock solutions (WSSA)

Sample Code	Concentration (µg/ml)	Volume of SSA to be taken (µl)	Volume of mobile phase to be made up to (ml)
WSSA 2	5	1000	10
WSSA 2	1	200	10
WSSA 3	0.25	50	10

Table 3b: Preparation of Mobile Phase standards (MPSA)

Sample Code	Concentration (ng/ml)	Volume of WSSA to be taken (µl)	Volume of WISA to be added (µl)	Volume of mobile phase to be made up to (ml)
MPSA 6	100	100 of WSSA 1	500	10
MPSA 5	50	100 of WSSA 1	1000	10
MPSA 4	20	100 of WSSA 2	500	5
MPSA 3	10	100 of WSSA 2	1000	10
MPSA 2	5	100 of WSSA 3	500	5
MPSA 1	2.5	100 of WSSA 3	1000	10

(b) Calibration graph

Calibration and quality control (QC) samples of I from 2.5 ng/ml to 100 ng/ml in serum were prepared by adding varying volumes of stock and working standard solutions in appropriate volume of pooled drug free rat serum so that the volume ratio of organic phase added to serum was less than 2%. Calibration and QC standards were stored at -30°C until analysis. The detailed scheme of the preparation of calibration standards and QCs are shown in the **Table 4a and 4b**. Prior to HPLC analysis these analytical standards and QC samples were processed by the following method.

Table 4a: Preparation of Calibration standards (CSA)

Sample Code	Concentration (ng/ml)	Volume of WSSA to be spiked (µl)	Volume of NRS (ml)
CSA 6	50	20 of WSSA 1	1
CSA 5	50	20 of WSSA 1	2
CSA 4	20	20 of WSSA 2	1
CSA 3	10	20 of WSSA 2	2
CSA 2	5	20 of WSSA 3	1
CSA 1	2.5	20 of WSSA 3	2

Table 4b: Preparation of QCs

Sample Code	Concentration (ng/ml)	Volume of WSSA to be spiked (µl)	Volume of NRS (ml)
QCA Hi	100	10 of WSSA 1	0.5
QCA Med	20	10 of WSSA 2	0.5
QCA Lo	2.5	10 of WSSA 4	1

(c) Sample preparation

To drug free or spiked serum (0.1 ml) was added 10 µl WISA (0.5 µg/ml), 12.5 µl of potassium hydroxide solution (2N) and 1 ml extraction solvent (2.5% isopropanol in diethyl ether) in a 5 ml glass tube. The tubes were vortex mixed for 1 min and centrifuged at 1000 g for 5 minutes. The organic phase was transferred to another tube by freezing the aqueous layer in liquid nitrogen and evaporated to dryness in SVC Speed Vac Concentrator (Savant Instruments, NY, USA). The residue was reconstituted in 0.1ml of acetonitrile and injected into HPLC.

d) Method validation Program

The validation program for the HPLC/MS method included within and between precision and accuracy studies on three different days. These studies were carried out in triplicates at three different concentrations: low 2.5ng/ml, medium 20ng/ml, and high 100ng/ml concentration levels. The calibration curve was obtained by linear regression with a weighing scheme of 1/x2, of the ratio of peak areas of I, II, III with I. S versus concentration with Microsoft Excel version 5.0 on the IBM PC computer.

2.2.6 Validation of assay method for simultaneous estimation of I,II and III in rat liver S9 fraction using HPLC/MS (System B)

The method developed for the estimation if I, II and III in rat serum was applied to rat liver S9 fraction. The method was found to selective, sensitive and robust and validated before its application.

(a) Stock and standard solutions

Stock solutions (SSB) of I, II and III (1mg/ml) and I.S.B (200µg/ml) were prepared separately by dissolving 10 and 20 mg of the respective compounds in 10 ml and 100 ml acetonitrile. Subsequent working standards (WSSB) of I, II and III (200,

50, 10,5 µg/ml) and working IS (WISB) (2.5µg/ml) were made in acetonitrile from stock solutions by appropriate dilution. Further dilutions were prepared from working standard in mobile phase in the range of 50– 4000 ng/ml for plotting the standard curve and determination of recovery of I, II and III. The detailed scheme of the preparation of standards is given in the **Table 5a and 5b.**

Table 5a: Preparation of working stock solutions (WSSB)

Sample Code	Concentration (µg/ml)	Volume of SSB to be taken (µl)	Volume of mobile phase to be made up to (ml)
WSSB 1	200	1000	5
WSSB 2	50	500	10
WSSB 3	10	100	10
WSSB 4	5	50	10

Table 5b: Preparation of mobile phase standards (MPSB)

Sample Code	Concentration (ng/ml)	Volume of WSSB to be taken (µl)	Volume of WISB to be added (ml)	Volume of mobile phase to be made up to (ml)
MPSB 7	4000	100 of WSSB 1	1	5
MPSB 6	2000	100 of WSSB 1	2	10
MPSB 5	1000	100 of WSSB 2	1	5
MPSB 4	500	100 of WSSB 2	2	10
MPSB 3	200	100 of WSSB 3	1	5
MPSB 2	100	100 of WSSB 3	2	10
MPSB 1	50	100 of WSSB 4	2	10

(b) Calibration graph

Calibration and quality control (QC) samples of I from 50 ng/ml to 4000 ng/ml in serum were prepared by adding varying volumes of stock and working standard solutions in appropriate volume of pooled drug and cofactor free rat liver S9 incubation mixture so that the volume ratio of organic phase added to rat liver S9 fraction was less than 2%. Calibration and QC standards were stored at -30°C until analysis. Prior to HPLC analysis these analytical standards and QC samples were processed by the following method (**Table 6a, b**).

Table 6: Preparation of Calibration Standards (CSB)

Sample Code	Concentration (ng/ml)	Volume of WSSB to be spiked (µl)	Volume of S9 incubation mixture (ml)
CSB 7	4000	20 of WSSB 1	1
CSB 6	2000	20 of WSSB 1	2
CSB 5	1000	20 of WSSB 2	1
CSB 4	500	20 of WSSB 2	2
CSB 3	200	20 of WSSB 3	1
CSB 2	100	20 of WSSB 3	2
CSB 1	50	20 of WSSB 4	2

Table 6b: Preparation of QCs

Sample Code	Concentration (ng/ml)	Volume of WSSB to be spiked (µl)	Volume of NRS (ml)
QCB Hi	4000	10 of WSS 1	0.5
QCB Med	500	10 of WSS 2	0.5
QCB Lo	50	10 of WSS 4	1

(c) Sample preparation

To the drug and co-factor free or spiked rat liver S9 incubation mixture (0.1 ml), 10 µl of WISB was added. This was basified with 10 µl 2N KOH and was processed with 1ml of 2% IPA in ether according to the method mentioned in sec 2.2.5 (c) and injected into HPLC/MS.

d) Assay Validation

The assay was validated in terms of recovery, accuracy and precision as described for System B. The QC samples included 50 ng/ml (Low), 500 ng/ml (Medium), 4000 ng/ml (High). Calibration model was selected as mentioned in Sec 2.2.5 (d)

2.3 RESULTS AND DISCUSSION

a) Chromatographic conditions

Reversed-phase HPLC method (RPLC) was employed for separation and quantitation of I, II and III in the biological fluids. The decision to develop and validate the methods using different systems largely depended on the selectivity and specificity needed for the estimation of analytes in both *in vitro* and *in vivo*

conditions. A systematic approach was followed and each parameter like stationary phase, pH, buffer, molarity, mobile phase composition, flow rate etc., was carefully optimized for developing sensitive and reliable HPLC assay methods. The demands on the chromatography varied system to system. When adequate sample clean up was possible, chromatography was limited to rough separation and sufficient retention of the analytes from the unretained solvent front (system A), but when the specificity of the sample was low, more chromatographic selectivity was required (system B). The methods developed were largely based on analysis in preliminary oral and intravenous pharmacokinetic studies in rats. The chromatography of all the samples in the study was carried out at ambient temperature (25±2°C).

b) Stationary phase

Several stationary phases like octadecylsilane C18, cyano (CN), phenyl, amino and silica columns were tried for the separation of I, II and III in the biomatrices. Of all the stationary phases, C18 and CN columns were found to be suitable for eluting the compounds as sharp peaks. The choice of the stationary phase depended upon the type of mobile phases used in different systems and the resolution of the analytes obtained.

A stationary phase containing a reversed phase C18 column (Spheri-5, 5µm, 220 X 4.6 mm id) preceded by guard column packed with the same material (30 x 4.6mm id) was found to be suitable for optimum elution and resolution of I, II and III from endogenous interferences and unknown metabolites in normal rat serum for system A as compared to CN column. For system B (HPLC/MS), chromatographic resolution was not an important criterion. CN column (Ultremex 5, 5µm, 30 X 4.6 mm id) was utilized to obtain sufficient retention of the analytes without endogenous interference from the biomatrix.

c) Mobile phase

Mobile phases with varying proportions of organic and aqueous solvents at different pH conditions and molarities of the buffer salt were tested during assay development. The type of the buffer salt utilized in the mobile phase depended upon the function it performed in different systems.

In system A phosphate buffer (KH_2PO_4) of a molarity of 25 mM buffering a pH of 4.0 was utilized to provide strength for mobile phase to elute the analytes with proper peak shapes. Decreasing either molarity or increasing the pH of the buffer in the mobile phase resulted in increased retention times of the analytes and excessive tailing. Acetonitrile was selected as the organic solvent in the mobile phase due to its less viscosity and good elution strength. Solvent mixtures of acetonitrile and buffer were prepared in such ratios so as to make compromise among good baseline resolution, minimum analysis time and column stability. With mobile phase containing 65% acetonitrile and 35% buffer (pH 4.0) at a flow rate of 1.5ml/min the compound eluted as a sharp peak.

Although LC/MS is a very powerful analytical tool, there are some important issues that should be addressed to appreciate the results (6). The optimization of mobile phase systems for LC/MS is sometimes troublesome, since the number of solvents and additives that can be used is limited. In practice the, the majority of mobile phases used for LC/MS contain water, with methanol or acetonitrile as organic modifiers, and ammonium acetate or formate, which is adjusted to the preferred pH with ammonium hydroxide or acetic or formic acid. The use of conventional phosphate salts generally cause problems such as contamination of the ion sources or suppression of the ionization. Moreover the organic modifiers, which can be applied in, reverse phase chromatography result in suppression of the ionization (9). Therefore the optimization of mobile phase systems for LC/MS requires coupling of the "art" of HPLC with a thorough understanding of the "science" of API/MS techniques. System B utilizes potassium hydroxide of 1 mM molarity, pH adjusted to 4 with glacial acetic acid as the aqueous component of the mobile phase. This aqueous phase provided maximum facilitation of the ionization of the analytes resulting in $[M+H]^+$ compared to ammonium acetate buffer, which is the widely used volatile buffer. Increasing the molarity of potassium hydroxide resulted in suppression of the ionization and precipitation of the potassium acetate salts in the ion source. Since a buffer of very low strength was used, elution strength of the mobile phase could be achieved by the use of a mobile with a high percentage of organic solvent. Methanol was found to facilitate maximum ionization of the analytes but was not sufficient to provide better peak shapes, while use of acetonitrile resulted in the elution of the analytes as sharp peaks but with a decreased sensitivity. A mobile phase containing 45% acetonitrile, 45% methanol and 10% potassium hydroxide (1 mM), pH adjusted to 4.0 with glacial acetic

acid, at a flow rate of 1ml/min eluted the analytes with a run time of 10 min and without any loss in the sensitivity. The high percentage of acetonitrile in the mobile phase ensured rapid and complete elution of the analytes from the column, while the acidity of the of the mobile phase and methanol permitted better detection of the analytes as positive molecular ions.

d) Detection

The analytes I, II and III exhibited both UV and fluorescence. UV absorption maxima were observed in mobile phase at 270 and 240 nm. Excitation and emission maxima for fluorescence were found at 250 and 350 nm respectively. Fluorescence was more sensitive than UV and was therefore used for quantification of the analytes in system A. Fluorescence detection enabled quantification of 62.5 ng/ml of I, II and III.

Preliminary investigations showed that I, II, III and IS were good candidates for LC-ESI/MS and their full scan positive mass spectra showed protonated molecules [M + H]$^+$ at m/z 297.2, 369.3, 429.2 and 369.2 respectively, as shown in **Figure 1**. The intensities of the protonated molecules were checked at various cone voltages from 20-70 V and a cone voltage of 61 V was found to generate maximum intensity signal for the analytes and IS. Although the analytes were not completely resolved, however, their chromatograms were perfectly extracted from the total ion chromatogram (TIC) at m/z 297.2for II, 369.3 for III and IS and 429.2 for I respectively **Figure 2**. LC/MS with selected ion monitoring (LC/MS/SIM) of the analytes increased the quantitative ability by 62.5 times with a limit of quantitation of 2.5 ng/ml with the samples being injected onto the column using a 20 µl loop as compared to 50 µl loop in system A.

Figure 1: *Full scan positive mass spectra of a) I, b) II, c) III, d) I. S.*

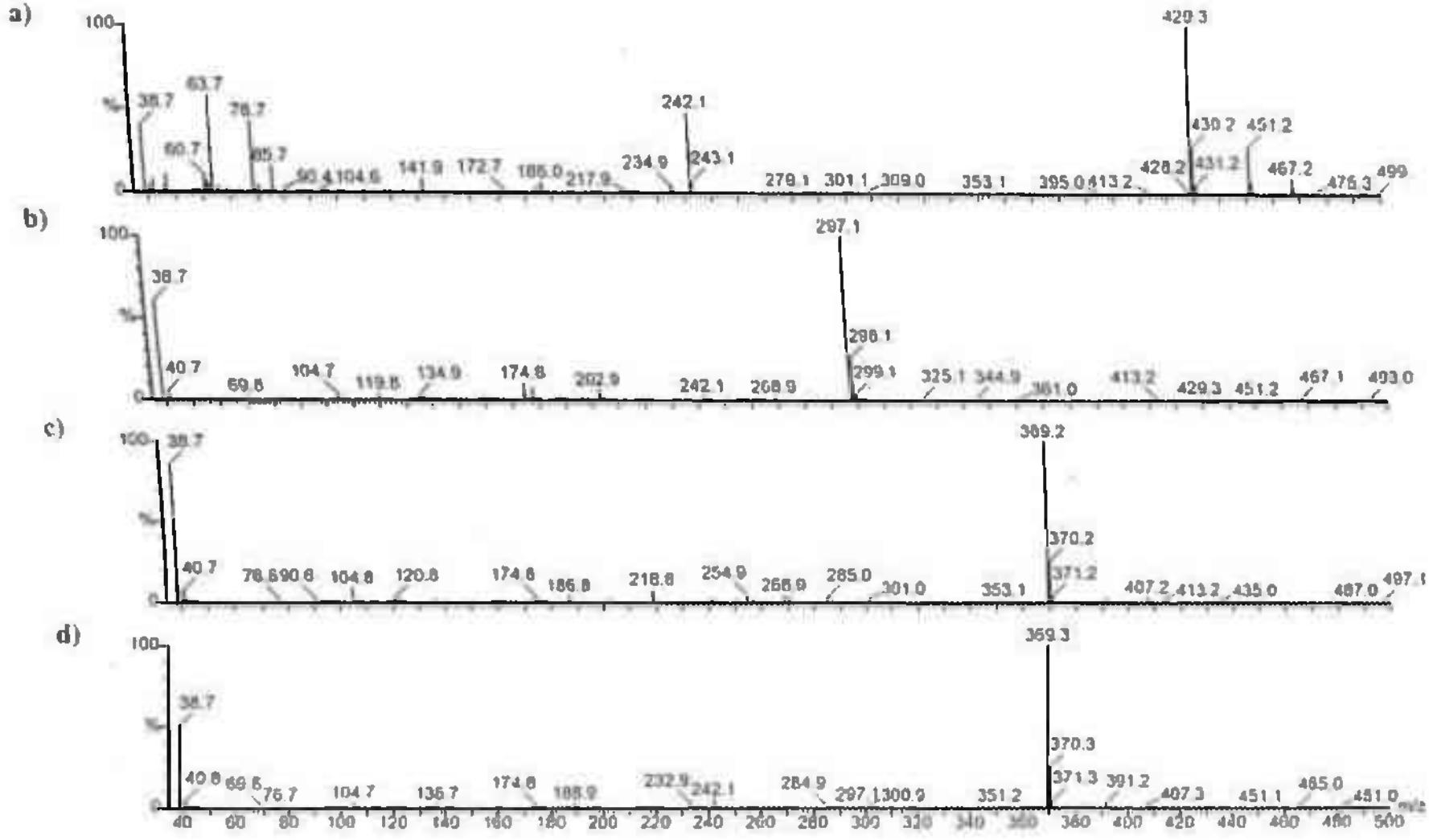
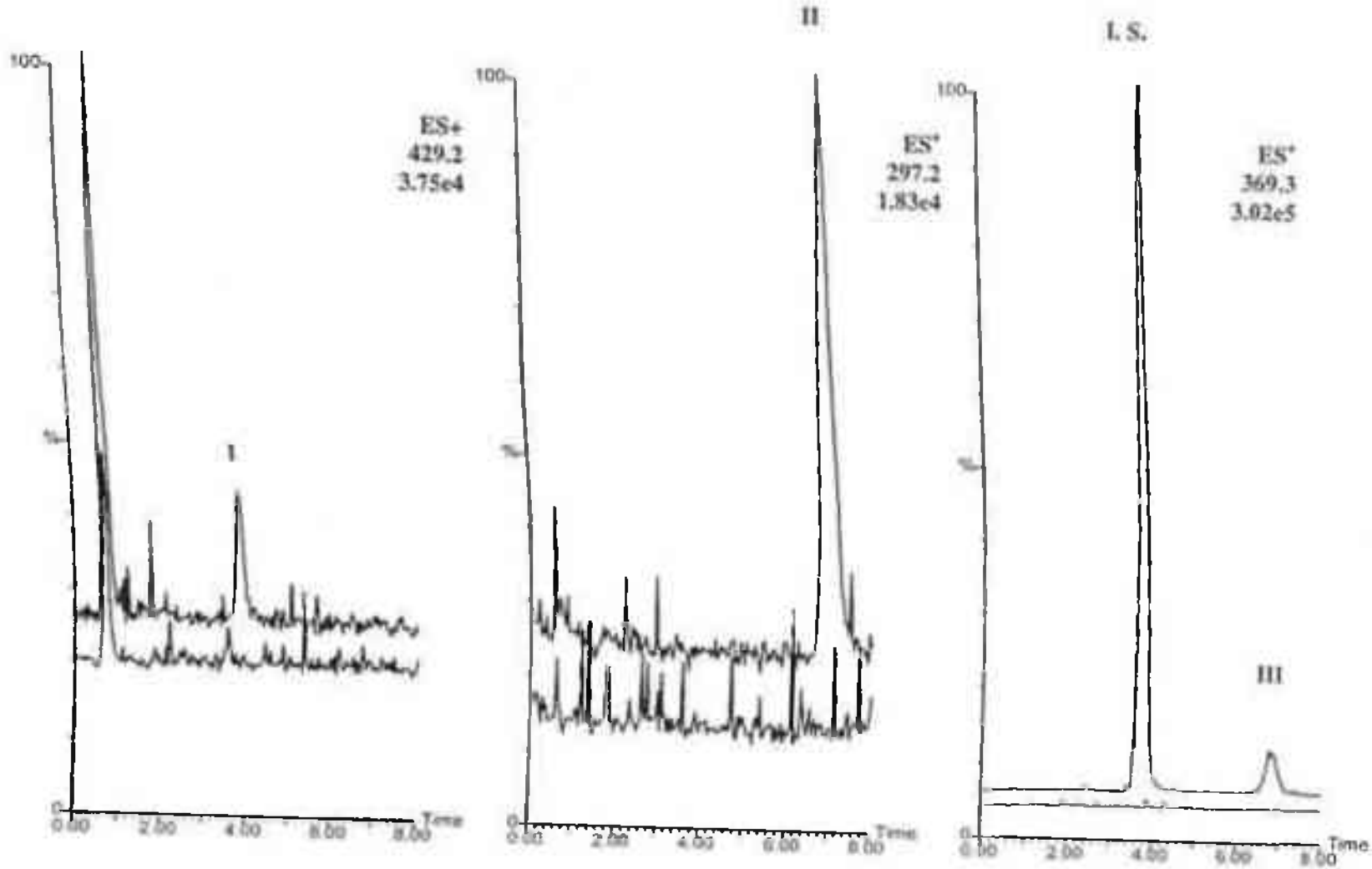


Figure 2: *Overlay of the LC/MS chromatograms of Mobile phase blank and analytical standard containing mixture of I, II and III (2.5ng/ml) and I. S. (50 ng/ml).*



(e) Standard curves in mobile phase

Standard solution containing I, II and III with or without IS were analyzed with each batch of bioanalysis to confirm recovery of analytes form biomatrix. The peak heights of all the three compounds varied linearly and were reproducible under the analytical conditions of system A, while the ratios of the peak areas of the analytes with that of IS varied linearly under analytical conditions of system B (Table 7).

Table 7: Calibration curve equation for I, II and III for the analytical standards in acetonitrile/mobile phase (n =3).

System	Concentration range (ng/ml)	Compound	Equation	Weight	Slope	Intercept
System A normal rat serum	62.5 - 2000	I	y = mx	-	170.8	-
		II	y = mx	-	231.2	-
		III	y = mx	-	23.5	-
System B rat liver S9 fraction	50 - 4000	I	y = mx+c	$1/x^2$	0.0023	-0.011
		II	y = mx	-	0.0049	-
		III	y = mx	-	0.0079	-
System B normal rat serum	2.5 - 100	I	y=mx +c	$1/x^2$	0.0066	0.0006
		II	y = mx+c	$1/x^2$	0.0179	0.006
		III	y = mx+c	$1/x^2$	0.0228	0.0122

2.3.1 Estimation of I, II and III in biomatrix

a) Method A

This method was followed for the sample clean up and extraction of the analytes from normal rat serum when system A was used for the detection of the analytes. The sample cleaning of 0.5ml serum was tested using various solvents. Direct extraction of the biomatrix resulted in endogenous interference in either one of the regions of interest or low extracting efficiency. Therefore a sample preparation method involving double sample cleanup followed by single extraction was developed. The endogenous serum interference was partially removed by initial precipitation with acetonitrile, which also improved extraction efficiency. The acetonitrile layer containing the analytes of interest were transferred into a different tube and evaporated to dryness. The residue so obtained was acidified with 200 µl of 0.5 N HCl. Since the analytes were weak bases addition of 0.5 N HCl converted them into salts/charged species resulting in appreciable solubility in polar solvents like water and little

solubility in nonpolar solvents like n-hexane. Back-extraction of the acidified layer with n-hexanne resulted in the removal of lipophillic endogenous serum components. Finally the compounds of interest were re-extracted from the basified aqueous phase with 2% IPA in ether. The dry residue was reconstituted in 0.1 ml of acetonitrile. Ultimately the analytes were enriched by 5 times. The assay method was found to be specific for I, II and III with the analyte regions free from endogenous interferences. The chromatogram of the blank control serum (500µl) is given in **Figure 3a**. An HPLC chromatogram of the control rat serum spiked with all the three compounds is shown in the **Figure 3b**. Linear regression of the calibration data demonstrated linearity for the peak heights of I, II and III. The calibration curve was linear over the range of 12.5 ng/ml to 400 ng/ml. An unweighted (y = mx) linear equation was used to perform standard calibration, which is represented in the **Table 8**. The LOD determination demonstrated that all the analytes gave a signal-to-noise ratio of 3 and above for 12.5ng/mL extracted/injected level.

b) Method B

The basic method for the extraction of the analytes in serum followed in system A was adapted to system B. When the analytes along with IS were quantified using MS in SIM mode, no interference of the matrix was observed on direct extraction with 2% IPA in ether after the basification of the aqueous phase with 2 N KOH. This high specificity of LC/MS/SIM enabled direct extraction of the samples without the need for sample clean up by acetonitrile or back extraction with n-hexane. This resulted in marked decrease in time and labor spent in extraction without sacrificing the sensitivity for the estimation of the analytes in serum. Moreover, the high sensitivity of the method resulted in minimal sample requirement of 0.1 ml and eliminated the necessity of sample concentration. 0.1 ml of the sample was spiked with the IS and basified with 12.5 µl of 2 N KOH. The analytes of interest in the basified matrix were extracted twice with 2% IPA in ether. The selectivity of the method is represented in **Figure 4**, which depicts an overlay of the blank extracted serum chromatogram with blank control spiked rat serum (I, II and III 2.5 ng/ml, I. S. 50 ng/ml). Linear regression of the calibration data demonstrated linearity between peak area ratio of the analytes and IS. The calibration curve was linear over the range of 2.5 ng/ml to 100 ng/ml is represented by the equation shown in **Table 8**.

The same sample preparation method was applied to rat liver S9 fraction. The extraction method resulted in clean analyte regions as shown in **Figure 5**. Linear regression of the calibration data demonstrated linearity between peak area ratios of the analytes and IS over range of 50 ng/ml to 4000 ng/ml represented by the equation shown in the **Table 8**.

Table 8: Calibration curve equation for the standards in normal rat serum (n=3).

Extraction method	Concentration range (ng/ml)	Analyte	Equation	Weight	Slope	Intercept
Method A		I	y = mx	-	822.89	-
normal rat	12.5 - 2000	II	y = mx	-	1092.76	-
serum		III	y = mx	-	83.140	-
Method B		I	y = mx+c	$1/x^2$	0.0027	-0.0356
Liver S9	50 - 4000	II	y = mx	-	0.0055	-
fraction		III	y = mx	-	0.0092	-
Method B		I	y=mx +c	$1/x^2$	0.0115	0.0025
normal rat	2.5 - 100	II	y = mx+c	$1/x^2$	0.02289	0.0090
serum		III	y = mx+c	$1/x^2$	0.02566	0.0039

Figure 3: *HPLC/Fluorescence chromatograms of a) blank control normal rat serum b) spiked control rat serum (80 ng/ml).*

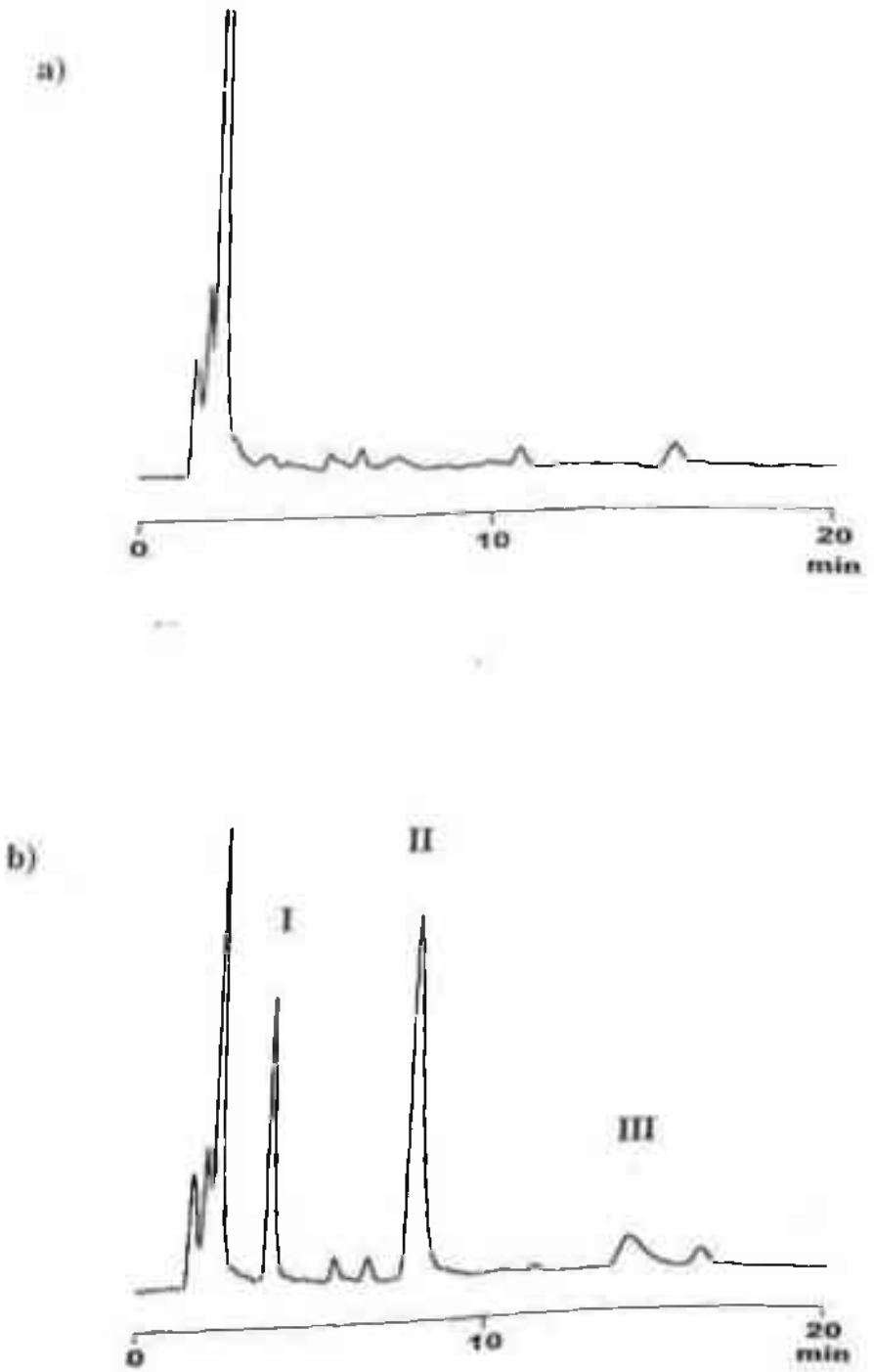


Figure 4: *Overlay of the LC/MS chromatograms of normal rat serum blank and spiked rat serum blank of I, II and III (2.5ng/ml) .5ng/ml) and I. S. (50 ng/ml).*

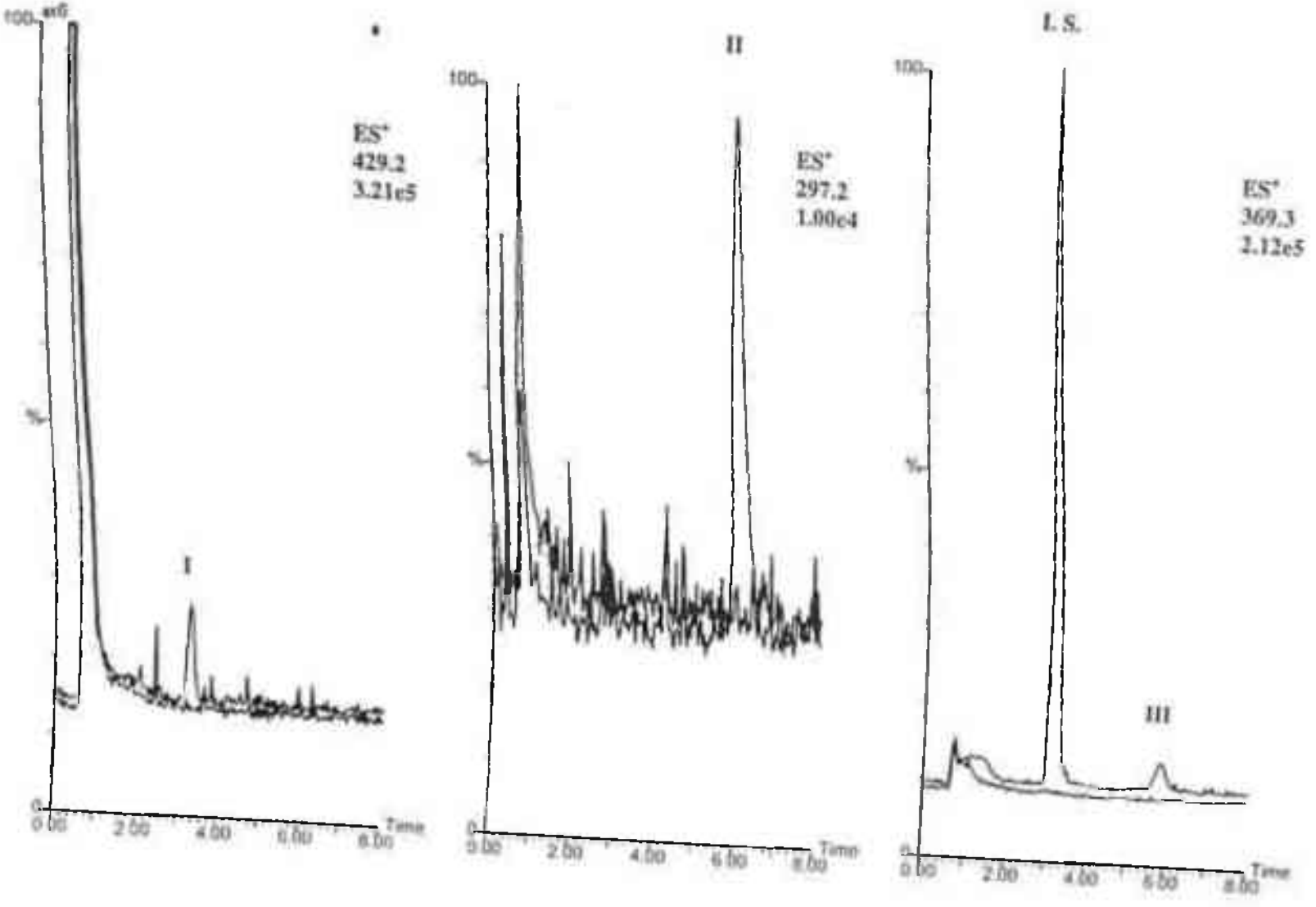
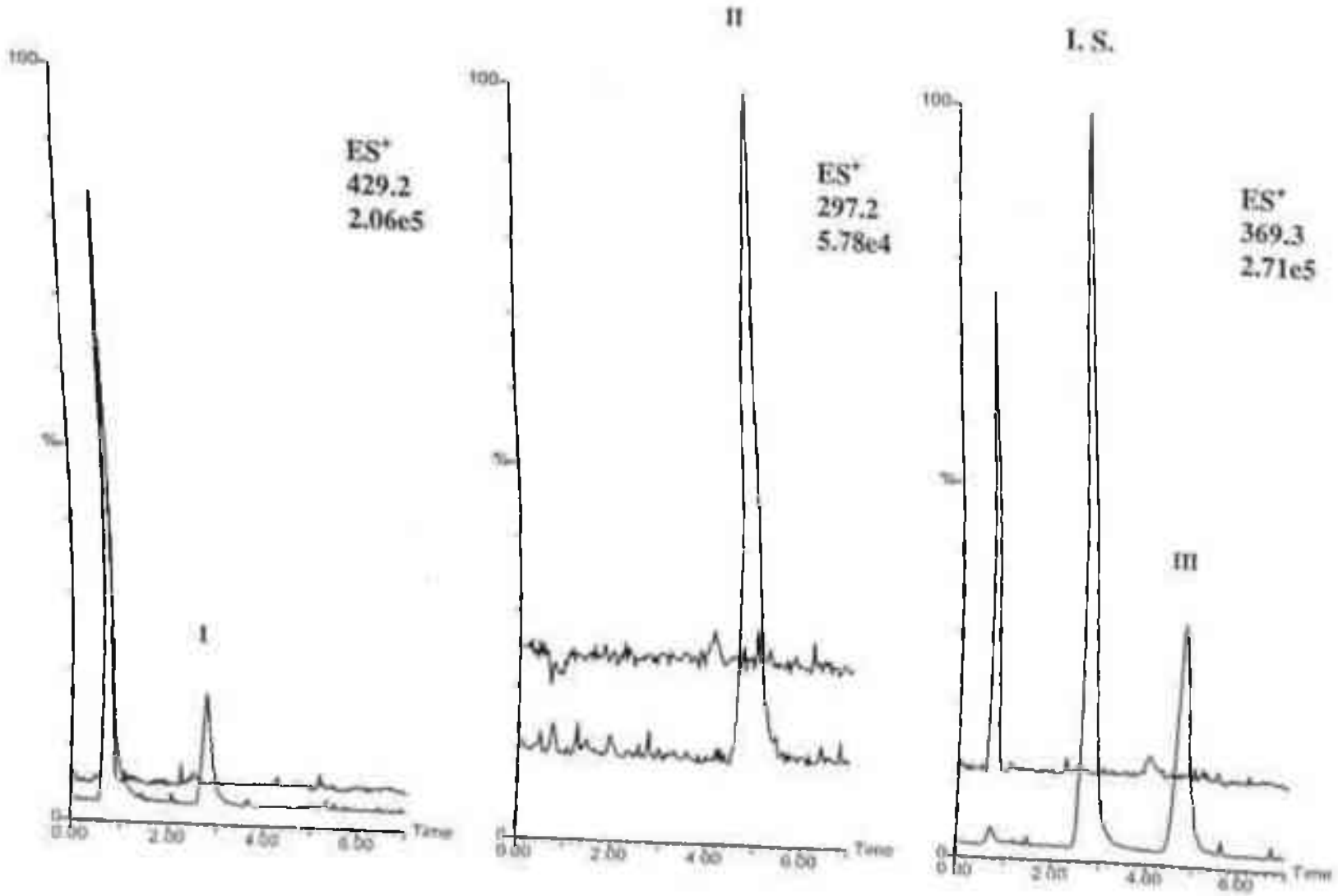


Figure 5: *Overlay of the LC/MS chromatograms of normal rat liver S9 fraction blank and spiked rat liver S9 fraction blank of I, II and III (50 ng/ml) and I. S. (500 ng/ml).*



2.3.2 Assay validation by methods A and B

The assay methods were validated in terms of recovery, intra/inter batch accuracy and precision at low, medium and high concentrations in rat serum. The various aspects of the assay validation for the developed methods (A, and B) is discussed in detail under the following headings:

(a) LOD and LOQ

The LOD for system A was 12.5 ng/ml for all the analytes while the LOQ was 16 ng/ml. When method B was applied to rat serum, the LOD and LOQ were 2.5 ng/ml while, for rat liver S9 fraction, they were found to be 50 ng/ml.

(b) Recovery

The recovery of I, II and III at different concentration levels were consistent. The recoveries of I, II and III ranged from 85-100%, 70-90% and 45-70% respectively in rat serum. In rat liver S9 fraction, the recoveries ranged between 55-70% for all the analytes. The recoveries for all tha analytes for all the systems are summarized in **Table 9**.

(c) Accuracy and precision

Table 9 summarizes the within- and between run precision and accuracy for the determination of the analytes in various methods. Variations in intra and inter-batch accuracy and precision were within acceptable limits of ± 20 % at LOQ and ± 15% at higher concentrations (**7**).

Table 9: Precision, accuracy and recovery of I, II and III in spiked biomatrix (n = 3 days).

Method	Analyte	Concentration (ng/mL)	Recovery	%Bias		%RSD	
				Intra batch	Inter batch	Intra batch	Inter batch
A	I	16	101.56±4.70	3.38	3.21	6.58	3.82
		80	96.68±6.80	6.23	6.71	0.53	1.47
		400	97.54±8.61	1.66	0.72	6.42	3.89
	II	16	98.28±5.60	6.24	8.94	1.41	5.88
		80	97.18±0.89	3.43	3.43	1.56	3.45
		400	91.47±0.52	4.92	0.4	8.53	5.75
	III	16	73.08±1.41	6.42	6.62	1.05	0.44
		80	66.55±0.22	5.29	5.31	1.11	1.39
		400	73.95±4.76	-2.96	-6.16	6.47	12.89
B (serum)	I	2.5	102.02±15.6	2.33	2.53	5.31	6.61
		20	92.13±12.07	-1.41	-1.60	2.10	1.60
		100	87.54±10.43	8.47	8.49	5.08	2.01
	II	2.5	92.77±16.7	-1.71	-2.34	9.21	5.14
		20	75.05±0.30	-5.01	-7.98	10.56	1.27
		100	67.14±1.88	-3.74	-3.87	11.39	5.36
	III	2.5	66.94±1.77	4.31	8.03	10.03	8.03
		20	57.2±9.16	-4.51	-3.96	12.96	0.99
		100	47.63±10.43	-5.69	-5.73	3.83	2.08
B (rat liver S9 fraction)	I	50	56.2±8.4	12.72	13.10	2.46	3.08
		500	62.82±4.39	-3.20	-2.05	8.92	0.72
		4000	68.40±7.07	9.29	7.91	9.38	2.39
	II	50	64.14±0.13	-13.58	-12.2	10.13	2.63
		500	62.01±8.30	-9.03	-8.23	1.45	2.26
		4000	68.87±12.50	-8.35	-7.83	5.01	4.11
	III	50	64.38±0.25	-3.35	-7.85	13.45	1.66
		500	65.01±13.16	0.30	-1.50	8.47	0.43
		4000	65.49±6.71	0.74	1.86	1.81	2.77

2.4 Conclusion

Validation of bioanalytical methods is an important task to assure reliability of the assay data and also a mandatory requirement for various regulatory agencies. Hence, in the various assay methods developed and validated, for I, II and III, the specificity, sensitivity, calibration linearity, extraction recovery, precision and accuracy have been adequately demonstrated. Appropriate model selection was also performed after observing the data obtained during assay validation. Thus, for the application of the concept of cassette dosing for I, II and III the analytical methods are specific without any interferences from endogenous and exogenous substances and the have a low limit of quantification capable of accurate measurement to provide meaningful results.

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Chapter 3

APPLICATION OF THE CASSETTE CONCEPT TO *EX VIVO* STUDIES

In vivo studies are not always ideal in pinpointing the main issues as PK parameters such as bioavailability are based on a number of variables (1). Under these situations, the controlled environment of *in vitro* situation means that multiple factors influencing PK and drug metabolism *in vivo* can be investigated as single components. This can be seen as an advantage in that it enables a complex problem to be broken down into its constitutive parts. In terms of throughput and cost, *in vitro* screens represent an intermediate level of filtering between *in silico* and *in vivo* (2-4). Moreover, the *in vitro* models used to predict aspects of drug absorption and metabolism are popular owing to their simplicity (5). A trickier task is to use these *in vitro* systems to predict *in vivo* behavior of the drug. In addition, a good understanding of pharmacokinetic principles will help *in vitro/in vivo* extrapolation. In recent years, there has been a large expansion in both the range and use of *in vitro* systems to study absorption and metabolism. Moreover, *in vitro* screens have the advantage that human tissues can be used, thus providing early data on potential hits in the target species (3-4). Furthermore, cross-species comparisons can be rapidly be made, which could assist in the selection of appropriate species for future *in vivo* studies. The progress in utilizing *in vitro* screens during drug discovery stage has been rapid due to the ability to automate most of the *in vitro* tests. This has been achieved by some major technological developments and advances in automation, software and analytical hardware (1). For the *in vitro* screening to be most effective, it is important that the screens be applied at the right stage of the drug discovery process. Applying such screens before any potency has been achieved can be counterproductive, but conversely trying to optimize the DMPK of the compounds after other characteristics have been optimized gives less scope for improvement. Finally, it should be remembered that no lead strategy can ignore the utility of *in vivo* models and it is anticipated that however elaborate or predictive the *in vitro* approach becomes, the permeability and metabolism determined in vitro are not subjected to full range of influences experienced in vivo. Therefore, in vitro screens are commonly regarded as ranking tools used to prioritize compounds for relatively slower throughput in vivo investigations (6). The confidence in the validity of the screens has to be established with targeted in vivo experiments.

The concept of cassette (N-in-One) was applied to various *ex vivo* screens to explore for the ability to provide information regarding the *in vivo* behaviour and ultimately rank the compounds on the basis of their PK characteristics. The subsequent sections discuss in detail

various *ex vivo* techniques utilized to explore the behaviors of I, II and III. Protein-binding studies of the compounds was performed to search for the possibility of displacement reactions, when the compounds were administered as a cocktail. The validity of the selection of the compounds in the cassette is established only if there is no displacement of one or more compounds in the presence of the other compounds. Since oral delivery of the compounds was desirable, evaluation of the capability of the compounds to cross the gastro intestinal tract (GIT) barrier was carried out using *in situ* recirculation technique. This was followed by *in vitro* screening for the metabolic stability of the compounds in rat liver S9 fraction.

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Chapter 3

Section 1

PROTEIN BINDING STUDIES

3.1.1 Introduction

Drug molecules circulate in blood in two forms: bound to the blood components such as proteins, red cells, or platelets and those, which are unbound and are dissolved in plasma water (1-3). The extent of drug binding to plasma proteins varies widely among different types of drugs and depends on the physicochemical properties of the drug. The protein-bound fraction ranges from less than 0.1 for caffeine and ethosuximide to greater than 0.99 for highly bound drugs such as warfarin and dicumerol (4). The effects of changes in the extent of protein binding on free drug fraction and on drug metabolism are significant for highly bound drug (greater than 80% bound), since even a relatively small change in the degree of binding has a dramatic effect on the free fraction. Albumin is quantitatively the most important drug binding protein for many drugs, particularly the neutral and anionic (acidic) drugs such as warfarin, phenytoin, and valproic acid (5). In the recent years, cationic (basic) drugs such as propranolol, quinidine, and the tricyclic antidepressants have been shown to bind not only to albumin, but also to other blood proteins such α_1-acid glycoproteins (AAG) and lipoproteins (6-8). In some cases binding to AAG exceeds that to albumin. Fluctuations in plasma levels of AAG and lipoprotein fraction such as low-density lipoproteins are common and point to importance of understanding the interactions of cationic drugs with these proteins (6, 9, 10).

Protein binding has an important effect on the pharmacokinetics of the drugs. The alterations in the protein binding have an impact on the clearance characteristics of a drug. This phenomenon is generally observed in those drugs that are removed from the plasma by glomerular filtration through the kidneys or by passive uptake by the liver. The clearances of such drugs are sensitive to alterations in protein binding. A higher free fraction will lead to higher clearance rates, shorter elimination half-lives and a larger fluctuation in peak and trough levels. Drugs exhibiting such behaviour are called as restrictively cleared drugs Eg: Theophylline and warfarin (11, 12-14). However, some drugs are extracted by active mechanisms in both free and bound forms by the liver or the kidneys. Their clearance rates are dependent on organ blood flow and relatively independent on protein binding (15). Such drugs are known as non-restrictively cleared drugs Eg. Lidocaine and propranolol (16-18).

A widely held view on the pharmacological effect of protein binding is that only the free form of the drug can cross the capillary, the cell membranes and reach the site of action. Thus, plasma-protein binding can influence both pharmacokinetic and pharmacodynamic properties of

a drug.

Plasma-protein binding is usually evaluated *in vitro* and found to be well correlated with *in vivo* in majority of cases. Many techniques have been used to study the binding of drugs to proteins and to measure the free drug fraction. Among these are equilibrium dialysis, ultrafiltration, ultracentrifugation, and gel-filtration (**19-21**). The basic principle of all the techniques is physical separation of the free drug from the bound drug. Even though equilibrium dialysis has been employed most frequently, it has a major disadvantage of being laborious and may require many hours or even days to reach equilibrium. Therefore, ultrafiltration has been an attractive alternative to equilibrium dialysis. Furthermore, centrifugation to obtain ultrafiltrates from multiple specimens is technically simpler and more rapid than equilibrium dialysis, making ultrafiltration more suitable for the free drug measurement in the clinical laboratory environment.

3.1.2 Experimental

In ultrafiltration a pressure gradient is generated by centrifugation, which forces plasma water and small molecules to pass through the semipermeable membrane. In general drug binding to proteins is inversely proportional to the temperature, thus an increase in the latter leads to a decrease in binding and vise verse. Since the physiological temperature is about 37°C, thus *in vitro* determination of protein binding is carried out by maintaining the surrounding temperature to 37°C. Pressure gradient is maintained low but sufficient to generate acceptable filtration rate and is best maintained by low speed centrifugation (1000-2000 g). Sieve effects due to polarization of protein on the membrane may be minimized by centrifugation in a fixed angle rotor. Prior to determination of protein binding using ultrafiltration devices, it is very essential to determine the nonspecific adsorption of the candidate drug onto these devices. In such a case where the drug adsorbs onto the membrane or the device it is very difficult to estimate the protein binding (**22**).

3.1.2.1 Materials

Pure reference standards I, II and III were procured from Medicinal Chemistry Division, Central Drug Research Institute (CDRI), Lucknow. Ultracentrifuge tubes (cap., 1 ml) were obtained from Centrifree®, Amicon Micropartition System, Amicon, USA.

3.1.2.2 Drug formulations

i) Drug formulation (µg/ml) in K_2HPO_4 buffer (0.1 mM, pH 7.4)

K_2HPO_4 buffer (5 ml) was spiked with 125 µl of the stock dilution (200 µg/ml) of I, II and III as discrete and cassette to give a final concentration of 5 µg/ml.

ii) Drug formulation (2.5, 5, 10 µg/ml) in normal rat serum (NRS)

30 µl of stock solutions (250, 500, 1000 µg/ml) of I, II and III as discrete as well as cassette were transferred to test tube and evaporated to dryness the residue was dissolved in 3ml NRS to give a final concentration of 2.5, 5, 10 µg/ml.

3.1.2.3 Adsorption studies to ultracentrifuge tube

The experiment was performed at 37°C. 1 ml aliquot (spiked buffer) was transferred into an ultracentrifuge tube and placed in the centrifuge maintained at 37 °C. The samples were centrifuged at 4000-4500 rpm for 10 min to collect ~10% (100 µl). The ultrafilterate was processed and percentage of adsorption was calculated by the following equation:

$$\% \text{ adsorption} = \frac{\text{Initial concentration} - \text{Final concentration}}{\text{Initial concentration}}$$

3.1.2.4 Protein binding study

The experiment was performed at 37°C. 1 ml aliquot (spiked serum/2.5 min i.v. dosed sample) was transferred into an ultracentrifuge tube and place in the centrifuge maintained at 37 °C. The samples were centrifuged at 4000-4500 rpm for 10 min to collect ~10% (100 µl) and analyzed. The percentage of adsorption was calculated by the following formula.

$$\% \text{ Bound} = \frac{Ct - C_u \times 100}{C_t}$$

where, C_t = total concentration and C_u = unbound concentration

3.1.2.5 Sample analysis

Samples were diluted 1:1 with acetonitrile and analyzed by HPLC method reported for System A in 2.2.1 and 2.3.1.

3.1.2.6 Protein determination

The protein content of the serum utilized in the experiment was determined by Lowry method (23).

3.1.3 Results and discussion

The present *in-vitro* studies were carried out to determine the extent of serum protein binding of I, II and III by using Cassette Dosing approach. The study was utilized as a criterion to determine the validity of the selection of the compounds in cassette for *in vivo* studies. Bound and unbound compounds from spiked serum were separated by ultra-filtration technique. Nonspecific binding of the compounds were found to be less than 5%. The percent of binding of I ranged from 54 – 59% **(Table 1)** while that of II and III were found to be above 95% over a concentration range of 2.5 – 10 µg/ml and also in 2.5 min *i.v.* dosed samples. Moreover, the binding of all the three compounds as cassette were found to be comparable with the results obtained by carrying out experiments on discrete. The protein content was found to be 75.2±2.8 mg/ml and remained unaltered in both discrete and cassette.

Table 1: Comparison of the % of I bound to proteins in normal rat serum in cassette and discrete (n = 3).

I	% Protein bound	
Concentration (µg/mL)	Discrete	Cassette
2.5	57.25±1.24	57.11±0.55
5.0	58.94±0.37	57.51±0.05
10	54.03±0.40	58.61±0.09
2.5 min sample	58.56±.28	58.01±0.65

3.1.4 Conclusion

The above results prove that there is no alteration of protein binding when all the three compounds are present together. Moreover, the protein binding remained unaltered in test sample indicating absence of displacement *in vivo* owing to metabolite formation. Thus the approach of Cassette Dosing can be used to determine the protein binding of the drugs. Furthermore, it can be concluded that the compounds selected for the cassette would not alter their PK characteristics owing to their protein binding characteristics.

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Chapter 3

Section 2

IN SITU ABSORPTION STUDIES

3.2.1 Introduction

Oral route of administration is the most convenient and popular route for drug delivery. Hence, it is essential to estimate its absorbability for developmental novel drugs/ dosage forms or both. The success of an oral drug delivery depends on both the physicochemical and biological factors (1-3). The physicochemical factors include formulation factors (dosage form) and drug factors (solubility, chemical and enzyme stability, lipophilicity, hydrogen bonding potential, conformation, pKa, molecular size, and affinity for endogenous transporters). The biological considerations that will determine the success of an oral delivery include gastric emptying rate, intestinal motility, the composition (e.g. pH, enzymes, food) of the intestinal lumen are the most significant biological barriers to oral drug delivery.

Experimental techniques for studying the transport of drugs by the intestine include the use of isolated cells (4), everted gut sacs (5), perfused preparations (6), and whole animal methods involving intraduodenal, -portal, -venous, and -arterial routes of administration (7). Among these methods only the perfused organ preparation has the property of allowing precise control of experimental conditions such as blood flow. Some of these methods require large animals such as dogs (8), or involve gut perfusion of only the gut lumen (9), other methods involve complex surgery in the rat. Other methods, which provide the advantages of high-throughput, are cell monolayers – most commonly, Caco-2 cells, which are derived form human colon adrenocarcinoma and Mandin-Darby canine kidney cells (MDCK) (10-12).

In situ recirculation (13) or *in situ* closed loop method (9) is usually used for estimation of absorbability of drugs. The information obtained from these studies, e.g. absorption rate constants, k_a, is the average value through the intestinal tract. These systems better mimic *in vivo* physiological process and hence provide more realistic experimental conditions. The main advantage is that specific areas of GIT may be isolated and studied while venous supply of GIT remains intact. The perfusion of drug solution through the intestinal lumen at constant rate permits the measurement of rate of disappearance of drug from intestinal lumen. This technique has been used to differentiate drugs exhibiting rapid, moderate or slow absorption rates and is applicable to various animals like rats, rabbits and dogs. It has the advantages that the half-lives for expressing the first order rates of absorption are realistic and are scalar quantities comparable to those estimated from blood concentration data. In many cases this approach is satisfactory but may overestimate absorption rate constants for substances that are metabolized/degraded or

bound extensively to the lumen. The absorbability of drugs has usually been estimated by *in-situ* recirculation studies or *in-situ* closed loop studies. The information obtained from these studies, e.g. absorption rate constants, k_a, is the averaged rate constant through the intestinal tract. There are, however, site differences in absorbability of drugs and the concentration observed in the plasma is the sum of the amounts of the drug absorbed from each segment. It has been reported that these techniques yield absorption rate constants that are realistic and comparable to those observed following oral drug administration. In addition a reduced time is required for obtaining accurate, reproducible and quantitative kinetic absorption data.

The concept of Cassette Dosing was applied to screen I, II and III, in house CDRI molecules, for ranking their ability to cross the intestinal barrier utilizing the *in situ* recirculation studies.

3.2.2 Experimental

The experimental technique used a fixed length of intestine, with an intact blood supply of an anaesthetized *Sprague Dawley* rat weighing 200±25 g. Intestinal absorption of I, II and III were examined by means of an *in situ* recirculation experiment. Usefulness of this experimental technique stems from the fact that plots of log drug concentration versus time give a detailed profile of the drug disappearance process, which often simply reflects the drug absorption. Although the data observed in these studies depict drug disappearance rates from the gut lumen fluids, the terms disappearance and absorption will be used interchangeably since absorption is the driving force for the disappearance process in the gut just as it is for the appearance process in the rising blood concentration curve. The rats were fasted for 16-20 hours prior to the experiment. However, drinking water was readily accessible.

3.2.2.1 Materials

Pure reference standards I, II and III were procured from Medicinal Chemistry Division, Central Drug Research Institute (CDRI), Lucknow. A peristaltic pump (Wiz peristaltic pump, Isco Inc., Lincoln, Nebraska, USA) fitted with silicone tubing (1/16" i.d. x 3/16" o.d.) was used for pulse free movement of perfusion/drug solution. Urethane for anesthesia was procured from S.D's Lab-Chem Industry, Mumbai, India.

3.2.2.2 Reagents

(i) Sorensen buffer (pH 7.4): The buffer was prepared by adding 19.2 ml of 0.067 M KH_2PO_4 solution and 80.8 ml of 0.067M Na_2HPO_4 solution (11).

(ii) Perfusion solution: The perfusion solution was prepared using NaCl: 1.45 x 10^{-1} M, KCl: 4.56 x 10^{-3} M, $CaCl_2$: 1.25 x 10^{-3} M, NaH_2PO_4: 5.00 x 10^{-3} M (11).

All buffer solutions were filtered through 0.22 µm filtration membrane before use.

3.2.2.3 Drug formulation

1 mg of I, II and III were weighed and transferred to 250 ml conical flasks containing 100 ml of Sorenson buffer pH 7.4 respectively. For the concept of Cassette Dosing 1mg of each of the compounds were weighed into a 250 ml conical flask containing 100 ml Sorenson buffer pH 7.4 (Formulation A). The flasks were placed in a thermostatic incubator shaker for 12 h at 37°C slowly agitated. The suspended drug particles were filtered off and the filtrate was analyzed for drug content by HPLC using System A.

3.2.2.4 Adsorption studies

To confirm the nonspecific binding of the compounds to the silastic cannula, 10 ml of drug solution was transferred to 25 ml beaker. Using the peristaltic pump the drug solution was recirculated through the silastic cannula for 60 minutes. 100 µl samples were withdrawn from the reservoir at 10 min intervals and were analyzed by HPLC. Adsorption to the cannula was calculated using the equation

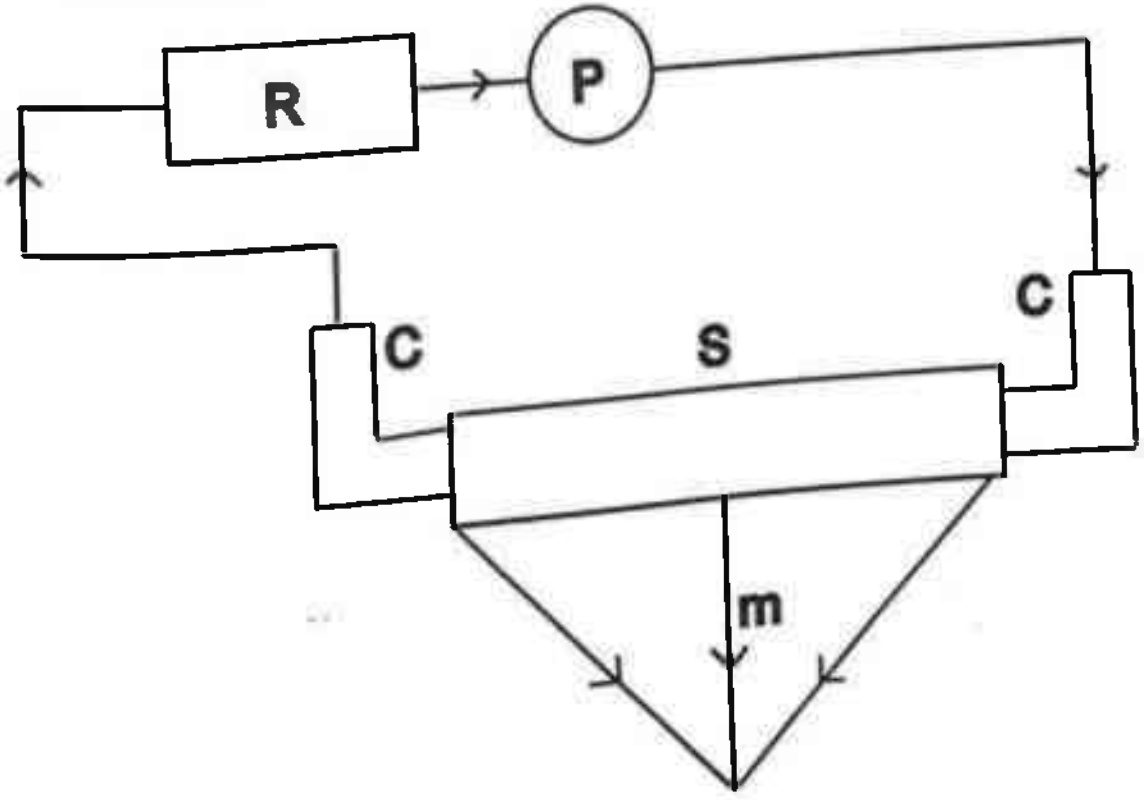
$$\% \text{ binding} = [(\text{Initial Concentration} - \text{Final Concentration}) \div \text{Initial Concentration}] \times 100$$

3.2.2.5 Cannulation and Sampling

Young, healthy, male Sprague Dawley rats weighing 200±25g were procured from the Laboratory Animal Division of the institute. Rats were housed in-groups of three in plastic cages with free access to standard pellet food (Goldmohar Laboratory animal feed, Lipton India Ltd., Chandigarh, India) and water. Required numbers of rats were fasted for 16-20 h prior to the performance of the experiment. However, drinking water was supplied ad libitum. The rats were

anaesthetized by administering 1mg/ml of urethane solution intraperitoneally (11), thirty minutes prior to the surgery. The small intestine of anaesthetized rat was exposed by midline incision on the abdominal region. A 10 cm intestinal loop was prepared (13) by inserting two silicone cannulae, one isoperistaltically at the proximal end of the duodenum and the other antiperistaltically at the distal end of the ileum and tied with silk suture. The loop was washed with 30 ml perfusion solution to clear the intestinal contents. The free ends of the cannula were dipped into the reservoir as illustrated in Figure 1. The perfusion solution was then expelled and 10 ml of formulation (containing the respective compounds)/or Formulation A (containing all the three compounds as Cassette) was filled into the system. The drug solution was recirculated within the system at a flow rate of 0.9 ml/min. Samples of the intestinal solution (100 µl) were withdrawn at 0, 2.5, 5, 10, 15, 20, 25, 30 and 40 minutes from the reservoir and were stored at – 60 °C till further analysis. The entire experiment was performed under a closed hood in which heating lamps was positioned to maintain the preparation at 37±2 °C while performing the absorption studies.

Fig 1. Schematic diagram representing the experimental setup for recirculating perfusion technique. Key: (S) perfused intestinal segment; (C) cannula; (M) jejunal mesenteries; (P) peristaltic pump; (R) reservoir.



3.2.2.6 Sample analysis

Samples were diluted 1:2 with acetonitrile and vortexed for 1 min. The samples were centrifuged at 10,000 rpm and the supernatant were analyzed by System A (**Section 2.3.1**) and the concentrations of the unknowns were read from the standard curves for system as reported earlier in **Section 2.3 (e)**

3.2.2.7 Data analysis

During recirculating perfusions, the absorption of the compounds was evaluated by measuring its disappearance from the perfusate, which followed first-order kinetics. The time dependence of the luminal concentration, C can be written as:

$$\ln \frac{C_t}{C_o} = -k_{ab}t$$

Where C_o and C_t are the luminal concentration of the model compound at time 0 and t, respectively, and k_{dis} is the first-order disappearance rate constant (min^{-1}). The disappearance rate constant k_{dis} was calculated from plots of ln (C_t/C_o) verses t by linear regression (12).

3.2.3 Results and discussion

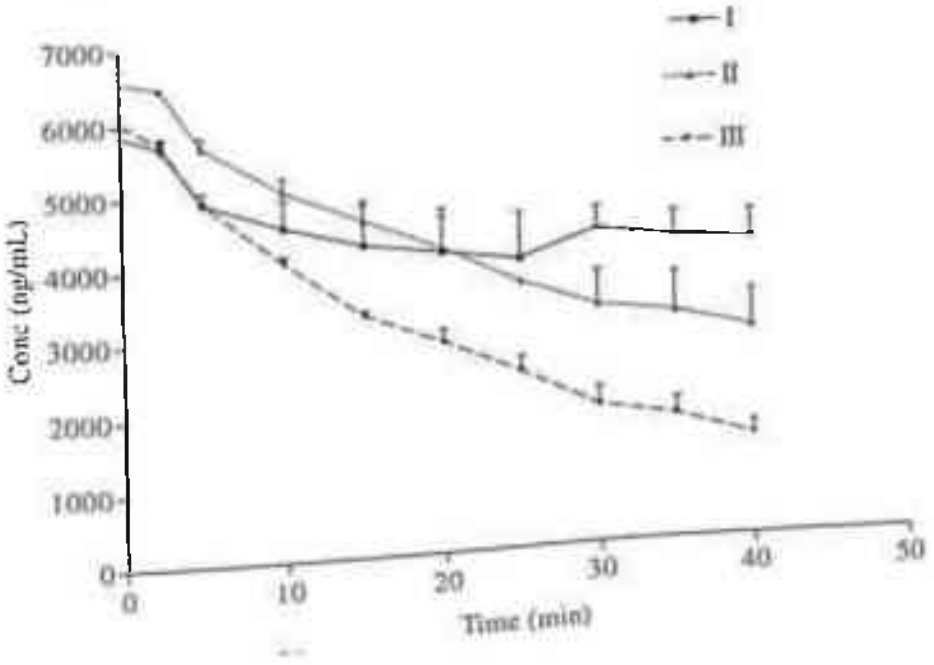
Typical plots showing the disappearance of I, II and III from the solution in intestinal lumen are shown in the **Figure 2a and 2b.** The absorption rate constant was calculated from the slope of logarithmic plots of mean [C_t/C_0] time profile by linear regression method, **Figure 3.** The disappearance and absorption rate are used interchangeably because absorption is the driving force for the disappearance of the compounds from the intestinal lumen. Nonspecific binding of the compounds to the cannula was also confirmed and no appreciable drug was found to bind to it. The absorption rate constants of the compounds determined from discrete as well as cassette are given in the following **Table 1.**

Table 1: Comparison of disappearance rate constants of I, II and III as discretes and cassette (n = 3).

Compounds	k_{dis} (min^{-1})	
	Discrete	Cassette
I	0.025±0.01	0.022±0.004
II	0.053±.0.02	0.040±.0.009
III	0.087±0.02	0.068±0.006

Fig 2: C vs t plot of I, II and III as a) Cassette b) Discrete

a)



b)

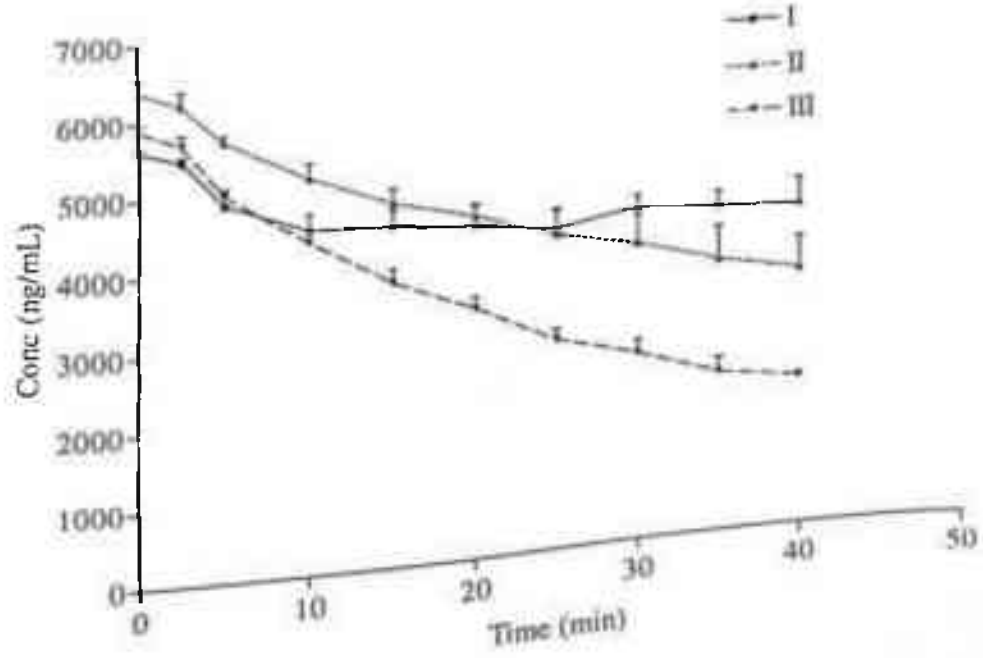
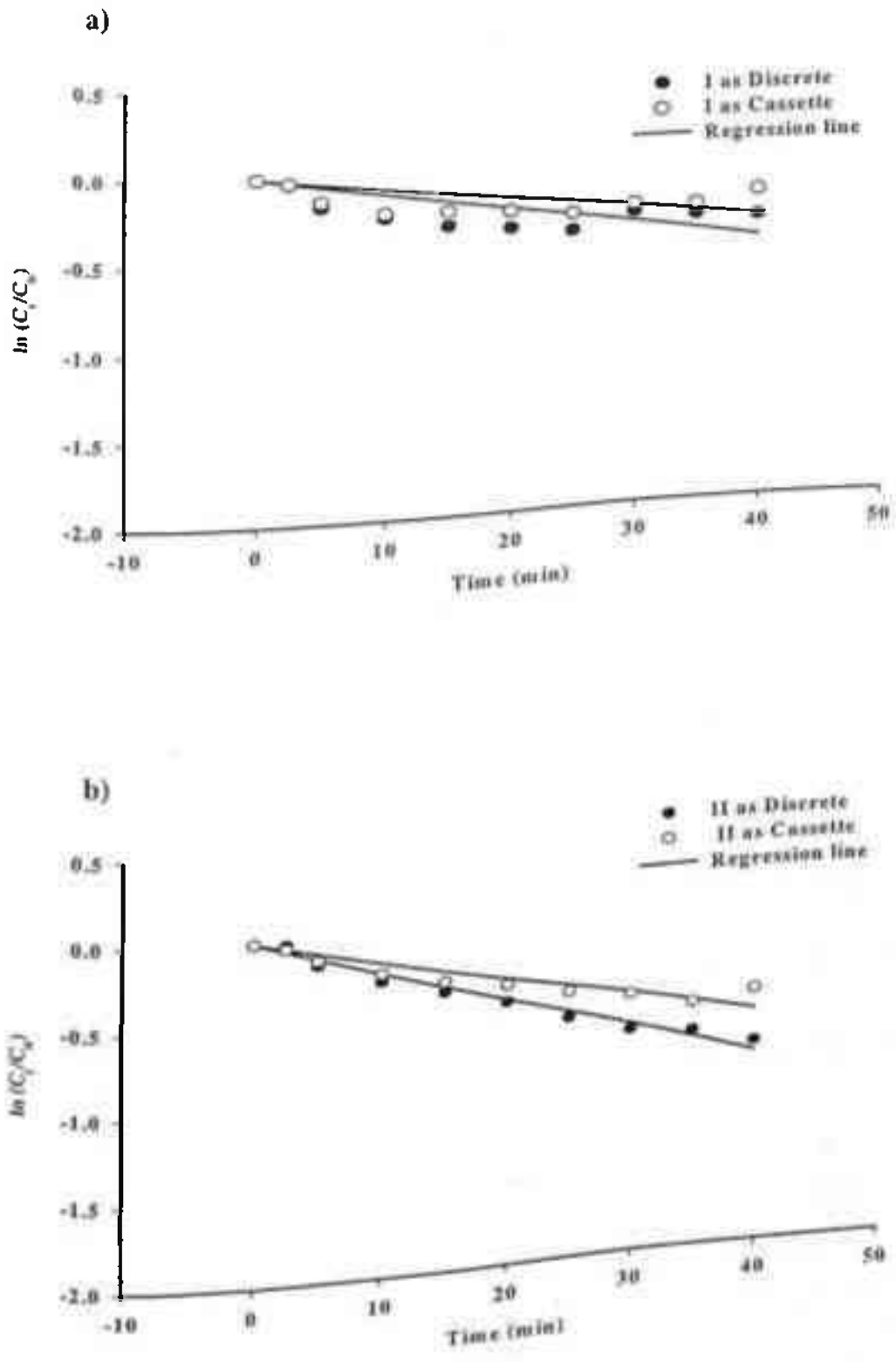
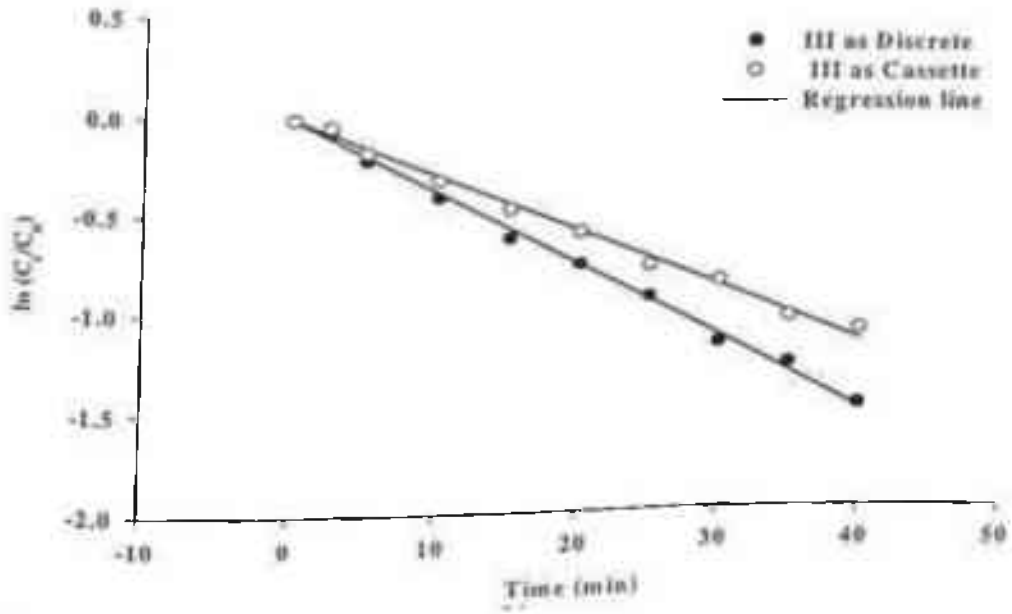


Figure 3: Comparison of *In situ* absorption from rat small intestinal lumen between Cassette and Discrete of a) I, b) II and c) III.



c)



The concentration of I, II and III remaining in the reservoir at various time points when the experiment was carried out using a cocktail of I, II and III showed a very good correlation with the values obtained when the experiment was performed discretely as evidenced by linear regression analyses. The equations governing and the coefficient of correlation of the regression analyses is shown in **Table 2**. This shows that the pattern of the disappearance of the compounds as discrete and as cassette remains same. Moreover, overlapping confidence intervals and statistical significance test performed on the absorption rate constants from the *in situ* experiments of the compounds as discrete and cassette showed no appreciable statistically significant difference ($p<0.05$).

Table 2: Regression analyses of the mean concentration of I, II and III in the reservoir as discrete and cassette.

Compound	Equation	R^2
I	$y = 0.83x + 656.9$	0.9086
II	$y = 0.78x - 1225.6$	0.9962
III	$y = 0.86x - 777.6$	0.9970

3.2.4 Conclusion

From the data obtained, it can be concluded that among the three compounds, III has the fastest rate of absorption across the intestinal barrier followed by II and I. Moreover, the concept of cassette can be successfully applied to obtain the same ranking. Application of the concept of cassette not only reduces the number of cannulation experiments to 3 as compared to 9 when the experiment is performed as discrete, but also decreases the number of samples to be processed and the analysis time by a factor of 3.

This ranking of the permeability of the compounds across the GIT barrier does not necessarily mean that the compound with the fastest ability to permeate will have the highest bioavailability, since, upon oral administration apart from GIT, liver is also a major barrier which plays a crucial role in determining the bioavailability of a molecule. Therefore, one could predict oral behavior of the compounds to a good extent from the GIT permeability studies along with data regarding metabolic stability and clearance from the body (obtained on i.v. administration).

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Chapter 3

Section 3

IN VITRO METABOLIC STABILITY STUDIES

3.3.1 Introduction

A drug that is absorbed orally is transported via the portal circulation to the liver, where it may be subjected to hepatic metabolism followed by excretion in bile or via the kidneys. A typical drug metabolism pathway is the oxidation of the parent drug (phase I oxidation) followed by the conjugation of the oxidized moiety with highly polar molecules such as glucuronic acid, glucose, sulfate, methionine, cysteine or glutathione (phase II)(1). The key enzymes for the phase I oxidation are the isoforms of cytochrome P450 (CYP) family. The major human CYP isoforms involved in drug metabolism are CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 (2). Of these, CYP3A4 is responsible for xenobiotic metabolism in almost 50% of the known pharmaceuticals. The key phase II enzymes include UDP-dependent glucuronosyl transferase (UGT), phenol sulfotransferase (PST), estrogen sulfotransferase (EST), and glutathione-S-transferase (GST). Like CYP, the phase II enzymes also exist in isoforms(1).

Drug metabolism is a key determinant of several important drug properties:

a) Metabolic stability: a drug with low metabolic stability *i.e.*, rapidly metabolized, will require multiple daily dosing or continuous infusion to maintain an adequate therapeutic plasma level. Likewise, a highly stable drug, *i.e* not readily metabolized and eliminated, could have prolonged half-life.

b) Drug-drug interaction: One of the major causes of drug-drug interactions is the interference of the metabolism of one drug by a co-administered drug.

c) Drug toxicity: a drug might be rendered pharmacologically inactive or more toxic by metabolism.

The evolution of drug metabolism field has reached a very exciting stage, primarily as a result of three major factors: first, advances in our understanding of the different drug-metabolizing enzyme systems and the ability of the various *in vitro* models for studying drug metabolism (3); second, the realization that these models can be used in conjunction with conventional *in vivo* ADME models; third, the development of analytical methods such as LC/MS, LC/MS/MS, LC/NMR, Capillary Zone Electrophoresis (CZE) that can be coupled and automated(4-8). Today, the emphasis is more on the appropriate integration of these tools in exploring the metabolic fate of NCEs. This has resulted in most pharmaceutical companies to increasingly making use of in vitro information and include it in their submissions to various regulatory agencies (3). In turn, these agencies have acknowledged the utility of the information.

both in terms of assessing drug safety and approvability. Ultimately, FDA has recently prepared a guidance package entitled "Drug Metabolism/Drug Interaction studies in drug development process: Studies *in vitro*." Therefore, where preclinical drug metabolism investigators solely relied on animal models, the same scientists now use a variety of *in vitro* models.

Since liver is the major organ for drug metabolism, a number of assay models have been developed. These include liver slices, hepatocyte cultures and subcellular fractions such as S9 fractions and/or microsomes(9). Liver microsomes or postmictochondrial S9 fractions are being commonly used in the pharmaceutical industry in the early selection of drug. The throughput of this screen has been increased with the advent of trans-well technology in combination with LC/MS analytical technologies (10). The screen is compatible with automated dispensing and sampling technologies. LC/MS is typically used to monitor the disappearance of compounds as a result of incubation without detailed investigation of the structure of the metabolites in screening model (11-12). The results of the metabolic stability are generally expressed as percentage of the parent compound disappeared.

3.3.2 Experimental

In the present study, the metabolic stability of the compounds I, II and III in rat liver S9 fraction has been evaluated and ranked by applying the concept of Cassette. The validity of the concept of the cassette has been tested by comparison with the ranking obtained from discrete.

3.3.2.1 Materials

Pure reference standards I, II and III were procured from Medicinal Chemistry Division, Central Drug Research Institute (CDRI), Lucknow. A shaking water bath (Vam 908D, Vam Instruments Pvt Ltd, India) operated at 37 °C shaking at 50 rpm for incubation. D Glucose-6-phosphate monosodium salt, Glucose-6-phosphate Dehydrogenase(Type XV from Bakers Yeast), β-Nicotinamide Adenine Dinucleotide Phosphate Sodium salt (βNADP) were obtained from Sigma Chemicals, St. Louis,MO, USA. All other chemicals used for the experiment were either analytical grade or HPLC grade.

3.3.2.2 Reagents

(i) Buffer for incubation: 8.75 g Di potassium hydrogen othophosphate (0.1 M) was dissolved in 500 ml MilliQ water and the pH adjusted to 7.4 with orthophosphoric acid (40%, v/v). The solution was filtered through 0.22 µm membrane filter.

(ii) Buffer for Homogenization of rat liver: 0.875 g Di potassium hydrogen orthophosphate (0.01M) and 5.75 g Potassium Chloride (1.15%w/v) were dissolved in 500 ml MilliQ water and the pH was adjusted to 7.4 with orthophosphoric acid (40%, v/v). The solution was filtered through 0.22 µm membrane filter.

3.3.2.3 Drug formulation

10 mg each of I, II and III were accurately weighed into a 10 ml volumeteric flask. The compounds were dissolved in methanol and the volume made up to 10 ml to prepare 1mg/mL of cassette spiking solution. Similarly 10 mg of each of I, II and III were weighed separately into respective 10 ml volumeteric flasks. The compounds were dissolved in methanol and the volume to 10 ml to prepare 1 mg/ml of discrete spiking solutions.

3.3.2.4 Preparation of rat liver S9 fraction

Young, healthy male SD rats were procured from Laboratory Animal Services Division of the institute. Animals were fasted for 12h but allowed water *ad libitum*. The rats sacrificed by decapitation and then the liver isolated. The liver was placed on ice-cold homogenizing solution and the adhering non-liver tissues were carefully removed. The liver was washed with the homogenizing solution free of traces of blood. The isolated and washed liver was homogenized with ice cold homogenizing solution (1:3 w/v). The homogenate was centrifuged at 9000 g, with rotor maintained at 4 °C for 30 min. The supernatant (S9 fraction) was used for the preparation of incubation mixture (13). The S9 fraction was maintained at 4 °C till use. The protein content of the preparation was determined by standard method (14).

3.3.2.5 Preparation of β-NADPH regenerating system

The incubation mixture was prepared by dissolving 1.25 nM of NADP, 2.5 mM of Glucose-6-phosphate, 0.75 U.ml-1 of glucose-6-phosphate dehdrogenase, and 6.25 mM of $MgCl_2$ in 0.1 M potassium phosphate buffer (pH 7.4). The β-NADPH regenerating system was kept at 4 °C till further use (13).

3.3.2.6 Preparation of incubation mixture

To 2 mL of ice-cold S9 fraction, 10 mL of ice-cold β-NADPH regenerating system was added and incubated at 37°C for 5 min on a shaking water bath before spiking with drug solution (13).

3.3.2.7 Incubation and sampling

48 µl (48 µg) of the cassette and discrete formulations were spiked into the respective incubation mixtures (12 ml) after 5 min of preincubation at 37°C. 200 µL of the incubation mixture were sampled at 0, 5, 10, 15, 20, 30, 60, 90, 120 min respectively into eppendorff tube containing 25 µl of 2N KOH. The eppendorff tubes were vortexed for 30 s and stored at -80°C till further use.

3.3.2.8 Sample analysis

The samples were processed according to the method B described in **Section 2.2.5(c)** and analyzed using LC/ESI-MS with system B **(2.2.3.2)** as mobile phase. The concentration of the analytes in the test samples were read from the calibration curves prepared according to the scheme described in **Section 2.2.6(b)**

3.3.2.9 Data analysis

The metabolic stability was evaluated by measuring the disappearance of the compounds from the incubation mixture. The results of the metabolic study was expressed as the percentage of the compound remaining, represented by the equation:

$$\% \text{ Parent compound remaining} = \frac{[\text{Concentration of the parent compound after incubation}] \times 100}{[\text{Concentration of the parent compound before incubation}]} \quad (1)$$

Non-linear regression analysis of a plot of percentage of parent compound remaining versus time was performed using GraphPad Prism ver 3.00 software. The equation defining the regression analysis is represented as follows:

$$\% \text{ Parent compound remaining} = \text{Span}.\ e^{-k_{in}t} + \text{Plateau} \qquad (2)$$

Span – Difference in the % Parent compound remaining at time zero to time t_{last}.

Plateau - % Parent compound remaining at time t_{last}.

k_{in}- metabolism rate constant

3.3.3 Results and discussion

The protein content of the S9 fractions was found to be 2.46±0.32 mg/ml indicating the uniformity in the preparations. HPLC/MS chromatograms showing the concentrations of compounds at 0 min and 20 min of incubation are shown in **Figure 1** and **Figure 2** for cassette and discrete respectively provide an indication regarding the metabolic stability

Figure 1: *Overlay of HPLC/MS chromatograms of 0 min and 20 min in vitro metabolism samples as Cassette of a) I, b) II, c) III.*

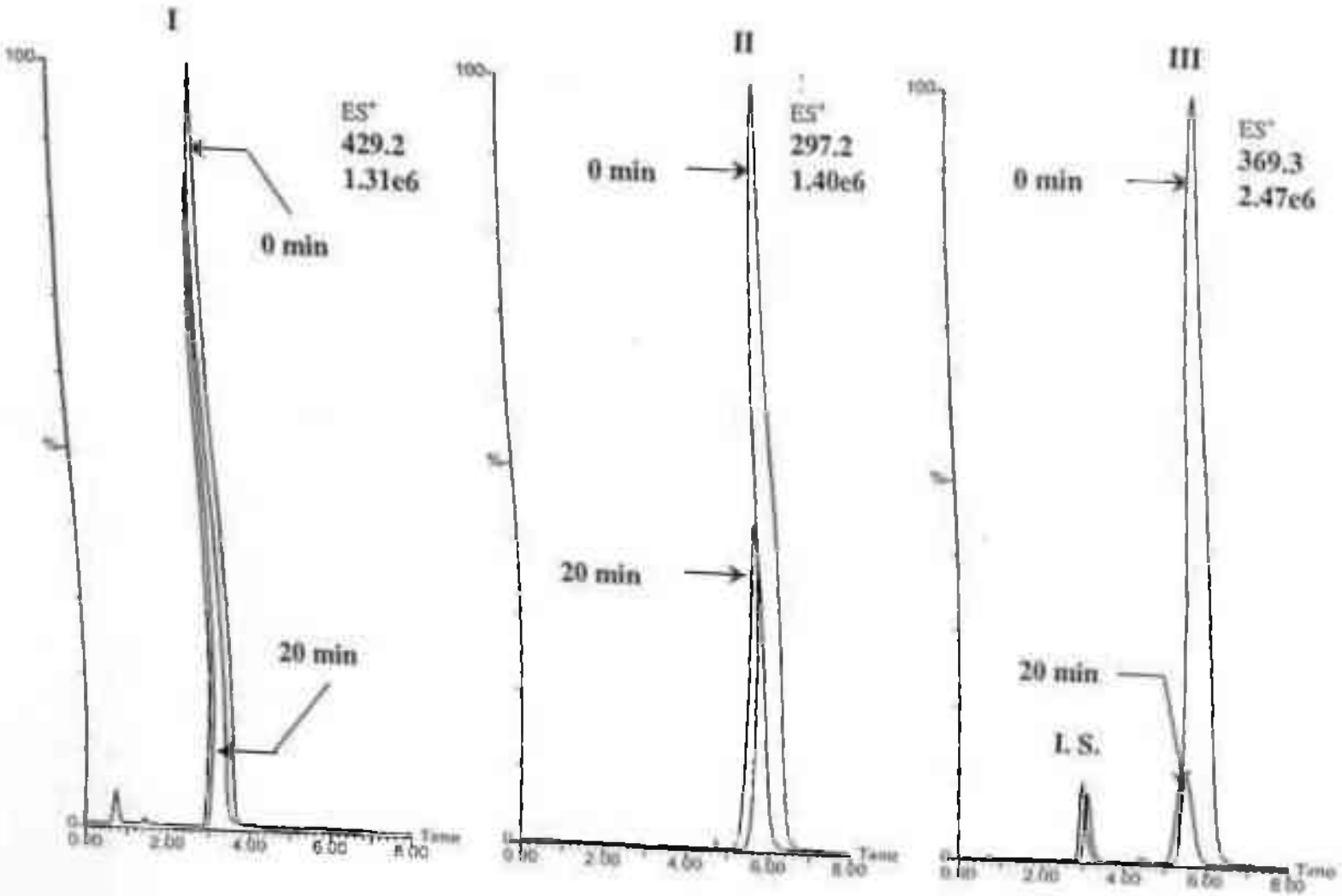
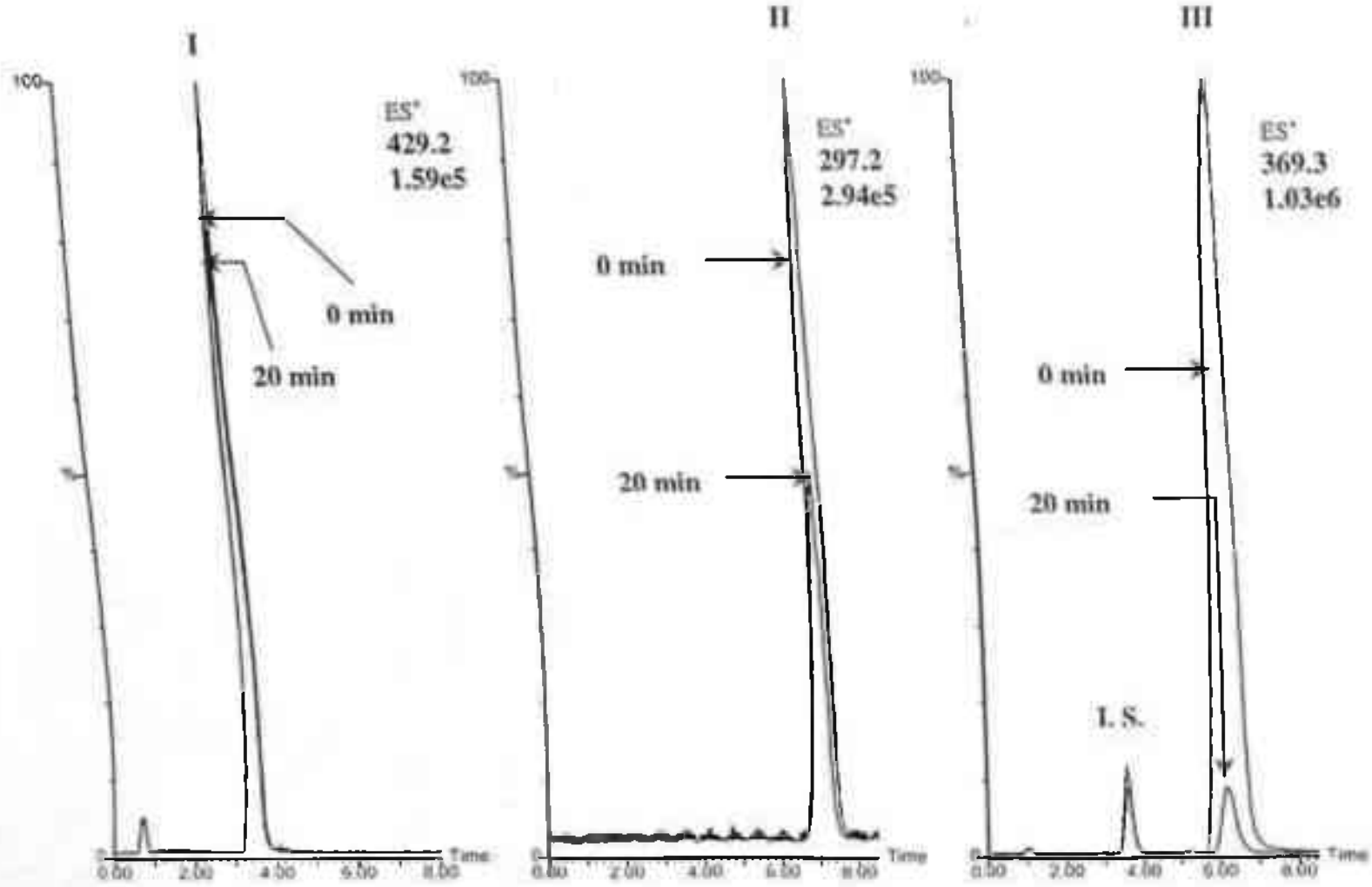


Figure 2: *Overlay of HPLC/MS chromatograms of 0 min and 20 min in vitro metabolism samples as Discrete of a) I, b) II, c) III.*



The metabolic stability of the compounds is indicated by the rate of their disappearance from the incubation mixtures. Typical plots showing a comparison of % Parent compound remaining in the incubation mixture versus time for I, II and III as discrete and as cassette is shown in **Figure 3a, b, c**. Non-linear regression analysis of the data showed good fitting for one phase exponential decay, which is shown in **Figure 4a, b, c**. The half-life ($t_{1/2}$) indicating the metabolic stability of the compounds was derived by the following equation:

$$t_{1/2} = 0.693/k_m \qquad (3)$$

where k_m is the metabolism rate constant obtained from the non-linear regression analysis obtained in the **Equation 2**. The k_m and $t_{1/2}$ were indicators of metabolism of parent when the experiment was carried out as discrete and cassette. The results are summarized in the **Table 1**.

Table 1: Comparison of the metabolism rate constants and half-lives of **I, II** and **III** in cassette and discrete experiments (n = 3).

Compound	Cassette		Discrete	
	k_m (min^{-1})	$t_{1/2}$	k_m (min^{-1})	$t_{1/2}$
I	0.027±0.003	25.5±2.91	0.038±0.003	18.263±1.489
II	0.055±0.003	12.7±0.601	0.063±0.001	10.97±0.211
III	0.123±0.002	5.65±0.080	0.107±0.002	6.49±0.094

Fig 3: Comparison of the % drug remaining versus time for cassette and discrete studies (n = 3) of a) I, b) II and c) III.

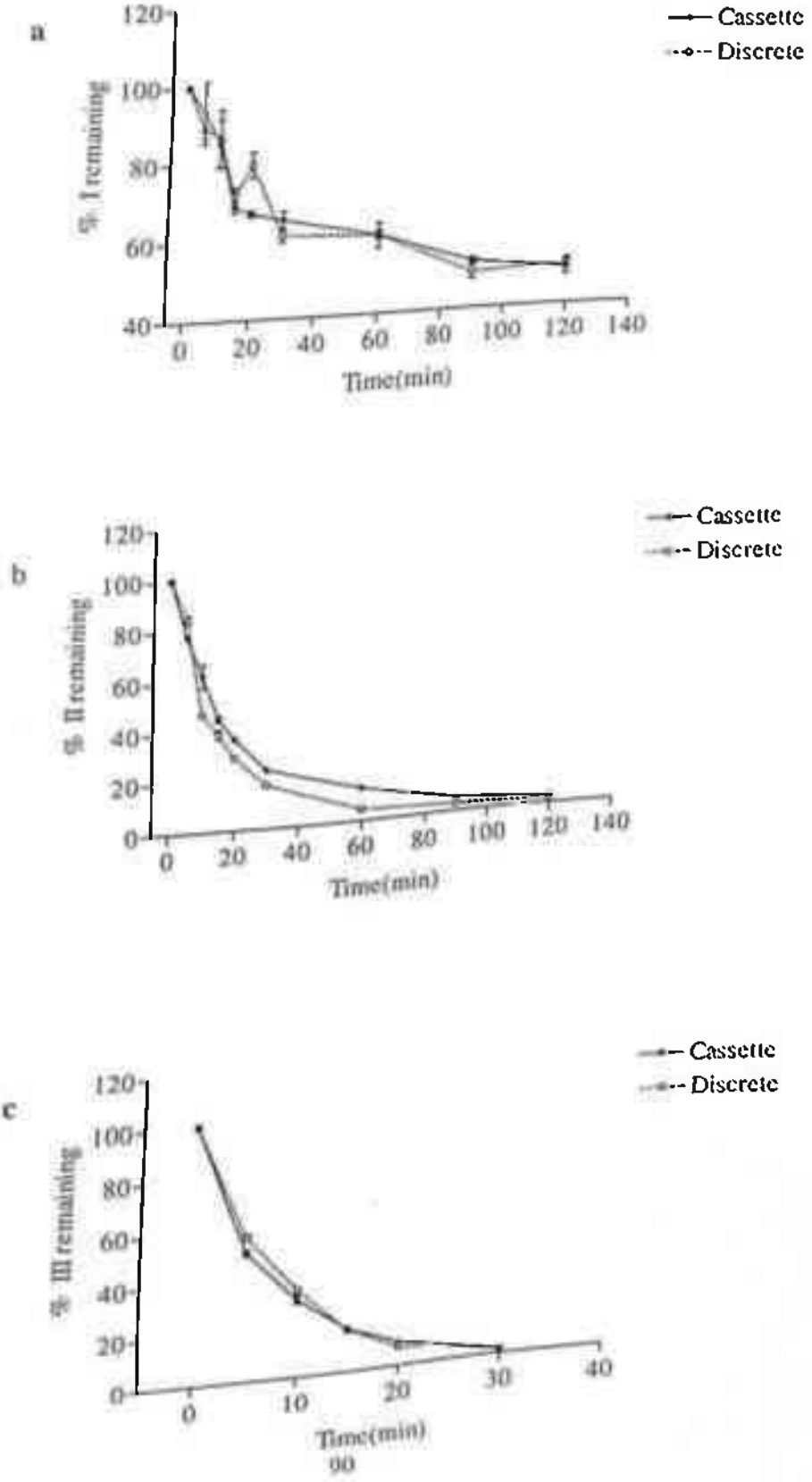
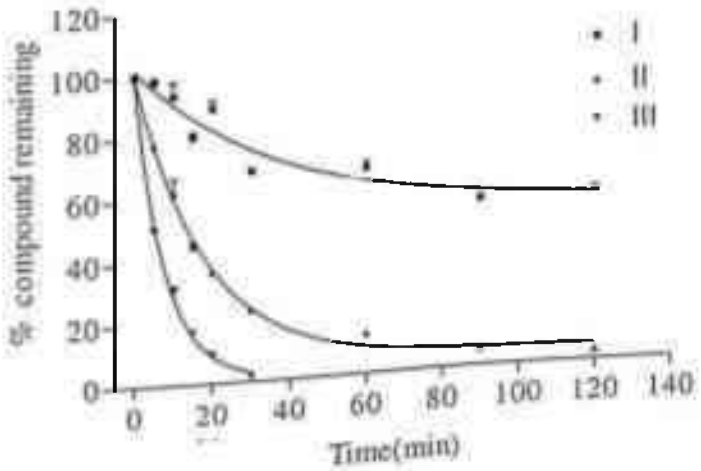
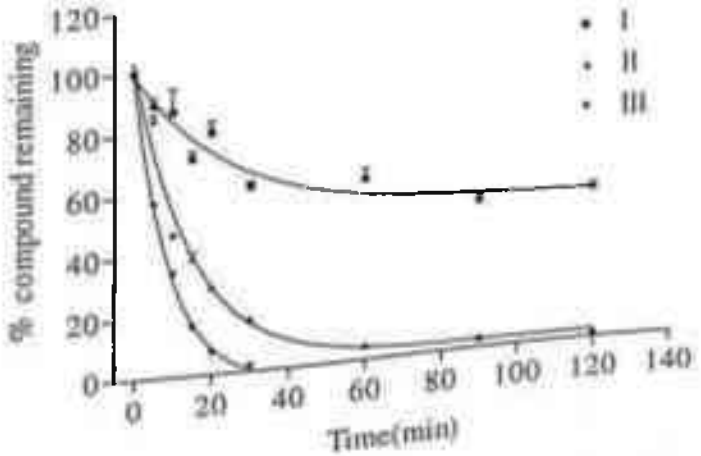


Figure 4: Non-linear regression analysis of % parent compound remaining versus time a) Cassette b) Discrete

a)



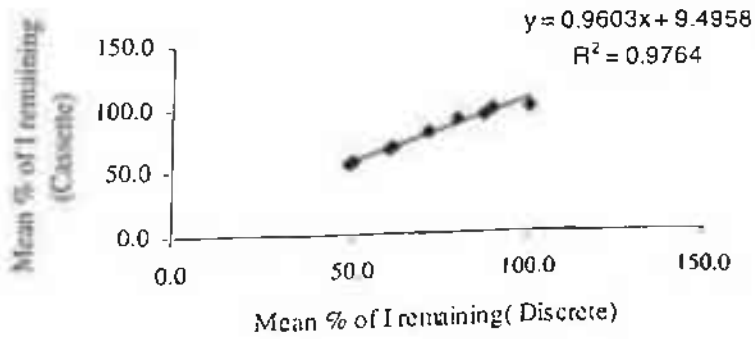
b)



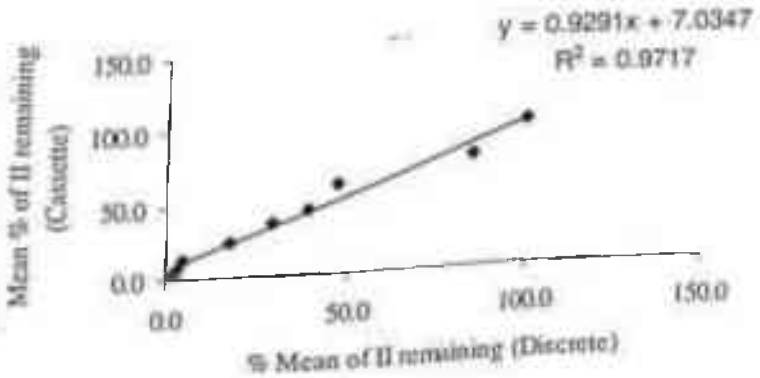
Linear regression analyses of the Mean % of the parent compound remaining for I, II and III in cassette and discrete showed linearity with good coefficient of correlation (**Figure 5a, b, c**). Moreover, linear regression analysis of the metabolism rate constants (k_m) and the t1/2 of the compounds I, II and III when the S9 *in vitro* metabolism experiment was carried out with discrete and cassette also showed linearity with a good coefficient of correlation (**Figure 6a,b**). These indicate that the data generated by the application of the concept of Cassette correlated with the data generated by carrying out the experiments on discrete. Even though, overlapping confidence intervals and statistical significance test performed on the metabolism rate constants and $t_{1/2}$ generated from the *in vitro* metabolism experiments of the compounds as discrete and cassette showed statistically significant difference ($p<0.05$) in few cases, the order of ranking the metabolic stability could still be achieved, which was the basic aim of the experiment. From the data obtained, it can be concluded that among the three compounds, III has the lowest metabolic stability followed by II and finally by I. Compound III has the fastest rate of metabolism with a high metabolism rate constant and shortest half-life followed by II and I among the compounds in the cassette. Furthermore, from the above data, it is evident that k and $t_{1/2}$ of II and III are comparable in cassette and discrete but the k of I in cassette was significantly lesser compared to that in cassette resulting in a higher half-life in cassette compared to discrete experiment. This suggests a possibility of inhibition of the metabolism of I in cassette by one/both of the compounds

Figure 5: Regression analysis of the Mean % of the parent compound remaining in Cassette versus Discrete a) I, b) II, c) III.

a



b



c

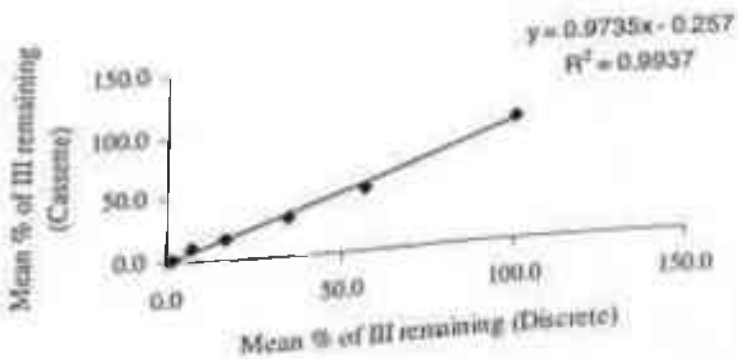
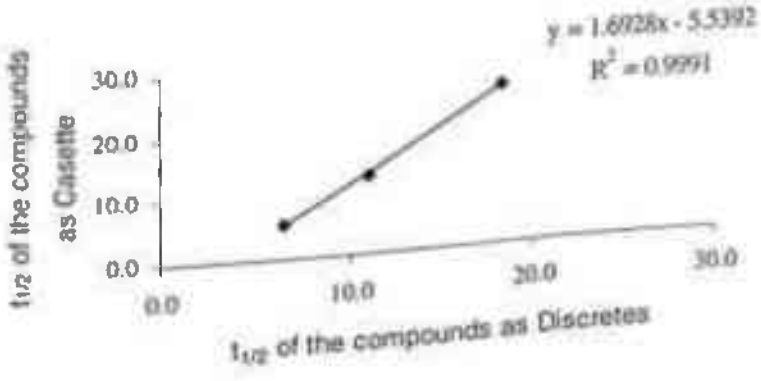
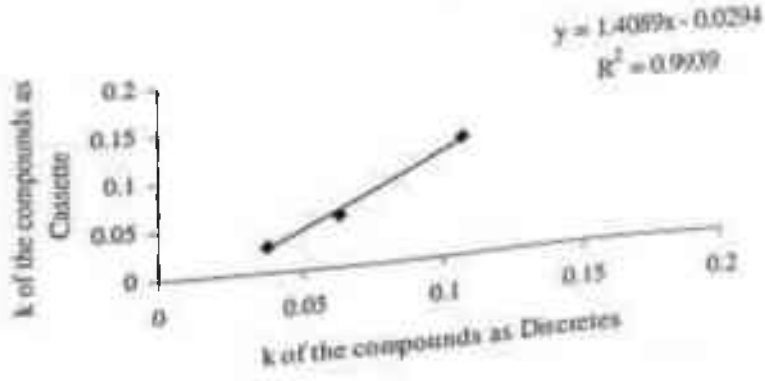


Figure 6: Regression analysis of I, II and III in Cassette versus Discrete a) $t_{1/2}$, b) k.

a)



b)



3.3.4 Conclusion

The results of *in vitro* metabolism when interpreted taking into account the role of *in situ* absorption studies leads to the following inference:

i) Compound I has a low gastrointestinal permeability and high metabolic stability. The limiting factor on oral administration would be its ability to cross the GIT, rather than liver.

ii) The GIT permeability of Compound II is better than I, but II is metabolized faster than I, which indicates liver to be more limiting than GIT.

iii) Compound III has high GIT permeability and a very low metabolic stability, suggesting that even though the compound can cross GIT barrier easily, its availability in the systemic circulation is severely limited by liver.

Therefore, it can be predicted as Compound II to possess optimum PK parameters such as high AUC and less clearance. The ranking of compounds upon oral administration can be predicted as the following order: II > I> III. The validity of the above results can only be confirmed with *in vivo* PK studies, which can support the above conclusion.

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Chapter 4

APPLICATION OF CASSETTE DOSING TO *IN VIVO* STUDIES

Despite the utility of the *in vitro* models, *in vivo* drug metabolism and pharmacokinetic (DMPK) studies are still an intrinsic part of the lead optimization processes in drug discovery. The approaches selected will depend very much on the issue arising, but common to many projects is the problem of achieving high enough systemic levels for sufficient periods to achieve appropriate efficacy (1). Poor bioavailability following an oral delivery can be due to poor absorption across the intestinal mucosa or P-gp mediated efflux, or hepatic first-pass metabolism. In addition to oral bioavailability, plasma elimination half-life, clearance and volume of distribution are all useful parameters that can be generated from the PK profiles following oral and intravenous dosing. Shortcomings of a lead identified this way can help steer optimization in right direction. However, *in vivo* methods are generally low-throughput because of the numbers of the compounds that can be dosed in unit time and the time required for analytical method development. Developments in the analytical instrumentation particularly mass spectrometry and software have resulted in decrease in the time required for the bioanalytical method development. Novel concepts such as 'cassette' or 'N-in-One' dosing protocols, result in generation of PK profiles more rapidly from fewer animals (2-4).

The concept of Cassette dosing was applied for generating PK data on intravenous and oral dosing of the compounds I, II and III, forming cassette. The data thus generated was utilized to rank the compounds on the basis of the PK criteria for the cassette such as high Area Under Curve (AUC), longer elimination half-life ($t_{1/2}$) and low systemic clearance (CL). Later, the PK data of the compounds dosed discretely was generated and ranked on the basis of the above mentioned criteria, so as to compare the data generated by cassette dosing.

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Chapter 4

Section 1

APPLICATION OF CASSETTE DOSING TO *I.V.* PHARMACOKINETIC STUDIES

4.1.1 Introduction

In the development of a new drug, pre-clinical trials are of immense importance, where a new chemical entity (NCE) gains importance or is discarded. Turning a chemical lead into a marketable drug requires a balance of potency, safety and PK, which are traditionally low throughput processes (**1**). PK studies usually begin with the establishment of single dose intravenous PK studies. Intravenous administration is the fastest and most certain route of administration involving direct injection of the drug into the bloodstream, where it rapidly equilibrates with all the tissues in the body. The primary advantage of i.v. dosing is that the whole dose is delivered into the bloodstream. The drug first reaches the right heart followed by lungs and ultimately systemic circulation. The concentration time profile is characterized by an initial high concentration followed by a steady decline. The basic aim of i.v. PK study is to estimate the clearance of the drugs. Clearance cannot be estimated after an oral dose, since the total dose does not necessarily reach the systemic circulation.

Increased throughput in *in vivo* pharmacokinetic screening has recently been reported by a) dosing mixtures of compounds to a single animal i.e., N-in-One dosing /Cassette Dosing and (b) by pooling samples from singularly administered compounds prior to analysis. Both the methods capitalize on tandem liquid chromatography/mass spectrometry (*LC/MS*) as a sensitive and specific method for analysis (**2-6**). As the availability of the *LC/MS* systems is limited, we have investigated an alternative increased throughput approach by simultaneously characterizing the *in vivo* pharmacokinetics of multiple compounds utilizing traditional HPLC. In the present investigation the basic concepts of cassette dosing was followed. Pharmacokinetic parameters of I, II and III following intravenous (i.v.) administration were determined by cassette dosing followed by discrete dosing of the compounds to generate data for comparing the feasibility of the concept.

4.1.2 Experimental

Approval of the protocol for the study was obtained from the Local Ethics Committee for Animal Experiments of the institute, prior to the start of the study. The pharmacokinetic studies of I, II and III as cassette and discrete were carried out in young and healthy male albino Sprague–Dawley rats (n=3 for each experiment/time point) weighing 250±25g, obtained from Laboratory Animal Division of CDRI. Animals were cared for in accordance with principles of The Guide for the Care Use of Laboratory Animals (Department of Health, Education and Welfare, no. [NIH] 85-23). All surgical procedures were carried out under ether anaesthesia, taking suitable pre and post – operative care. Euthanasia and disposal of carcass were executed in accordance with the guidelines of Local Ethical Committee for Animal Experiments. The animals were housed in plastic cages in standard laboratory conditions with a regular 12h day– night cycle using non-heat radiating lamp. Standard pelleted laboratory chow (Goldmohar Laboratory Animal Feed, Lipton India Ltd, Chandigarh, India) and water were allowed *ad libitum*. The rats were acclimatized to this environment for at least 2 days before conducting the experiment. In all the studies mentioned below, the dose was administered after overnight (12-16h) fasting. Blood samples were collected, serum was separated by centrifugation at 1200 rpm for 10 minutes at 4 °C and were stored at –60 °C till analysis.

4.1.2.1 Formulation

The intravenous dosing formulations of compounds were prepared for dosing 3mg/kg/compound/animal. Preparation of dosing solutions involved weighing 45mg of each of the compound individually/all the three (45mg of each of the compounds) for cassette/discrete formulation in 2ml of DMSO: PEG600 (50%: 50%). To 1ml of the above solution 2ml of 50% DMSO: PEG600 (50%: 50%) was added resulting in a final concentration of 7.5mg/ml of each compound either individually or in cassette.

4.1.2.2 Dosing

Rats were dosed with 0.4ml/kg (i.e., a 250 g rat received 0.1ml), with the dosing solution using tuberculin glass syringe fitted with a 26G needle via the caudal vein, after the dilation of the tail with xylene in restrainer cages.

4.1.2.3 Sampling

Blood samples for pharmacokinetic studies were collected at different time points up to 6 h post dose. The samples (2.5-45 min) were collected by cardiac puncture under light ether anesthesia. Terminal samples (60-360 min) were collected from inferior venacava. All blood samples were allowed to clot at room temperature for 30 min. Serum was separated by centrifugation at 1000 x g for 10 min at 4°C and were stored at –60°C.

4.1.2.4 Analysis

The concentrations of I, II and III in serum samples of dosed rats were estimated by the sample preparation as mentioned for System A in **Section 2.2.4(c)**

4.1.2.5 Pharmacokinetic and statistical analysis

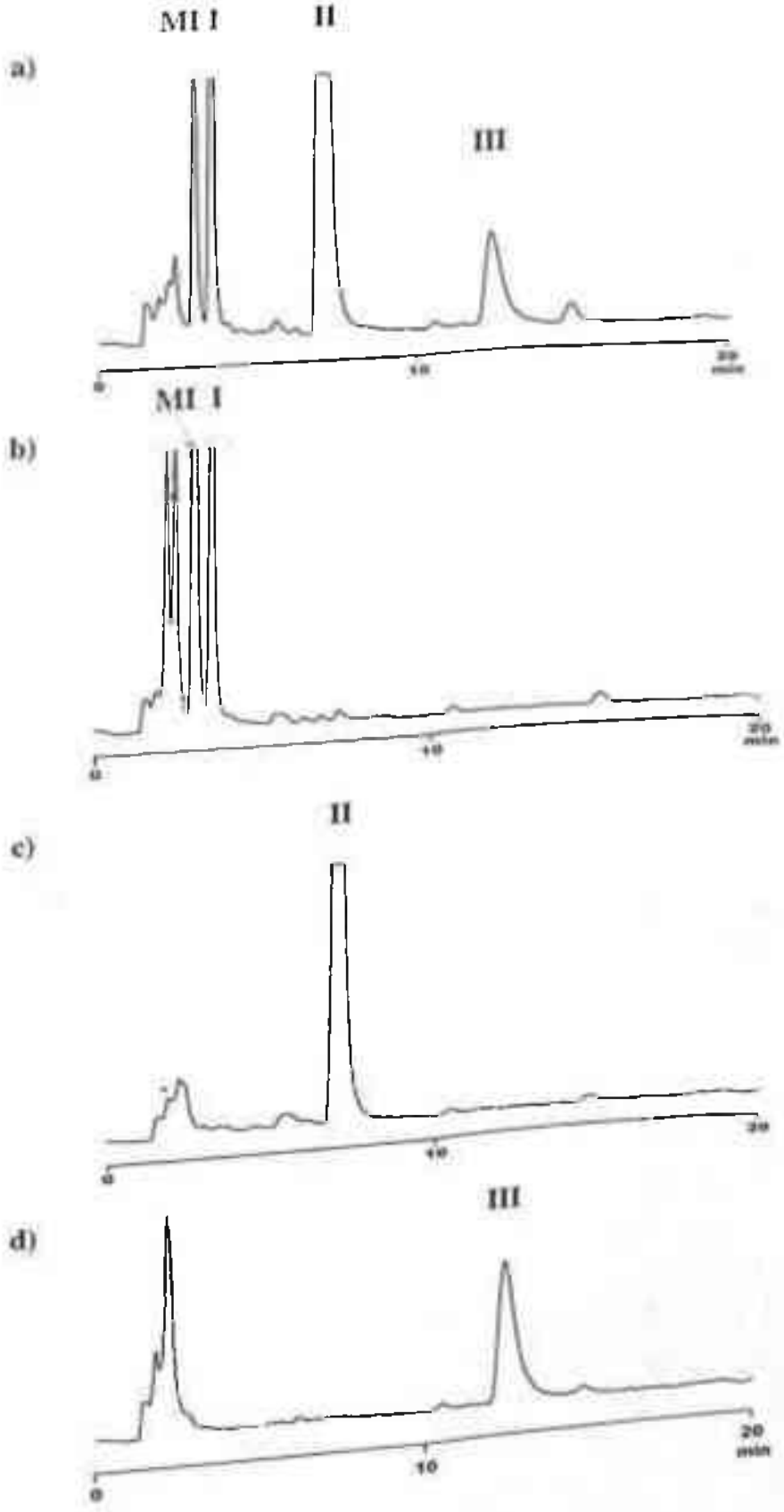
The clearance (CL), steady-state volume of distribution (Vss), mean residence time (MRT), elimination phase half-life (t1/2), and area under curve (AUC) of each of the compounds were determined from respective serum concentrations versus time curve by applying noncompartmental methods using WIN NONLIN software (SCI consultants, USA). Various weighting schemes (uniform, 1/y, 1/(y*y)) were applied and the model with the least residuals (uniform weighting) was selected for the estimation of the parameters. Overlapping confidence intervals and statistical significance tests were performed on the mean concentrations at each time point. Linear regression analyses were performed on the data generated by cassette and discrete dosing for correlation of the data generated in either forms of the study.

4.1.3 Results and discussion

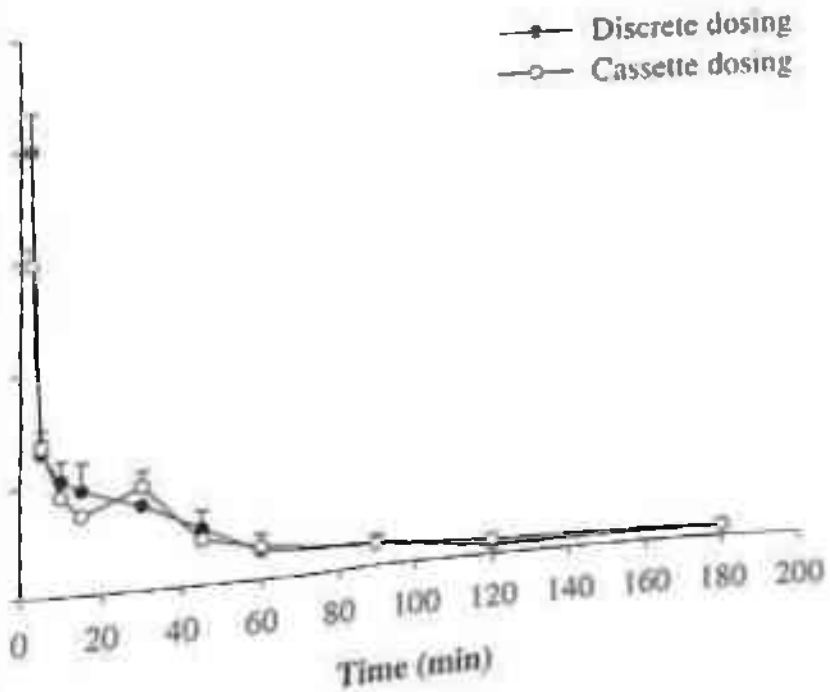
Substantial amounts of PK information for all the three compounds are simultaneously available. The chromatogram of a 2.5 min sample following cassette

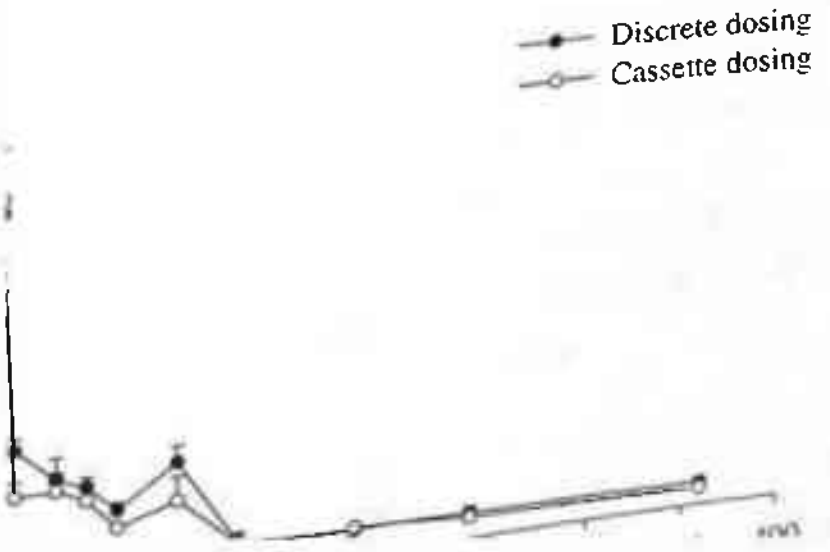
dosing and discrete dosing are shown in **Figure 1a** and **Figures 1b, c, d** respectively. It is evident that the regions of interest were devoid of any endogenous interference as compared to blank serum chromatogram (**Chapter 2, Figure 3**). Both in discrete as well as cassette, the retention times of I, II and III were 3.43±0.04, 6.99±0.15 and 12.37±0.15 min respectively. In cassette dosing, it is interesting to note the appearance of a metabolite at 2.09±0.01min, **Figure 1a,** which was absent in the chromatogram of blank serum sample (**Chapter 2 Figure 3**). Examination of the chromatograms of I, II and III (**Figure 1b, c, d**) when dosed individually, confirms the formation of the metabolite from I. The mean (n=3) serum concentration time profile of I, II and III following cassette and discrete dosing are shown in **Figures 2a, b, and c** respectively.

Figure 1: HPLC chromatograms of 2.5min test samples following i.v. dosing (3 mg/kg) as a) Cassette, b) I, c) II, d) III.



omparison of the concentration (ng/ml) versus time (min) profile
llowing Discrete and Cassete Dosing (n = 3) for a) I, b) II and c) III





The PK parameters obtained for all the three compounds following discrete dosing, **Table 2** were similar to that obtained earlier by cassette dosing

Table 2: Pharmacokinetics of I, II, and III in discrete dosing studies (n = 3).

Compounds	Half-life (min)	Clearance (ml/min/kg)	Stead-state volume of distribution (L/kg)	MRT (min)	AUC (ng.min/ml)
I	71.51	163.54	9.502	33.43	16532
II	163.01	79.83	15.89	98.73	29976
III	141	117.67	16.72	81.83	22301

The overlapping confidence intervals and statistical significance tests performed on the mean concentrations at each time point showed $p > 0.05$ at all time points except for one time point each in I and III, indicating the pharmacokinetic parameters obtained by cassette and discrete dosing are statistically not different. Moreover, the regression analyses of the serum concentration-time data for all the three compounds when dosed individually as well as Cassette followed linear trend **Figure 3a, b, c.** The regression analysis curves for I, II and III showed a good correlation. Therefore, pharmacokinetic parameters obtained from these concentration-time profiles are also not statistically different. There was a good correlation of the pharmacokinetic parameters, particularly that of MRT, Vss and clearance obtained after Cassette and individual dosing, while a modest correlation was obtained for $t_{1/2}$ and AUC_{all} as shown in **Figure 4a, b, c, d, e.** Based on the criteria set for the selection of the compound with the most optimum PK attributes, II emerges to be the compound of choice followed by III and I.

Figure 3: Regression analysis of Mean serum concentration in Discrete and Cassette Dosing (n = 3) of a) I, b) II and c) III.

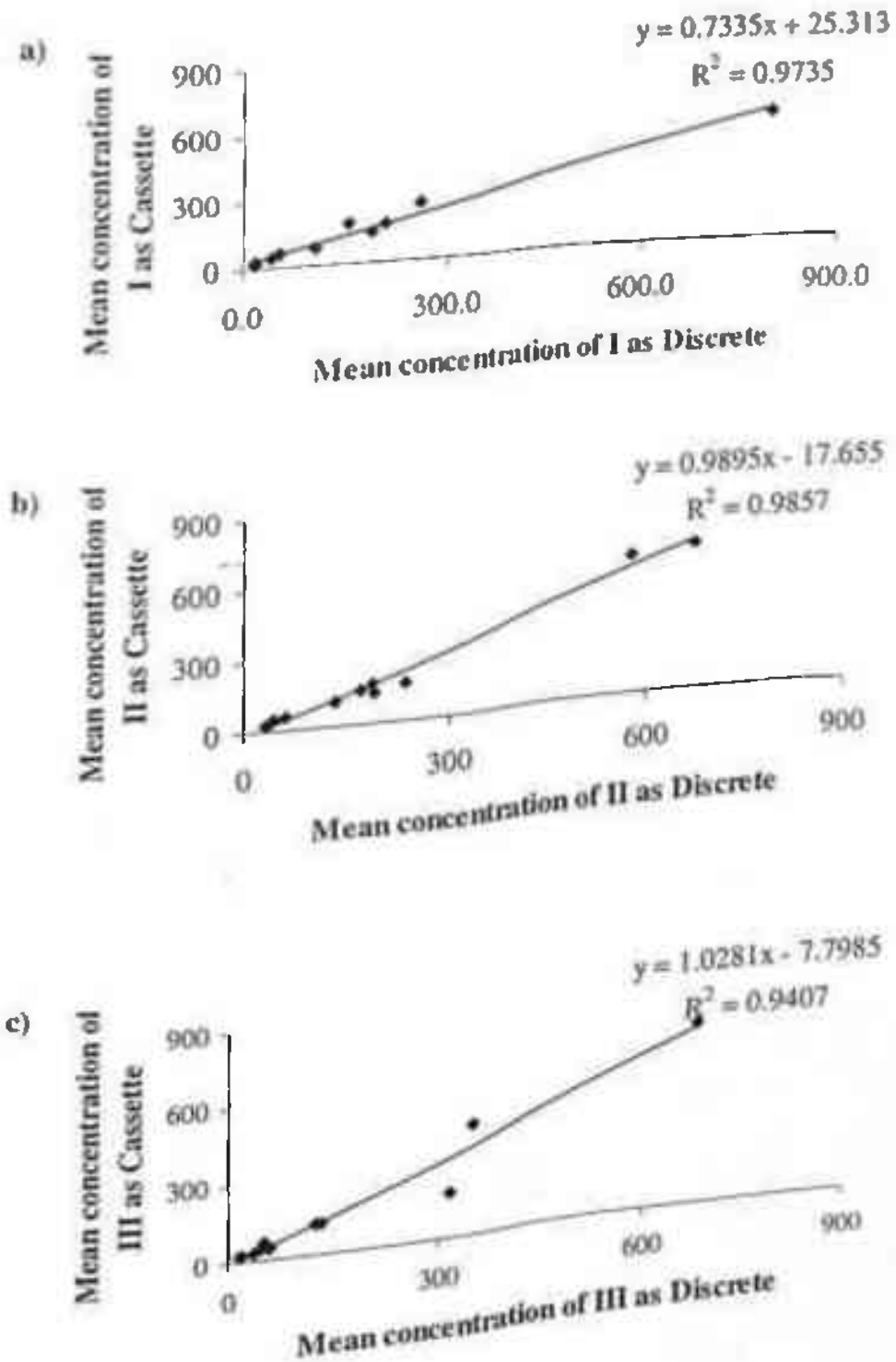
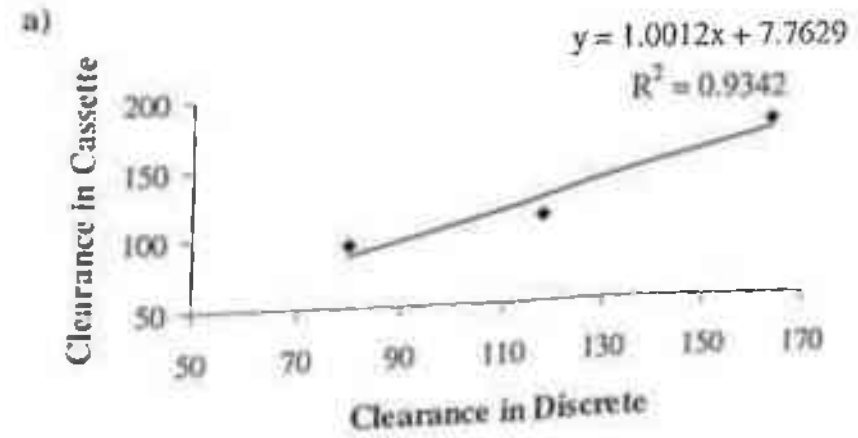
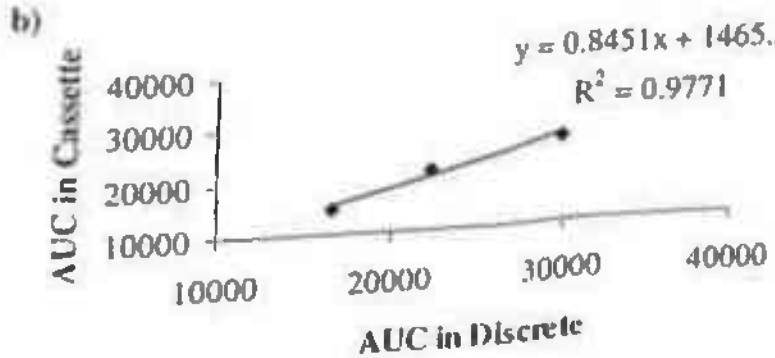
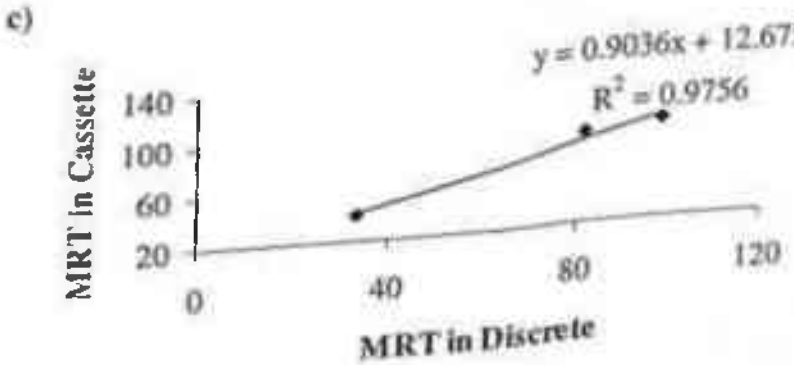


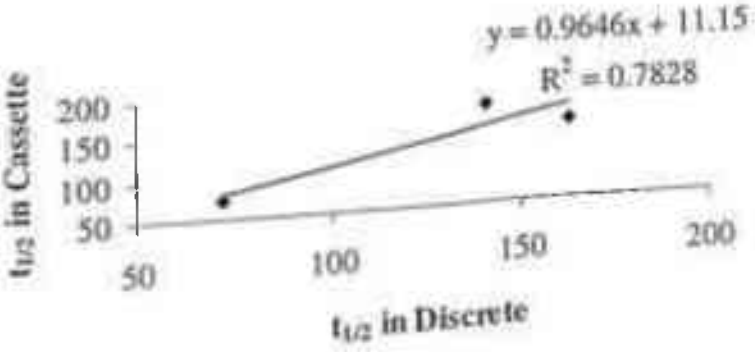
Figure 4: Regression analysis of I, II and III in Cassette versus Discrete a) Clearance, b) AUC, c) MRT, d) $t_{1/2}$, e) V_d.



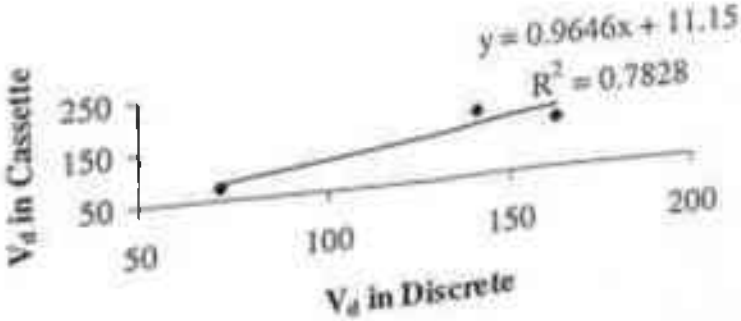




d)


y = 0.9646x + 11.15
R² = 0.7828
t1/2 in Cassette
200
150
100
50
50
100
150
200
t1/2 in Discrete

e)


y = 0.9646x + 11.15
R² = 0.7828
Vd in Cassette
250
150
50
50
100
150
200
Vd in Discrete

4.1.4 Conclusion

Taking into account the results of *ex vivo* and i.v. studies, attempt was made to predict the bhaviour of the compounds following oral administration. The inferences can be summarized as follows:

i) Clearance of I is high with a lesser half-life and residence time, which are contrary to the selection criteria. Furthermore, the formation of metabolite from 2.5 min post i.v. administration and higher *in vitro* hepatic stability indicate extra-hepatic metabolism, thereby reducing the metabolic stability. Moreover, with *in situ* studies low permeability indicates poor PK upon oral administration.

ii) Clearance of II is lowest (94.7 ml/min/kg), while the AUC highest (26368 ng.min/ml). The half-life and residence time are comparable with III (**Table 1**) and II possess comparatively satisfactory GIT permeability and metabolic stability as evident from *ex vivo* studies. These results indicate comparatively better PK characteristics for II compared to I and III.

iii) Even though the half-life of III is comparable to II, but it has comparatively higher clearance and lower AUC. Since III is shown to have severe metabolic instability *in vitro*, the PK characteristics of III can be predicted not to be better than II upon oral administration with respect to the required criteria of high AUC, low clearance and longer MRT.

However, *in vivo* oral study is required to confirm the extrapolation, as *ex vivo* results do not always necessarily correlate well with *in vivo* studies. Further more, Cassette dosing reduced the workload by several folds compared to discrete dosing. This could be achieved despite the fact that the time required for the development of the assay method and the run time of cassette was longer compared to that of individual compounds. However, the time spent in the validation of the assay method was directly reduced by three times as the analytical method was validated for all the three compounds simultaneously. Moreover, the numbers of test samples analyzed were reduced by 67% i.e., from 108 samples to 36 samples (12 time points, n =3 per time point, per compound). N-in One dosing also facilitates the overall reduction in the number of rats, from 54 rats (6 rats per study, n=3 per compound) for individual dosing to 20 rats, used in the study. The end result of cassette dosing is, reduced assay workload, and decreased number of

animals involved in the studies resulting in enhanced and economic throughput of the PK characterization. It should be remembered that this was achieved by traditional HPLC. However, it should be noted that the selectivity of compounds that can be achieved in a cassette depends upon the system capacity. It is recognized that application of LC-MS would further accelerate and economize the PK process, once they are more readily available, more user friendly, more efficient and reproducible in detection.

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Chapter 4

Section 2

APPLICATION OF CASSETTE DOSING TO ORAL PHARMACOKINETIC STUDIES

4.2.1 Introduction

Pharmacokinetic studies gain importance from the fact that the rate and extent of availability of the administered therapeutic agent will result in pharmacological response of desired magnitude and response. Oral route is the most convenient, painless, economical and safe for the administration of the drugs. It involves swallowing of the drug, which is absorbed from the gastrointestinal tract by the way of mesenteric circulation and proceeds from the hepatic portal vein to the liver and into systemic circulation. However, the oral route has some disadvantages. The absorption of the drug from the GIT is influenced by a number of factors such as pH, gastric motility, surface area that might limit the complete absorption of the drug. It is also known that many drugs are subject to the first-pass effect when absorbed from the GI tract into the systemic circulation. Since, *i.v.* PK studies neglect the above-mentioned influences; an oral PK study is necessitated to determine the relative bioavailability.

An attempt has been made to apply the concept of cassette dosing for the generation of the PK data following oral administration so as to determine the bioavalabilities of I, II and III. Simultaneously PK data was generated by discrete dosing which will serve for comparing with the data generated by cassette dosing and ultimately decide on the feasibility of the concept.

4.2.2 Experimental

The pharmacokinetic studies of I, II and III as cassette and discrete were carried out in young and healthy male albino Sprague–Dawley rats (*n*=3 for each experiment/time point) weighing 250±25g, obtained from Laboratory Animal Division of CDRI. Prior to the start of the study, approval of the protocol was obtained from the Local Ethical Committee for Animal Experimentation of the institute. Animals were cared for in accordance with principles of The Guide for the Care Use of Laboratory Animals (Department of Health, Education and Welfare, no. [NIH] 85-23). All surgical procedures were carried out under ether anaesthesia, taking suitable pre and post – operative care. Euthanasia and disposal of carcass were executed in accordance with the guidelines of Local Ethical Committee for Animal Experiments. The animals were housed in plastic cages in standard laboratory conditions with a regular 12h day– night

cycle using non-heat radiating lamp. Standard pelleted laboratory chow (Goldmohar Laboratory Animal Feed, Lipton India Ltd, Chandigarh, India) and water were allowed *ad libitum*. The rats were acclimatized to this environment for at least 2 days before conducting the experiment. In all the studies mentioned below, the dose was administered after overnight (12-16h) fasting. Blood samples were collected, serum was separated by centrifugation at 1200 rpm for 10 minutes at 4 °C and were stored at –60 °C till analysis.

4.2.2.1 Formulation

Single dose oral pharmacokinetic studies of I, II and III were performed at a dose of 15mg/kg/compound/animal. 45mg of each of the compound individually or all the three for cassette/discrete formulation were dissolved in 1ml of DMSO: PEG600 (50%: 50%). To 1ml of the above solution 2ml of 50% DMSO: PEG600 (50%: 50%) was added resulting in a final concentration of 7.5mg/ml of each compound either individually or in cassette.

4.2.2.2 Dosing

Rats were dosed with 2ml/kg (i.e., a 250 g rat received 0.5 ml), by intragastric gavage needle (Perfectum, TW, 20 guage).

4.2.2.3 Sampling

Blood samples for pharmacokinetic studies were collected at different time points up to 6 h post dose. The samples (5-60 min) were collected by cardiac puncture under light ether anesthesia. Terminal samples (120-360 min) were collected from inferior venacava. All blood samples were allowed to clot at room temperature for 30 min. Serum was separated by centrifugation at 1000 g for 10 min at 4°C and were stored at –60 °C.

4.2.2.4 Analysis

The concentrations of I, II and III in the serum samples of dosed rats were estimated by the sample preparation as mentioned for **System b** in **Section 2.2.5(c)**

4.2.2.5 Pharmacokinetic and statistical analysis

The clearance (CL), volume of distribution (V_d), mean residence time (MRT), elimination phase half-life (t1/2), and area under curve (AUC) of each of the compounds were determined from respective serum concentrations versus time curve by applying noncompartmental methods using *WIN NONLIN* software (SCI consultants, USA). Mean Absorption Time (MAT) was obtained from the difference in MRTs derived from oral and i.v. PK studies. Various weighting schemes (uniform, 1/y and 1/(y*y)) were used in model fitting and the model with the least residuals was selected for the estimation of the parameters. Linear regression analyses were performed on the data generated by cassette and discrete dosing for correlation of the data generated in either forms of the study.

4.2.3 Results and discussion

The bioanalytical method developed on LC/MS in SIR provides higher selectivity and sensitivity to carry out the oral PK study. This selectivity was not available in LC/Fluorescence, where the analyte regions had interference from the metabolites of other compounds in the cassette. The LC/MS chromatogram in SIR mode of test sample (t_{max}) following oral administration of I, II and III as Cassette is shown in **Figure 1**. The mean (n=3) serum concentration time profile of all the compounds when administered discrete as well as cassette is shown in **Figure 2**. The pharmacokinetic parameters of I, II and III following oral administration obtained by non-compartmental analysis (uniform weighting) are given in **Table 1**.

than I and III as evidenced by MAT and k_a. From the criteria of minimum clearance, long MRT and high AUC involved in selection of compound with most optimum PK parameter, the primary objective of II>III>I rank order can be achieved..

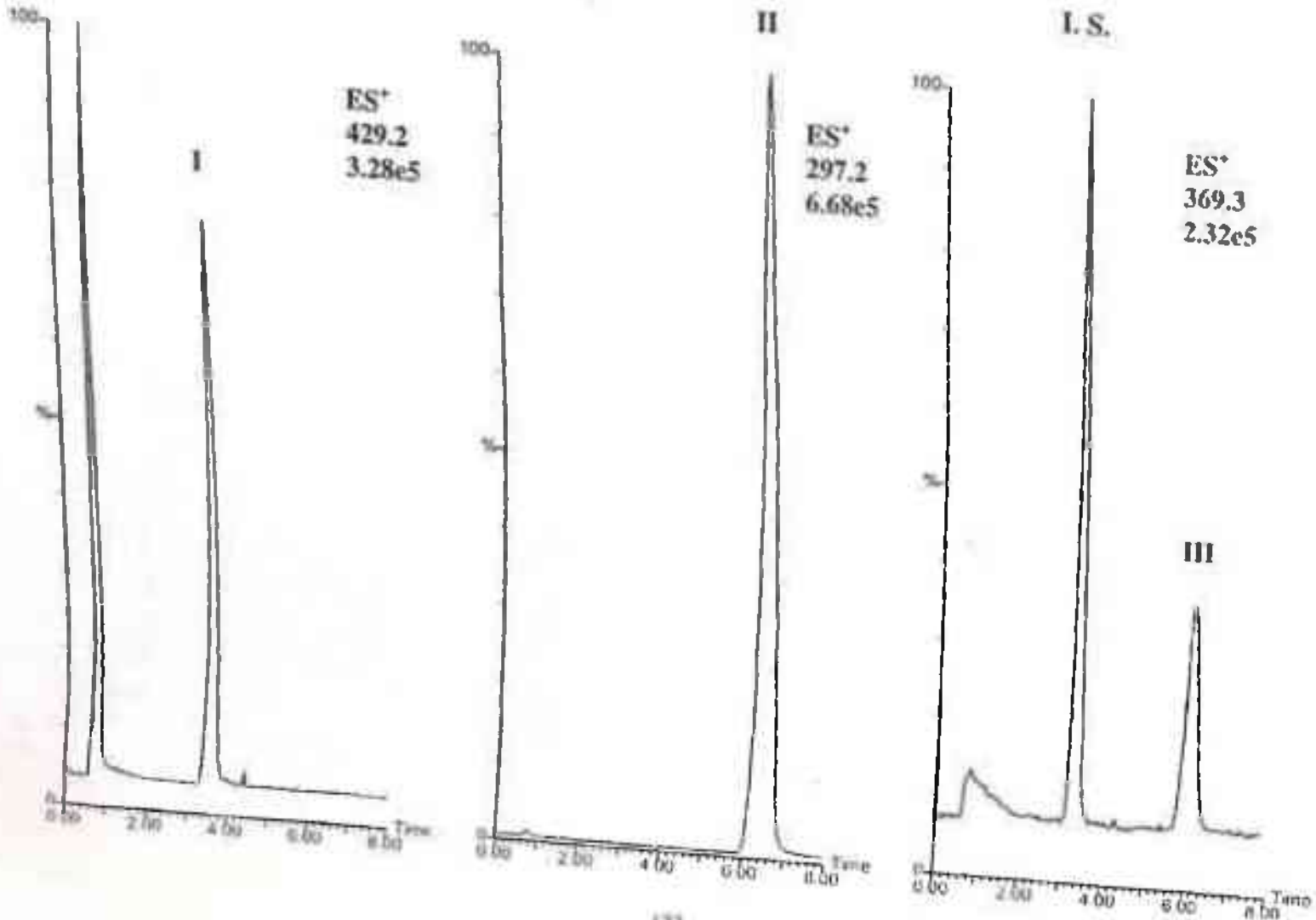
Table 1: Pharmacokinetics of I, II & III in oral Cassette dosing (30 mg/kg) studies ($n = 3$).

Compounds	I	II	III
T_{max} (min)	15	15	20
C_{max} (ng/m)	140.05	212.7	96.29
k_a (min^{-1})	0.024	0.036	0.0227
MAT (min)	41.48	27.34	44
$t_{1/2}$ (min)	71	159.67	72
MRT (min)	83.29	125.33	136
CL (L/min/kg)	122.30	17.73	29.55
V_d (L/kg)	12530	4099	3071
AUC (ng.min/ml)	2823	10114	7172
Bioavalability (%)	3.79	7.67	6.7

It is evident from the concentration-time profile (**Figure , Table**), I, II and III are rapidly absorbed with concentrations peaking at 15, 15 and 20 min respectively. Although the compounds are rapidly absorbed, the bioavailabilities were very low. This phenomenon suggests that initially, the observed Cmax may be due to rapid absorption from the stomach owing to bioenhancing effect of PEG 600 and DMSO in the formulation. This was followed by an irregular concentration-time profile for compounds II and III, while the levels in case of I decreased rapidly by 210 min and at later time points the levels were below LOQ. A justification to such behavior might not be possible with the above data generated but various possibilities can be suggested. The low absorption of the compounds may be due to precipitation followed by slow redissolution in the later non-absorptive portions of the intestine. Irregular time-concentration profiles has been reported for Cimitidine (**1**), Ranitidine (**2, 3**), Pencillamine (**4**) where delayed gastric emptying has been proposed as the reason for the double peak phenomenon. Other possible mechanisms of this phenomenon include enterohepatic recirculation (**5**), storage and subsequent release of drug from a post-absorptive depot site (possibly liver parenchymal cells) (**6**), and variable absorption rates along the gastrointestinal tract (**7**). The probability of enterohepatic recirculation for II increases owing to the fact that a similar phenomenon could be observed in the intravenous concentration-time profile.

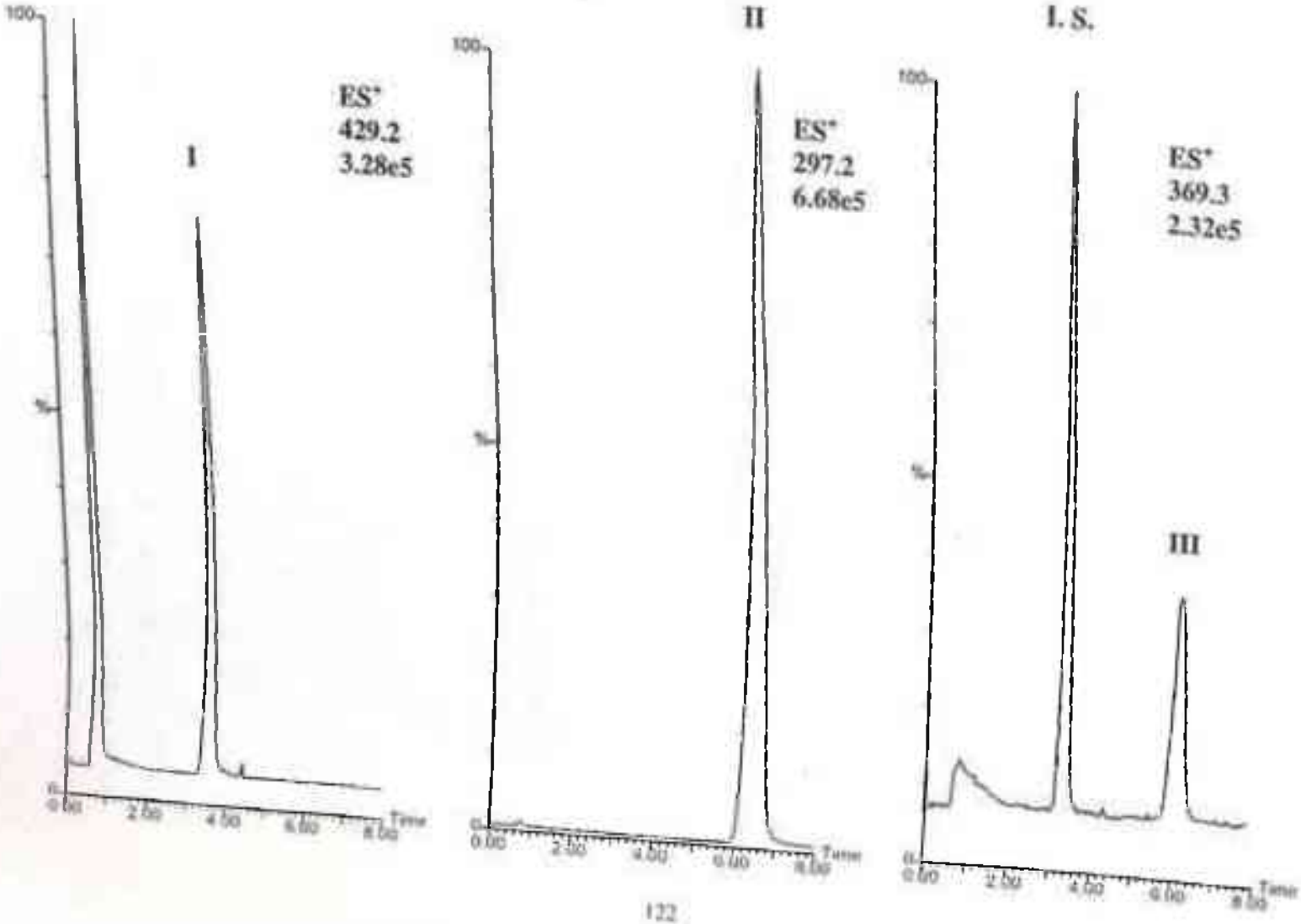
The pharmacokinetic profile of oral cassette dosing indicates the bioavailabilities of II and III are comparable,. While the clearance of II is 1.66 times less than III. Cmax of II is 2.28 times the concentration of III. Furthermore, II is absorbed 1.6 times faster

Figure 1: *LC/MS* chromatograms of test sample in SIR mode following oral administration of I, II and III (15 mg/kg) as a Cassette.

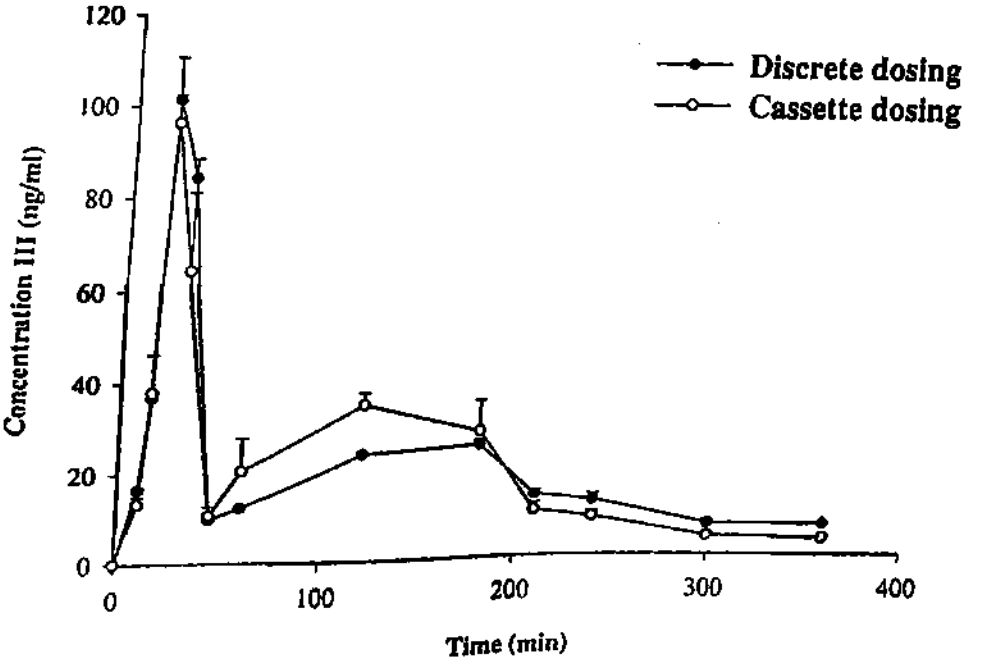


than I and III as evidenced by MAT and k_a. From the criteria of minimum clearance, long MRT and high AUC involved in selection of compound with most optimum PK parameter, the primary objective of II>III>I rank order can be achieved..

Figure 1: LC/MS chromatograms of test sample in SIR mode following oral administration of I, II and III (15 mg/kg) as a Cassette.



c)



The concentration-time profile generated by discrete dosing was found to be comparable with that of cassette as evident from **Figures 2a, b and c.** Similarly the PK data generated by discrete dosing (**Table 2**) was found to be comparable with cassette dosing.

Table 2: Pharmacokinetics of *I, II & III* on oral discrete dosing (30 mg/kg) studies (n = 3).

	I	II	III
		15	20

The LC/MS chromatogram in SIR mode of test sample (t_{max}) following oral administration of I, II and III as Discrete is shown in **Figure 3**. The regression analyses of the mean serum concentration-time data for all the three compounds when dosed individually as well as Cassette followed linear trend indicating a good correlation of the data generated between Cassette and Discrete dosing **Figure 4a, b, c.** There was a good correlation of the pharmacokinetic parameters, particularly for Clearance, AUC, V_d and MRT obtained after Cassette and individual dosing, while a modest correlation was obtained for $t_{1/2}$ and MAT as shown in **Figure 5a, b, c, d, e, f.** These results show that the conclusions drawn on the basis of the PK parameters generated will hold valid for either dosing scheme. Ultimately the linear regression analysis of bioavalabilities for I, II and III obtained by Cassette and Discrete dosing show a very good correlation, **Figure 6**, confirming the applicability of the concept. The ranking of the compounds on the basis of their bioavailabilities shows II>III>I, **Figure 7**, which is in line with the predictions from *ex vivo* studies and *in vivo* i. v pharmacokinetics. From the criteria of lesser clearance, longer MRT and higher AUC involved in selection of the compound with most optimum PK parameters, II>III>I rank order can be achieved by discrete dosing as well.

Figure 3: *LC/MS chromatograms (20 min) in SIR mode following oral administration of I, II and III (15 mg/kg) as Discrete.*

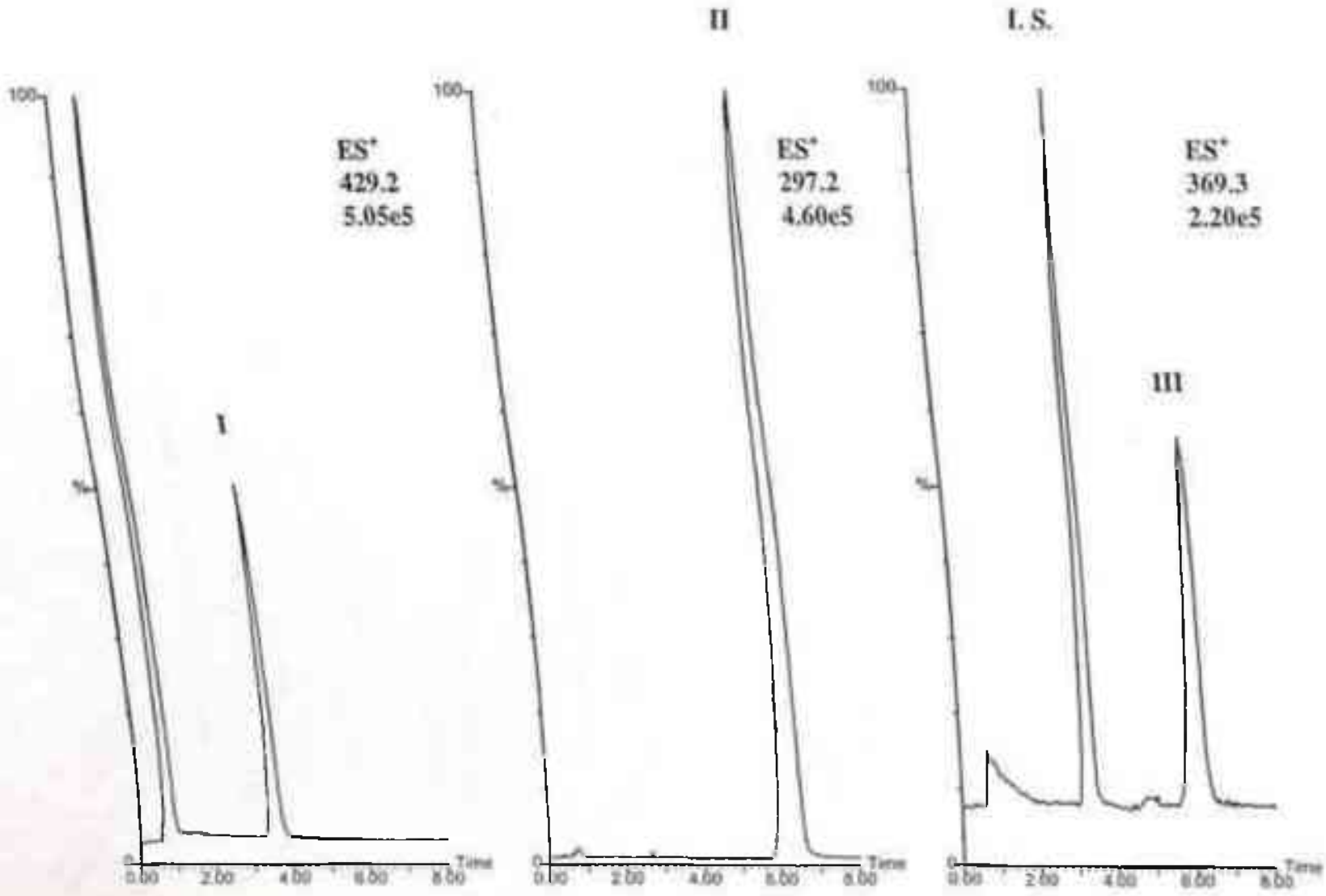
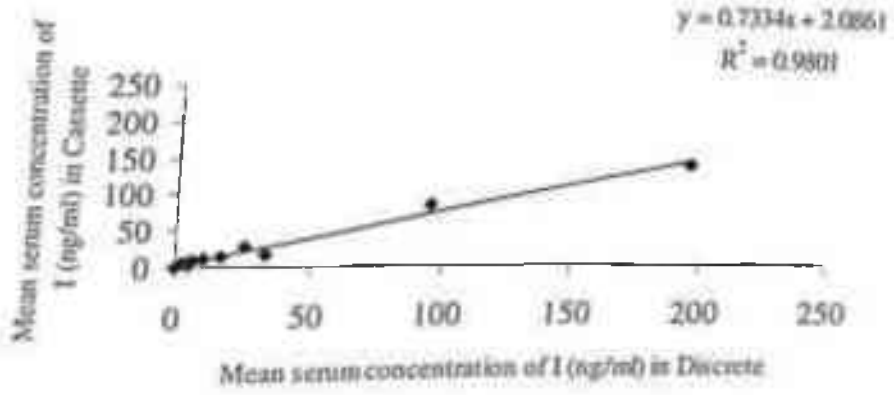
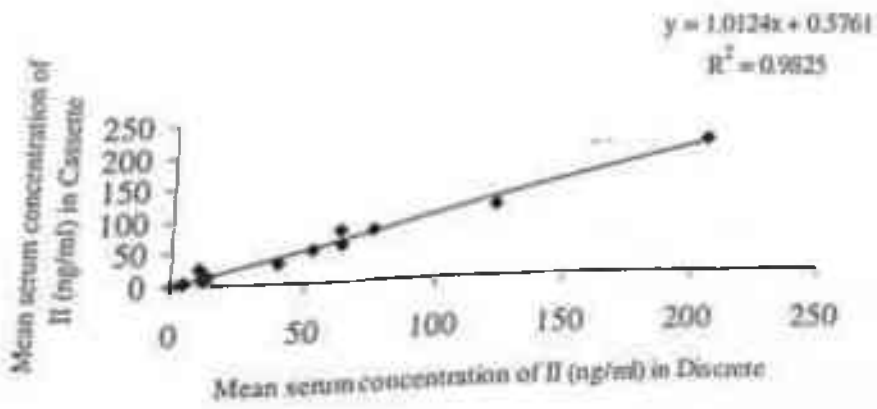


Figure 4. *Regression analysis of Mean serum concentration in oral 15 mg/kg Discrete and Cassette Dosing (n = 3) of a) I, b) II and c) III.*

a)



b)



c)

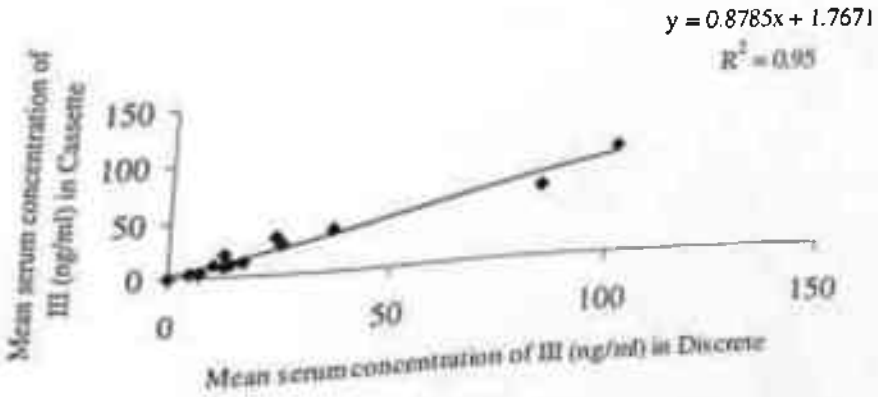
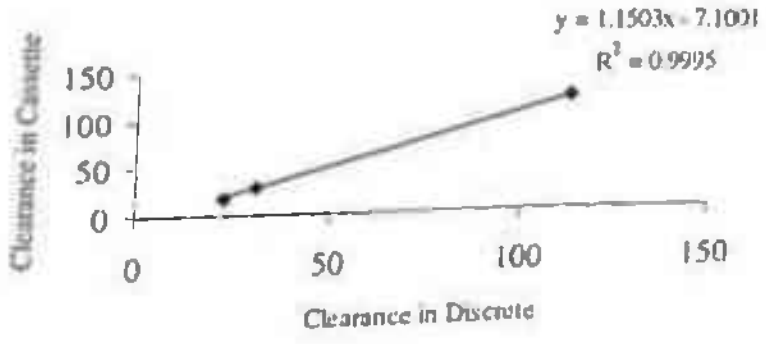
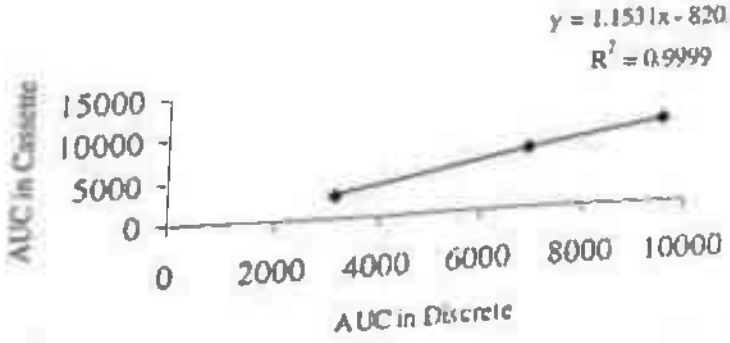


Figure 5: Regression analysis of I, II and III in Cassette versus Discrete a) Clearance b) AUC c) MRT d) V_d e) $t_{1/2}$ f) MAT

a)



b)



c)

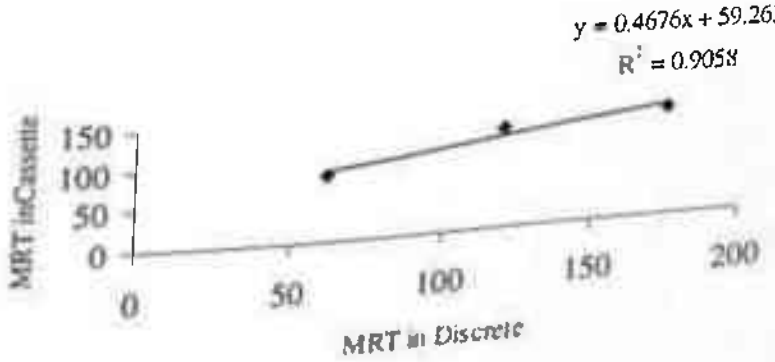


Figure 6: Regression analysis of the bioavailabilities for Cassette versus Discrete dosing (n = 3) of I, II and III.

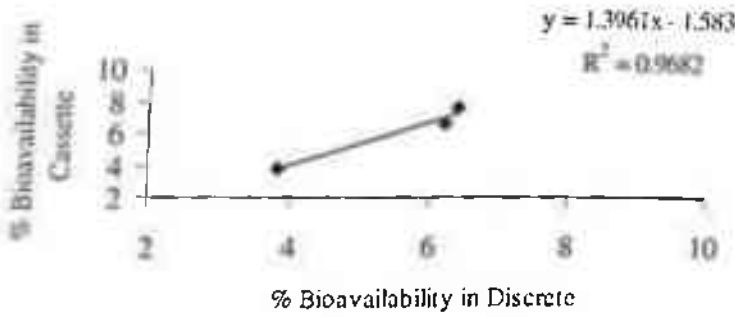
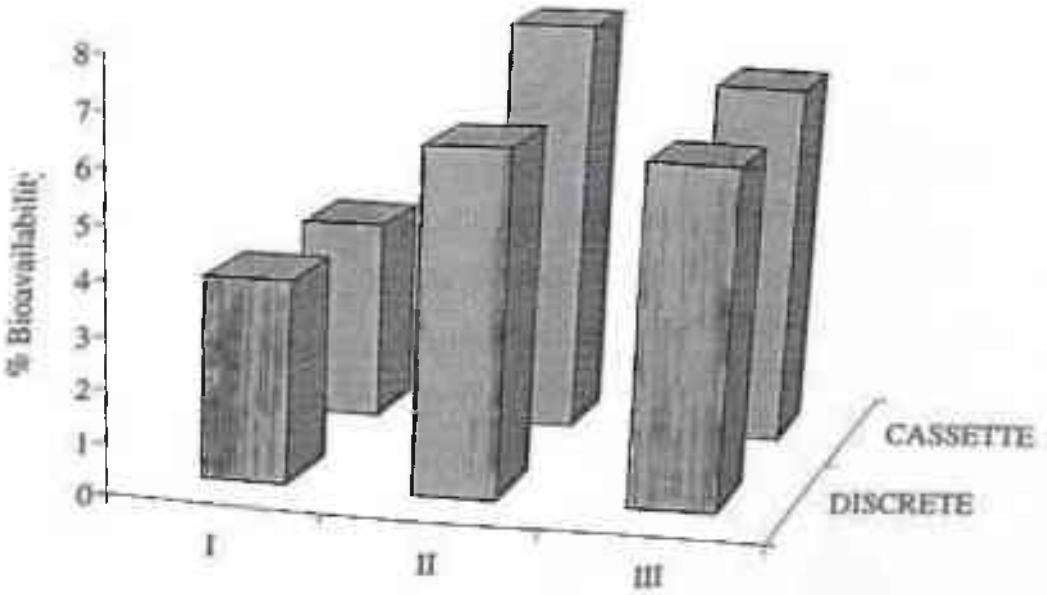


Figure 7: Comparison of the bioavailabilities of Cassette and Discrete Dosing (n = 3) of I, II and III.



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4.2.4 Conclusion

The results of oral PK studies confirms the predictions obtained from the *ex vivo* and i. v. PK studies (**Section 4.1.4**). Moreover, the data generated by Cassette dosing correlates well with that obtained by discrete dosing. The number of test samples analyzed, bioanalysis time and the number of animals involved in the study were reduced by a factor of 1/3 as compared to Discrete dosing studies. This shows the applicability of the concept of Cassette Dosing to increase the throughput of pharmacokinetic screening. Furthermore, conventional analytical methods may not always be useful for the application of the concept. The predictive ability of the concept and the size of the cassette increase with the application of sensitive and selective bioanalytical techniques such as LC/MS as compared to conventional methods.

References:

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3. R. L. Oberle and G. L. Amidon. The influence of variable gastric emptying and intestinal transit rateson the plasma curve of cimetidine: An explanation for the double peak phenomenon. *J. of Pharmacokin. Biopharm.*, 15 :529-545 (1987).
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SUMMARY

Selection and validation of novel molecular targets have become of paramount importance in the light of the plethora of new potential therapeutic drug targets that have emerged from human gene sequencing. In response to this revolution within the pharmaceutical industry, the development of high throughput methods in both chemistry and biology have been necessitated. This has resulted in the need for developing newer strategies and analytical methods to rapidly screen PK behavior of NCEs at required rates. Various concepts developed for rapid generation of PK data are Cassette Dosing and Sample Pooling, which utilize the high sensitivity and selectivity of LC/MS/MS to achieve the purpose.

In the present endeavour, we have investigated the possibility of utilizing traditional analytical techniques for increasing the throughput in preclinical PK screening. The concept of cassette dosing was taken up for the investigation owing to two reasons: i) Cassette Dosing involves lesser number of animals compared to Sample pooling ii) Even though both concepts depend upon a bioanalytical technique that is able to quantify selected "n" number of NCEs simultaneously, sample pooling requires a method which is "n" times sensitive as the samples are diluted "n" times before processing. Furthermore, present investigation also targeted the extent to which traditional bioanalysis would serve the purpose and also investigate the role of advanced bioanalytical techniques in increasing the throughput of PK screening and explore the application of the concept of cassette to various *ex vivo* techniques, which would be valuable in predicting *in vivo* PK. For illustrative purposes, a Cassette of n = 3 compounds 1-(4-propionyl phenoxy)-3-[N4-3',4'-dimethoxyphenyl)-piperazinyl]-N1-propan-2-ol, 1-(phenoxy)-3-[N4-phenyl piperazinyl0]-N1-propane, 1-(2-isopropyl phenoxy)-3-[N4-(2'-methylphenyl)-piperazinyl-N1-propan-2-ol belonging to the class of aryloxy substituted arylpiperazines was constructed and its PK characteristics explored. The feasibility of the concept was explored by comparison of the results obtained with the results obtained by studying discrete compounds. For the selection of the compound with the most optimum PK parameters, a criterion was defined as required by the drug discovery research program of the institute prior to the start of the studies. The desired criteria for the PK variables were, low clearance, high AUC and long residence time.

In the quest for increasing the throughput of PK screening, a sensitive bioanalytical method was developed using isocratic HPLC on a RP-18 stationary phase and a mobile phase consisting of acetonitrile and buffer with fluorescence detection for the simultaneous estimation of I, II and III. The extraction efficiency and sample clean up were achieved utilizing a combination of extraction solvents for extraction and back extraction. This method provided optimum extraction efficiency and reproducibility with shorter run times at a flow rate of 1.5 ml/min. The assay method was validated using accuracy and precision as the parameters so as to facilitate accurate collection of PK data. The method was used to apply the concept of Cassette for simultaneous determination of protein binding at three different concentrations and PK parameters after single intravenous dose of 3mg/kg/compound/animal.

The results of the application of the concept to protein binding show that there is no alteration of binding when all the three compounds are present together over a concentration range of 2.5-10 µg/ml. The percent binding of II and III was above 95% while that of I ranged from 54-59% both in cassette and discrete. Moreover, the protein binding remained unaltered in test sample indicating absence of displacement *in vivo* owing to metabolite formation. Furthermore, it can be concluded that the compounds selected for the cassette would not alter their PK characteristics owing to their protein binding characteristics.

The original goal of the effort, in essence, was achieved by the fact that of the three compounds, one compound emerged out as the one with optimum PK parameters on i.v. dosing of cassette using conventional HPLC/fluorescence detection. Based on the earlier defined criteria, II emerges as the compound with most favourable PK attributes. The clearance can be ranked as I>III>II, while the MRT and AUC as II>III>I. Linear regression analyses were performed to determine how well the data generated by cassette dosing correlated with that of discrete dosing. A very good correlation was obtained for the mean serum concentration at various time points, model independent parameters MRT, V_{ss} and a modest correlation for CL, $t_{1/2}$ and AUC. The end result of Cassette dosing was reduced assay workload (by 67%) and reduced number of animals involved in the study (34 less) resulting in enhanced and economic throughput of PK characterization.

However, the ultimate ranking of a compound depends on the extent of oral bioavailability. Further studies were directed to explore into the various aspects involved in oral administration. Investigations into this aspect were initiated by exploring for GIT permeability of the compounds. The concept of cassette was applied to enhance the rate of throughput and to explore for the pit falls associated with the concept. The GIT permeability was determined using *in situ* duodenal recirculation perfusion studies. The rate of disappearance of the compound from the reservoir was utilized as a parameter to rank the permeability. Based on the absorption rate constants, the compounds can be ranked as III>II>I. III emerges as the compound with best ability to cross the GIT barrier.

However, the extent of oral bioavailability cannot be determined solely on the basis of intestinal permeability as hepatic elimination is another major barrier which the compounds have to cross before becoming available in the system. Hence, the investigation focused on the determination of metabolic instability. *In vitro* rat liver S9 fractions were utilized to incubate the compounds and the rate of disappearance of the parent compounds was utilized to determine metabolic instability. The concept of cassette was applied to enhance the throughput. The samples obtained from the *in vitro* incubation experiments could not be analyzed utilizing the existing bioanalytical method owing to interference in the regions of interest by the metabolites formed. This called for the need of a more selective method of analysis. LC/MS was found to be a suitable method for bioanalysis without sacrificing the basic essence of the investigation i.e., of enhanced throughput.

The compounds were found to be suitable candidates for LC/ESI-MS in positive mode showing protonated molecules. A mobile phase containing high percentage of acetonitrile and methanol with a CN stationary phase provided rapid and complete elution. The low pH aqueous content of the mobile phase facilitated the formation of positive molecular ions for enhanced sensitivity. The chromatograms of I, II, III and I.S. were perfectly extracted from the TIC and when operated in SIR mode provided the required selectivity to carryout *in vitro* metabolism. The method was validated in rat liver S9 fraction with accuracy and precision being the parameters. Optimum sample cleanup and extraction efficiency were achieved. The method was applied to test the concept of cassette on *in vitro* metabolism experiments. The metabolic stability was evaluated by

measuring the disappearance of the parent compounds from the incubation mixture. Based on the disappearance rate constant and t1/2 determined by non-linear regression analysis, the metabolic stability can be ranked as follows III>II>I. Similar ranking could be obtained by carrying out the experiment with discrete compounds. But, the rate constants determined were significantly different indicating the possibility of the influence of one or more compounds on the activity of enzyme systems. Ultimately, based on *in vitro* metabolism studies, I was found to be most optimum followed by II and finally III. This result contradicts the findings of *in situ* absorption studies, which supports the earlier statement that prediction cannot be made solely on the basis of either of the experiments. However, a close observation of the results of both the experiments in conjunction with the results if i.v. studies gives a more realistic prediction. Based on the results, it could be predicted as II to possess the most favorable PK characteristics. II has a better ability to cross the G.I. T barrier compared to I and is found to posses better metabolic stability compared to III.- Moreover, i.v. PK studies, the clearance for II is the lowest and residence is highest amongst the compounds. This inference enables the prediction of better oral availability for II as compared to I and III. The validity of these predictions can be verified by carrying out an oral PK study applying the concept of cassette.

The LC/MS method developed in rat liver S9 fraction was modified for quantification of I, II and III in normal rat serum. Apart from the existing selectivity and specificity, the sensitivity of the method was increased by 62.5 times as compared to HPLS-Fluorescence detection, achieving an LOQ of 2.5 ng/ml, which was necessary to meet the demands of an oral PK study. The assay was validated using accuracy and precision as the parameters and they were found to be within the required limits. This sensitive and selective assay method was adopted for the generation of oral PK data by the application of the concept of cassette dosing. Oral cassette and discrete dosing at a dose level of 15 mg/kg/compound/animal were carried out. The primary objective of ranking the compounds by cassette dosing could be achieved. II was found to emerge as the compound with better PK parameters. Even though the bioavailability and MRT of II and II were comparable, the clearance of II was 1.66 times less than III and the Cmax of II was 2.28 times that of III, with II being absorbed 1.6 times faster than III. The mean serum concentration – time data obtained by cassette dosing showed a good correlation with

discrete dosing. Moreover, a good correlation was achieved for MRT, V_d, CL, and AUC in cassette and discrete dosing showing the feasibility of the concept of cassette dosing in its totality. Based on the above studies, results, inference and criteria, it can be concluded that the concept of cassette dosing can be applied to increase the throughput of PK screening. It should be noted that the limitations of traditional analytical techniques become more exposed especially while carrying out *in vitro* metabolism and oral PK studies, where the selectivity and sensitivity of LC/MS become a necessity. It is recognized that with the application of LC/MS/MS would further accelerate and economize the PK process.