CHAPTER – 1

Introduction

TITLE:- New Approaches in Developing Bioanalytical Assay Methods for Some Selected Drugs in Combination Therapy

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1.1 The perspective for the title

Bioanalysis is an essential part in drug discovery and development. Bioanalytical assay involves the chemistry dealing with the qualitative and quantitative analysis of drug substances and/or metabolites in various biological fluids or tissue. It acts as a significant tool leading to evaluation and interpretation of bioavailability, bioequivalence, pharmacodynamic and pharmacokinetic data. Bioanalysis has evolved over the decade to include toxicokinetics, the assessment of systemic exposures in animals undergoing toxicology studies. As many drug candidates show nonproportional dose–exposure relationships, underpinning toxicity assessments with plasma or target-organ drug levels is a significant advance.

Bioanalytical phases mainly comprises of method development, method validation and sample analysis (method application). Moreover, bioanalysis involves several steps from sample collection to sample analysis and data reporting. The first step is sample collection from clinical or preclinical studies, then sending the samples to laboratory for analysis. The second step is clean-up (sample preparation) and is considered a critical step in bioanalysis. In order to reach reliable results, a robust and stable sample preparation method should be applied. The role of sample preparation is to remove interferences from sample matrix and improve analytical system performance. Sample preparation is often labor intensive and time consuming. The last step is the sample analysis and detection. For separation and detection, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is considered as the method of choice in bioanalytical laboratories and thus has been selected for the present research. This is due to high selectivity and high sensitivity of the LC-MS/MS technique. In addition, the information about the analyte’s chemical structure and properties is important to be known before embarking upon the bioanalytical work.

The present research work is intended to provide further information on the recent advances made in bioanalytical assay methods by optimizing three fundamental steps namely, sample preparation, chromatographic separation and mass spectrometric detection using LC-MS/MS for drugs in combination therapy. Here, combination therapy has been referred to,
✓ drugs that are used in **fixed-dose combination (FDC)** and
✓ combination of drug and its active metabolite.

### 1.2 **Fixed-dose combination (FDC) therapy**

Innovative drug development requires science and regulation to advance in concert. Advances in genomics and cell biology have increased the opportunity for rational design of targeted drugs to inhibit the function of specific molecules. Although targeted therapies may offer enhanced efficacy and improved selectivity (and therefore less toxicity), more often their effects are not durable when they are used alone. Additionally, as the burden of chronic diseases has increased, which often require multiple the need for FDC has also increased consequently [1]. These products, which combine two or more drugs having complementary mode of action into a single tablet, have shown time and again to increase adherence to drug regimens [2]. A meta-analysis of research in this area found an even greater positive effect [3]. Synergistic combinations of two or more agents can overcome toxicity and other side effects associated with high doses of single drugs by countering biological compensation, allowing reduced dosage of each compound or accessing context-specific multitarget mechanisms [4-7].

The term “combination” includes virtually all the ways in which one medication may be added to another. The other commonly used terms are “augmentation” which implies an additive effect from adding a second medicine to that obtained from prescribing a first, an “add on” which implies adding on to existing, possibly effective treatment which, for one reason or another, cannot or should not be stopped. The issues that arise in all potential indications are: a) how long it is reasonable to wait to prove insufficiency of response to monotherapy, b) by what criteria that response should be defined; c) how optimal is the dose of the first monotherapy and, therefore, how confident can one be that its lack of effect is due to a truly inadequate response? Before one considers combination treatment, one or more of the following criteria should be met;

- a) Monotherapy has been only partially effective on core symptoms;
- b) Monotherapy has been effective on some concurrent symptoms but not others,
for which a further medicine is believed to be required;

c) A particular combination might be indicated *de novo* in some indications;

d) The combination could improve tolerability because two compounds may be
employed below their individual dose thresholds to prevent any side effects.

Regulators have been concerned primarily with criteria ‘a’ and, in principle
with criteria ‘c’. In clinical practice, the use of combination treatment reflects the
often unsatisfactory outcome of treatment with single agents [8].

The challenge is to test novel combinations particularly for drugs that have not
been as yet licensed. Clinical trials are often influenced by the financial pressure
aimed at demonstrating product effectiveness for licensing rather than systematically
designing trials to evaluate the mechanisms of action of the therapy as well as
whether biological end-point are achieved or why treatment might have failed. Such
information could provide clues for the design of more sophisticated follow up
studies using novel agents. Thus, several drugs tested as single agents risk being
withdrawn and thus become unavailable for further research by early negative clinical
results from studies that had not been planned carefully to understand the potential
activity of the drug when administered to human beings. It may be important to
rescue drugs that “failed” when used as single agents to study their effectiveness in
combination with others that have different but potentially synergistic mechanisms of
action [9].

The concern has been that the policies of the Food and Drug Administration
(FDA) on the development of combination therapies, which heretofore have focused
primarily on FDCs of already marketed drugs, are a barrier to the development of
novel combination regimens using targeted therapies [1, 10].

**De novo**

*De novo, a term is for any method that makes predictions about biological features using only a computational model without extrinsic comparison to existing data.*

**Placebo**

*A placebo is a simulated or otherwise medically ineffectual treatment for a disease or other medical condition intended to deceive the recipient.*

FDA regulations for FDCs require demonstration of the contribution of each
component of the combination to the treatment effect. Often, a large clinical trial, using a multigroup
factorial design to demonstrate that the combination is superior to each of the individual
components alone, is needed to meet this requirement. For example, a factorial study for a
two-drug combination could have four groups so that the combination can be compared with each of
the individual components alone, as well as with
either the standard of care or placebo [1]. Furthermore, noncompliance to medication decreased by 26% when people took FDCs for conditions such as hypertension, HIV and tuberculosis, which concludes with a recommendation for increased use of combination drugs for chronic illnesses [2]. The FDA recognizes that for diseases in which innovative targeted combination therapies are likely to be used, such studies will often be unethical because of the potential for promoting the development of resistance and rendering a new therapy ineffective [1]. In addition to this, new research may be required if there is potential for the active agents to interact in a way that could prove harmful, or if there is the possibility that one component could chemically modify another component and compromise its effectiveness. But if there are no apparent safety issues, the manufacturer may not have to conduct any additional research [2].

To ensure that the regulatory expectations are clear, the FDA has drafted guidance about testing and developing two or more novel agents together in a single development program (termed “co-development” in the guidance) [11]. It also makes clear that the FDA’s regulations and policies pertaining to the amounts and types of data needed to demonstrate the contribution of each drug to the overall effect provide adequate flexibility to facilitate the development of novel targeted therapies for use in combination regimens in diseases for which a large factorial study (requiring monotherapy treatment groups) would not be possible. And it emphasizes that a range of potential data sources could be used to help establish the contribution of the individual drugs and provides examples of potential alternative study designs, including the use of data from in vivo models and pharmacodynamic studies. To date, in addition to the chronic diseases interest in combination development has also focused on cancer and infectious diseases like tuberculosis [1].

Here in the present work, three potent FDCs have been studied whose selective synergy depends on multi-target drug activity.
1. Losartan & Hydrochlorothiazide (Antihypertensive)
2. Metformin & Sitagliptin (Antidiabetic)
3. Metformin & Saxagliptin (Antidiabetic)

### 1.2.1 FDC of Antihypertensive Drugs

Hypertension is defined as systolic blood pressure (SBP) that exceeds 140 mmHg and/or diastolic BP (DBP) that exceeds 90 mmHg [12, 13]. It is currently one of the greatest global healthcare challenges. Associated with an increased risk of several
serious comorbid conditions, including stroke, myocardial infarction, congestive heart failure, and renal failure, its complications are responsible for 9.4 million deaths globally each year, including around 45% of all deaths due to heart disease and around 51% of deaths due to stroke [14].

Although many effective drugs are available, combinations of 2 or more medications are often required to meet clinical targets. Combination therapy may be theoretically favoured by the fact that multiple factors contribute to hypertension, and achieving control of blood pressure with single agent acting through one particular mechanism may not be possible [15]. Most patients require combination therapy with two or more drugs to adequately control blood pressure to targets recommended by European and International guidelines [16]. Classes of antihypertensive agents which have been commonly used are angiotensin receptor blockers, thiazide diuretics, beta and alpha blockers, calcium antagonists and angiotensin-converting enzyme inhibitors. The majority of currently available FDCs have one component which a diuretic.

Angiotensin II receptor antagonists (angiotensin receptor blockers; ARBs) and thiazide diuretics have an accepted place in the management of hypertension. ARBs and the thiazide diuretic hydrochlorothiazide have complementary modes of action. FDCs of an ARB and low-dose hydrochlorothiazide provide a convenient and effective treatment option for patients who do not achieve blood pressure targets on monotherapy, without compromising the placebo-like tolerability of ARBs [16]. FDCs with hydrochlorothiazide currently are available for the ARBs candesartan, eprosartan, irbesartan, losartan, telmisartan and valsartan (Table 1).

<table>
<thead>
<tr>
<th>Combination</th>
<th>Available strength (mg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candesartan + Hydrochlorothiazide</td>
<td>8/12.5, 16/12.5</td>
</tr>
<tr>
<td>Eprosartan + Hydrochlorothiazide</td>
<td>600/12.5, 600/25</td>
</tr>
<tr>
<td>Irbesartan + Hydrochlorothiazide</td>
<td>150/12.5, 300/12.5</td>
</tr>
<tr>
<td>Losartan + Hydrochlorothiazide</td>
<td>50/12.5, 100/12.5</td>
</tr>
<tr>
<td>Telmisartan + Hydrochlorothiazide</td>
<td>40/12.5, 80/12.5</td>
</tr>
<tr>
<td>Valsartan + Hydrochlorothiazide</td>
<td>80/12.5, 160/12.5, 160/25</td>
</tr>
<tr>
<td>Olmesartan + Hydrochlorothiazide</td>
<td>Country specific</td>
</tr>
</tbody>
</table>
The renin-angiotensin system (RAS) plays a central role in the maintenance of BP and is a major target for antihypertensive drugs. The principal pressor molecule of the RAS, angiotensin II, is involved in the control of BP, cardiac and vascular function, and sodium and water homeostasis. In the 1990s, orally-active, specific, non-peptide angiotensin II receptor antagonists (ARBs) that act by preventing the binding of angiotensin II to the angiotensin II type 1 \((\text{AT}_1)\) receptor (Figure 1) were used. ARBs may indirectly provide additional benefit as they do not oppose the potentially beneficial stimulatory effects of angiotensin II at the \(\text{AT}_2\) receptor. These selective antagonists effectively control BP in hypertensive patients, with tolerability comparable to that of placebo [17-24].

Although the treatment of hypertension provides a direct rationale for using drugs that target the RAS, angiotensin II has also been implicated in the pathophysiology of cardiovascular disease, diabetic nephropathy, and renal failure. Increasingly, the findings of large clinical trials have shown that targeting the RAS provides additional benefits, such as end-organ protection, beyond lowering BP. The greater selectivity of the ARBs in interrupting the potentially harmful effects of angiotensin II have made them a logical choice for the study [16]. The orally active ARBs share some pharmacologic characteristics. Most of them have a high affinity for \(\text{AT}_1\) receptors, display very high protein binding, and dose-dependently block the pressor response to exogenous angiotensin II [25].

Despite the range of treatment options available and a growing awareness of the need for achieving normal or optimal BP levels, BP control remains inadequate in many patients. International guidelines for the management of hypertension emphasize that most patients will require combination therapy with two or more antihypertensive drugs to achieve the increasingly stringent BP targets recommended for the control of hypertension (<140/90mm Hg, or <130/80mm Hg for patients with diabetes mellitus or chronic kidney disease) [26, 27]. The HOT (Hypertension Optimal
Treatment) study effectively demonstrated that it is possible to achieve such BP targets with simple combination therapy regimens [28]. Approximately 70% of patients in the HOT study required two or more antihypertensive agents to achieve BP targets (Figure 2) [29].

Thiazide diuretics, such as hydrochlorothiazide, have an established role in antihypertensive therapy and are recommended in international guidelines for the management of hypertension [26, 27]. When BP targets are not reached with monotherapy, the combination of a diuretic with ARBs is a rational treatment option, as these therapies have complementary mechanism of action. Thiazide diuretics act directly on the kidney, blocking renal tubular reabsorption of sodium, thereby increasing urinary sodium excretion, leading to a reduction in extracellular fluid volume and peripheral resistance (Figure 3). The resulting activation of the RAS and the sympathetic nervous system increases the sensitivity of the AT\(_1\) receptor and thus enhances the response to ARBs [16]. The use of ARBs and HCTZ in combination counteracts the potential adverse effects of these agents when given as monotherapy. Because of the tendency of ARBs to elevate potassium levels, this is less likely to be a problem when combined with diuretics. Likewise, many of the undesirable metabolic side effects of thiazide monotherapy, including hypokalaemia,
and elevated serum levels of uric acids, lipids and blood glucose levels, are minimised by the addition of an ARBs [30]. Available FDCs of ARBs with hydrochlorothiazide (Table 1) are rapidly gaining acceptance with physicians as an effective treatment option for hypertension [16].

Furthermore, diuretics are known to increase uric acid levels and, occasionally, to precipitate a gout attack in susceptible patients. Drugs that increase renal blood flow are known to have a slight uricosuric effect. Thus, in general, the addition of an ARBs to a diuretic will lower uric acid to some extent. However, in contrast to all other ARBs, losartan has been shown to have a distinct hypouricemic effect. The combination of losartan with a diuretic almost diminishes the hyperuricemic effect of the diuretic. This antagonism occurs with both FDCs of losartan and hydrochlorothiazide (50 mg/12.5 mg and 100 mg/25 mg) [31]. Thus, FDC of losartan and hydrochlorothiazide has been selected among all combinations for the present study.

1.2.2 FDC of Antidiabetic Drugs

Type 2 diabetes is a disease in which body loses its ability to produce and use insulin, a hormone made by the pancreas. After the breakdown of sugar and starch from food, insulin delivers the glucose to cells that absorb it and use it for energy. Insulin also helps to eliminate extra glucose from blood. With Type 2 diabetes, body cannot produce enough insulin to maintain normal glucose levels and cells fail to react properly to the insulin that is made – known as insulin resistance (Figure 4) [32].
Although the most desirable target for good glycaemic control remains in contention, there is much evidence to demonstrate that better glycaemic control, especially during early stages of the disease process, helps to reduce long term morbidity and mortality [33]. Most treatment algorithms for type 2 diabetes acknowledge this as a basis for their choice of target [34-37]. They also emphasize lifestyle (diet and exercise) advice as initial and ongoing therapy and generally suggest metformin as a first-line pharmacological agent, provided there are no contraindications or tolerability issues [34-37]. If appropriate, another type of antidiabetic agent such as an insulin secretagogue, thiazolidinedione or an $\alpha$-glucosidase inhibitor could be used. However, one antidiabetic agent alone is rarely sufficient to maintain acceptable glycaemic control. For example, in the United Kingdom Prospective Diabetes Study, less than one-quarter of patients treated with a single oral antidiabetic agent maintained a glycosylated haemoglobin A1c (HbA1c) level below 7% after 9 years [38]. When one agent does not achieve or sustain the desired glycaemic target, it is customary to add a second agent. If patients exhibit severe hyperglycaemia, this usually indicates the need for insulin therapy which can be introduced while continuing one or more oral agents (Chart 1).

As a rule of thumb antidiabetic agents with different modes of action can be used in combination to achieve additive or possibly synergistic glucose-lowering effects.
In principle, these differently acting agents should address different pathological factors, thereby increasing therapeutic breadth to combat the progressive nature of type 2 diabetes. Where lower doses of two agents can be used instead of a high dose of one agent, this can reduce the side effects that often occur with a high dose of the one agent. While these combinations have customarily been taken as separate tablets, several fixed-dose single tablet combinations are now available. These are based on bioequivalence with the separate tablets, giving similar efficacy to the separate tablets and necessitating the same cautions and contraindications that apply to each active component. They increase patient adherence compared with equivalent combinations of separate tablets, and this is associated with some improvements in glycaemic control [38].

Presently available antidiabetic FDCs include metformin combined with a sulphonylurea, thiazolidinedione, dipeptidylpeptidase-4 inhibitor or meglitinide as well as thiazolidinedione–sulphonylurea combinations, each at a range of dosage

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*Insulin secretagogue could be a sulphonylurea, meglitinide, DPP-4 inhibitor*
strengths to facilitate titration [34, 35]. The main fixed-dose single tablet combinations of antidiabetic drugs are listed in **Table 2**. Not all combinations or dosage strengths are available in all countries, and there are some variations in the names and approved indications.

<table>
<thead>
<tr>
<th>Tablet</th>
<th>Components</th>
<th>Strengths (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucovance</td>
<td>Metformin + glibenclamide</td>
<td>250/1.25, 500/2.5, 500/5</td>
</tr>
<tr>
<td>Metaglip</td>
<td>Metformin + glipizide</td>
<td>250/2.5, 500/2.5, 500/5</td>
</tr>
<tr>
<td>Avandamet</td>
<td>Metformin + rosiglitazone</td>
<td>500/4, 500/2, 1000/2, 1000/4</td>
</tr>
<tr>
<td>Competact</td>
<td>Metformin + pioglitazone</td>
<td>500/15, 850/15</td>
</tr>
<tr>
<td>Eucreas</td>
<td>Metformin + vildagliptin</td>
<td>850/50, 1000/50</td>
</tr>
<tr>
<td>Janumet</td>
<td>Metformin + sitagliptin</td>
<td>500/50, 1000/50</td>
</tr>
<tr>
<td>Kombiglyze</td>
<td>Metformin + saxagliptin</td>
<td>500/5, 500/2.5</td>
</tr>
<tr>
<td>Prandimet</td>
<td>Metformin + repaglinide</td>
<td>500/1, 500/2</td>
</tr>
<tr>
<td>AvaglimAvandaryl</td>
<td>Rosiglitazone + glimepiride</td>
<td>4/1, 4/2, 4/4, 8/2, 8/4</td>
</tr>
<tr>
<td>TandemactDuetact</td>
<td>Pioglitazone + glimepiride</td>
<td>30/4, 45/4</td>
</tr>
</tbody>
</table>

In the present work FDCs of metformin with one of the newly available secretagogues, namely a dipeptidylpeptidase-4 (DPP-4) inhibitor or an injectable glucagon-like peptide-1 (GLP-1) agonist have been selected for their bio-equivalence study by liquid chromatography-tandem mass spectrometry (LC-MS/MS). In practice, metformin can be combined with any other therapy. It improves glycemic control in type 2 diabetes by increasing insulin sensitivity in the liver and peripheral organs. This leads to decreased hepatic glucose production (the main effect) and increased glucose disposal in skeletal muscles. A systematic review of *in vivo* studies in humans showed metformin enhances insulin suppression of endogenous hepatic glucose production and clearance of glucose in the fasting state, but it did not enhance insulin-mediated glucose uptake in peripheral tissues. Metformin improves glycemic control through its action in the liver, small intestines, and skeletal muscles (*Figure 5*) [39].
Traditional glucose lowering agents include insulin – which has been in use for nearly a century now, sulfonylureas and metformin. The first two are associated with weight gain and hypoglycaemia – side effects which have largely restricted the ability to intensify therapy and attain stringent glycemic targets. Thiazolidinediones were frequently used in the early 2000’s, yet side effects such as weight gain, edema and increased risk of fractures have limited their use. The need for new agents which lower blood glucose efficiently and safely, with no weight gain, resulted in the development of new class of glucose lowering agents, namely DPP-4 inhibitors. The growing body of data demonstrating the efficacy, as well as overall and cardiovascular safety has greatly promoted the uptake and use of this new drug class [40]. In this work two potent DPP-4 inhibitors *viz.* sitagliptin and saxagliptin were chosen together with metformin.

In order to appreciate the mechanism associated with the DPP-4 inhibitors, we must understand the normal function of incretin hormones. Creutzfeldt first described the incretins in 1979. Incretin hormones are released from the gut in response to nutrient consumption, and they cause insulin release in a glucose-dependent manner. GLP-1 is one hormone that meets these criteria [41]. It is involved in glucose homeostasis via stimulation of insulin biosynthesis and secretion as well as inhibition of glucagon release. GLP-1 also improves satiety, slows gastric emptying, and may be associated with improved beta-cell function (*Figure 6*). However, GLP-1 is quickly inactivated by DPP-4, which is present in the kidneys, intestines, and bone marrow [42].
1.3 **Combination of drugs and their active metabolite(s)**

It has been estimated that for every 5000 chemicals evaluated in discovery programs, only one is approved for market. Among the major reasons for termination of the development of new chemical entities (NCE) other than efficacy, the dominating factor is poor toxicology and unfavourable pharmacokinetic properties including poor absorption, distribution, metabolism and excretion (ADME) characteristics [43]. The expense of terminated development processes can be extremely high. To increase the probability that an NCE will pass clinical tests, the ADME properties should be tested as early as possible in the discovery phase [44, 45]. This has forced the pharmaceutical industry to modify procedures during drug discovery and to develop new technologies to increase productivity and efficiency of the discovery phase. Among the ADME properties, metabolic characterization is a key issue and nowadays it is integrated into the early discovery phase.

*Figure 6. Role of GLP-1 in glucose homeostasis. GIP = glucose dependent insulinotropic peptide; PACAP = pituitary adenylate cyclase-activating polypeptide*
Drugs are xenobiotics to living organisms, which therefore biotransform them into less toxic, less active and more hydrophilic forms and so enhance their excretion in urine. However, biotransformation can also lead to some unwanted consequences, such as rapid clearance of the drug from the body, formation of active metabolites, drug–drug interactions due to enzyme induction or competition and formation of reactive or other toxic metabolites. In the early discovery phase, the metabolic fate of drugs is effectively studied using simple *in vitro* approaches, instead of laborious but more relevant *in vivo* studies that are used in the development stage and in clinical tests [46-48]. The information generated in the early discovery phase can be used to identify NCEs with undesirable metabolic behaviour and to optimize pharmacokinetic and safety profiles by means of synthetic chemical transformations.

Metabolic pathways are divided into phase I and phase II reactions, and both classes of reaction often occur in parallel for particular compounds (Chart 2). In phase I reactions enzymes modify the parent compound via hydrolysis, oxidation and reduction, which increase the polarity and also the excretion of the compound. The resulting phase I metabolites are not necessarily inactive yet, as is the case with codeine, which is demethylated to the more potent morphine. More often phase I reactions are preparative stages for further reactions, exposing reactive sites to the molecule structure for the subsequent phase II processes, i.e. conjugation reactions. Phase II reactions are often considered as ‘true’ detoxifying reactions [47], as the conjugation of bulky and polar group most often terminates the activity of the substrate and enhances elimination.

![Chart 2. General routes of metabolism](image)
The role of metabolites in bioequivalence (BE) studies, however, has been a controversial issue for decades. A number of reasons for use of metabolite data have been put forward, such as (i) the parent is an inactive prodrug, (ii) plasma concentrations of the parent drug are too low to monitor because of inadequate assay sensitivity, (iii) the parent drug is metabolized rapidly to an active metabolite, and (iv) the parent drug and a metabolite both have therapeutic activities but the metabolite is present in higher concentrations [49].

Reactive metabolites can be generated during the process of drug metabolism that can covalently bind to protein, RNA and DNA, resulting in toxicity. Identification of reactive metabolites helps medicinal chemists minimize the formation of reactive metabolites by optimizing the structure of the parent drug. This knowledge also provides valuable information on the mechanism of drug-induced toxicity. Retrometabolic drug design is mainly based on drug metabolism. For a drug with a low bioavailability because of extensive first-pass effect, a metabolism-based structural modification will block the major metabolic pathway of the drug and thus improve its bioavailability [50]. Pharmacologically active metabolites can contribute significantly to the overall therapeutic and adverse effects of drugs [51]. Therefore, to fully understand the mechanism of action of drugs, it is important to recognize the role of active metabolites. Active metabolites can also be developed as drugs in their own right.

Regardless of the therapeutic class of NCE being developed, the analysis of metabolites in both toxicology species and clinical samples may become necessary during the course of development of the NCE. In the initial assays, it is fairly possible to use the parent drug as the surrogate reference standard to predict the concentrations of the metabolites. This would enable to get an idea of the exposure and/or pharmacokinetics of the metabolites in the species tested and would guide the requirements for the development and validation of a new assay for the metabolites. However, it is important that pure reference standard of the metabolites be made available subsequently to obtain proper validation of the method as well as to obtain unambiguous analytical data of the metabolites to delineate the pharmacokinetic disposition of the metabolites. The metabolite analysis becomes critical if the metabolite possesses activity either superior to or comparable with that of the parent, in order to better understand the role of metabolite in producing pharmacological effects [52].
Additionally, metabolite exposure data are needed in both toxicology species and clinical samples to compute appropriate safety margins. It is prudent to collect data on metabolites wherever possible, unless it represents a significant problem in collection, processing and analyzing the metabolite concentrations. Regardless of the complexity of the assaying of metabolites, it is always practical to weigh in the benefits of obtaining such data in the clinical study being conducted. It becomes very critical and absolutely important to monitor the levels of metabolites in clinical studies where drug–drug interactions (pharmacokinetic/pharmacodynamic) are being investigated. The availability of metabolite data during the clinical study may provide additional evidence of interaction (or lack of it thereof). The metabolite data may become useful where a judgment call solely based on parent drug concentrations (or pharmacokinetic parameters) is not possible. In some instances, when the parent drug disappears rapidly from the central compartment (rapid clearance), the metabolites provide a greater opportunity for monitoring any potential drug–drug interaction effects. For example, in the case of development of prodrugs, the active moiety released from the hydrolytic cleavage may be ‘technically’ considered as a metabolite [52].

Knowledge of the sites of metabolic processes is of great importance, not only in the evaluation of the fate of the compound, potential drug–drug interactions and competitive metabolic routes, but also in the design of the test system, especially when determining the most representative sample matrix. The information required to determine the metabolic fate of an NCE includes detection of metabolites, structure characterization and quantitative analysis [48, 51]. In some cases the concentrations of the metabolites may be extremely low and highly specific and sensitive analytical methods are then required.

The analytical strategy for drug analysis is dependent on the information sought. In the early stages of discovery, metabolic stability, drug–drug interaction and enzyme kinetic studies are based on the quantitative analysis of a parent drug or a few of its metabolites. In these types of analyses, the key issue is of throughput and therefore the analytical method should be as fast as possible. However, the determination of single drug or its combination with metabolite profiles is usually performed for a limited number of lead molecules in vivo and in vitro, and in these experiments the key issues are high specificity and sensitivity rather than speed [48]. An analytical strategy for metabolite profiling by LC/MS is presented in Chart 3.
Drug Collection of biological samples after drug administration

**Production**

Collection of biological samples after drug administration

**Purification**

Extraction and isolation of metabolites; e.g. PP, LLE, SPE

**Detection**

- Detection of radioactively labelled metabolites by LC using radioactivity detection
- Detection of new peaks in LC/MS total ion and LC/UV chromatograms. Comparison to the chromatograms of blank
- Detection of expected metabolite mass fragments in ion chromatograms
- Detection of metabolites in ion chromatograms using stable isotope labelling, if available
- Detection of possible metabolites by neutral loss and precursor ion scans, and using specific marker ions
- Detection of possible metabolites by LC-high resolution MS

**Characterization**

- Comparison of product ion spectra of a metabolite and parent drug
- Detection of structure specific marker ions by MS/MS or MSn
- Determination of elemental composition by accurate mass measurements
- Complementary MS analysis; e.g. reversed polarity, different ionization method
- Synthesis of the presumed metabolites and comparison of retention times and spectra
- LC/NMR/MS or Off-line NMR analysis after production and purification of the metabolites in large scale

**Chart 3. Strategy and possibilities for metabolite profiling by LC/MS**
In the current research four drugs belonging to different categories have been quantified along with their active metabolite(s) in human plasma using LC-MS/MS.

1. Losartan & losartan carboxylic acid together with hydrochlorothiazide (Antihypertensive)
2. Saxagliptin & 5-hydroxy saxagliptin along with metformin (Antidiabetic)
3. Pyrazinamide & its two active metabolites, pyrazinoic acid and 5-hydroxy pyrazinoic acid (Antituberculosis)
4. Bupropion & its three major metabolites, hydroxybupropion, threo-hydrobupropion and erythrohydrobupropion (Antidepressant and smoking cessation aid)

The first two drugs along with their mode of actions have been discussed in Section 1.2. Further the mode of action for pyrazinamide and bupropion is explained in brief below.

Pyrazinamide (PZA) is a drug used to treat tuberculosis (TB). It has remarkable sterilizing activity on the so-called persistent bacterial population. The core mechanism of PZA in mycobacteria relies on the inactivation of the nicotinamidase/pyrazinamidase enzyme (PncA, encoded by the pncA gene), physiologically involved in the metabolism of nicotinamide and responsible of the conversion of PZA to its active form (pyrazinoic acid; POA) [53, 54]. Despite the importance of PZA in the treatment of TB, its mechanism of action is probably the least understood. The prodrug PZA has been reported to accumulate mainly in the caseum of granulomas and it is active only at acidic pH, after its conversion to POA. The main mechanism of mycobacterial killing by PZA was demonstrated to be the depletion of cellular adenosine triphosphate reserves caused by the inhibitory role of the POA on the proton motive force interfering with adenosine triphosphate synthesis [53]. The process can be summarized as follows and is shown in Figure 7:

(A) Membrane transport systems (passive or facilitated diffusion seem to play a role) are involved in transporting the prodrug into the TB bacillus; (B) the PZase activity of PncA transforms the prodrug into the active compound POA; (C) in acid conditions, POA is converted to protonated POA (HPOA); (D) HPOA enters and kills the TB bacterial cell by reducing membrane potential and affecting membrane transport (the membrane in

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**Prodrug**

A prodrug is a medication or compound that, after administration, is metabolized (i.e., converted within the body) into a pharmacologically active drug. Inactive prodrugs are pharmacologically inactive medications that are metabolized into an active form within the body.
yellow in the figure); (E) trans-translation inhibition by directly targeting the rpsA gene (30S ribosomal protein S1) was demonstrated in 2011. Other factors affecting/influencing the activity of PZA include, acid pH, culture age, starvation and microaerophilic/anaerobic conditions [53, 54].

Bupropion was initially developed and licensed for the treatment of major depressive disorder in the United States in 1989. It was licensed as a pharmacotherapy for smoking cessation in the United States in 1997 and in the United Kingdom in 2000, and for the prevention of seasonal major depressive episodes in patients with seasonal affective disorder in the United States in 2006 [55].

Most antidepressants act through increasing the synaptic levels of serotonin or noradrenaline (norepinephrine) through various pharmacological mechanisms. Bupropion is a trimethylated monocyclic phenylaminoketone second-generation antidepressant, which differs structurally from most antidepressants, and resides in a novel mechanistic class that has no direct action on the serotonin system. Its main mechanism of action is believed to be via dopamine and noradrenalin reuptake inhibition [56]. Figure 8 illustrates the plausible mode of action for bupropion.
1.4  **Bioanalysis concept: From vial to file**

Bioanalysis is well established in pharmaceutical companies to support drug discovery and drug development. It has an important role to perform in toxicokinetics (TK), pharmacokinetics (PK) and pharmacodynamic (PD) studies of new drugs. Bioanalysis is also established in clinical, preclinical and forensic toxicology laboratories [57]. Thus, bioanalysis is an important discipline in many research areas such as the development of new drugs, forensic analysis, doping control and identification of biomarkers for diagnostic of many diseases.

Bioanalysis is challenging due to the complexity of the sample matrix [58-61]. It is well known that complex matrices such as blood, plasma and urine need an intensive sample preparation prior to injection to analytical instrument. High throughput sample preparation and hyphenated analytical instruments are required in modern bioanalysis. Liquid chromatography (LC) combined with tandem mass spectrometry (MS/MS) have been used for a long time in drug bioanalysis. Method validation is most important part in regulated bioanalysis. Validation is necessary to demonstrate the bioanalytical method performance [62]. The bioanalytical workflow contains many steps from sample collection to sample analysis and data reporting.
The life cycle of a bioanalytical method evolves, and new findings may require additional method enhancements (Figure 9).

![Figure 9. Life cycles of bioanalytical method](image)

Typically a bioanalytical LC-MS method can be divided into four phases: method development, method validation, sample analysis and method enhancement. Information about the chemical properties of the analyte such as stability, volatility, reactivity and polarity are imperative before proceeding for the bioanalytical work. In addition, the expectation of the analyte concentration range and the nature of sample matrix are important to be known.

### 1.5 Strategic method development for bioanalytical assay

Before initiating the bioanalytical method development there are many key points to consider. These points are analyte chemical structure, pKa value, solubility properties; stability and adsorption properties. Bioanalytical method development includes two main sections, sample preparation and sample detection. Sample preparation has an important role in bioanalysis to get clean extract with high extraction efficiency. Additionally, selection of appropriate detector depends upon
the analyte concentration range. Moreover, choice of a suitable internal standard (IS) is an important issue in bioanalytical method development. The role of internal standard is to compensate for matrix effects and to get accurate results. The IS should be similar to the analyte chemical structure and chemical properties. The best IS for LC-MS/MS bioanalysis is a stable isotopically labelled compound [59]. Today LC-MS/MS is the most widely used instrument in quantitative bioanalysis. LC-MS/MS has replaced HPLC-UV in many clinical laboratories in high income countries. An overview of bioanalytical method development is presented in Table 3.

<table>
<thead>
<tr>
<th>Table 3. Bioanalytical method development and validation strategies</th>
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<tr>
<td><strong>Subject</strong></td>
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<tr>
<td>Analyte</td>
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<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td>Sample preparation</td>
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<td>Method validation</td>
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In addition, the analyte concentration in bioanalysis is a dynamic concept which can be determined in many investigations such as drug pharmacokinetics, drug toxicity, metabolism and absorption [62]. To have an efficient validated method, a stable dynamic concentration range is essential. For nonclinical or clinical studies the concentration range of the studied analytes (high/low range) should be approximately known. This is to decide the lower and upper limits of detection and to set up a calibration curve. **Chart 4** shows the general procedure to be followed for the development and optimisation of an LC/MS method.
1.6 Extraction techniques in drug bioanalysis

Sample clean-up (sample preparation) is a primary step before injection of complex matrices such as plasma, blood, urine and tissue into the analytical instrument. Selection of the most appropriate sample preparation step depends on the assay sensitivity required, duration of use of the assay, and the complexity of the task. If the assay lifetime is short, then it may not be appropriate to develop a highly sophisticated method; on the other hand, if the methodology is going to last for an extended period of time or will be applied to a high throughput scenario, then it makes good economic sense to expend the time to develop a robust, reliable sample clean-up step. Furthermore, reducing sample complexity will allow the LC cycle time to be significantly reduced without affecting the assay performance, and hence increase productivity [57]. The sample preparation process fulfils three major roles:
Removal of protein-related materials that may contaminate the chromatography column;

Eliminates endogenous compounds that are the major cause of ion suppression/enhancement in LC-MS/MS;

Pre-concentrate the sample to increase assay sensitivity

Routinely used sample preparation methods in many bioanalytical laboratories are protein precipitation (PP), liquid-liquid extraction (LLE), and solid phase extraction (SPE). In the following sections the major sample preparation techniques are summarized and discussed.

Precipitation is extensively applied for product recovery of biomolecules particularly proteins. PP is appropriate for plasma or blood samples especially at high analyte concentration. Precipitation is usually induced by addition of a miscible organic solvent (acetonitrile, acetone or methanol), salt (aluminium chloride), metal ions (zinc sulphate) or by changing the sample pH to alter the nature of the solution (acids such as trichloroacetic, perchloric, metaphosphoric and tungstic). PP as a fast and simple extraction approach can be applied for both hydrophilic and hydrophobic compounds [63]. Further, PP only addresses the removal of proteins. Phospholipids and other contaminants that remain in the matrix may cause ion suppression/enhancement, which can lead to inconsistencies or inaccuracies in the detection and quantitation of the compound of interest, as well as reduction in sensitivity. Nevertheless, PP remains a popular method for sample clean-up because it is relatively fast and cheap.

Despite of huge developments in sample preparation techniques, LLE is still an attractive technique in sample preparation and it has been widely used for the preparation of aqueous and biological samples. Basically, in LLE an aqueous sample (e.g., plasma, urine) and an immiscible organic solvent are mixed to remove the analyte into the organic phase for direct injection into an analytical system. This method can provide good recovery and clean sample. LLE has been used for the extraction of basic and acidic drugs from biological samples with high extraction recovery [57, 63]. However, selecting the right solvents for sample partitioning makes this extraction method laborious. Solvent cost and disposal are also factors that must be considered, as well as the lack of potential for automation.

Nowadays, SPE is one of the most widely used sample preparation method due to its high efficiency, cost-effectiveness, high-reproducibility, relatively green and easy to operate and automate. SPE is advantageous such as separating and
concentrating of trace analytes in biological samples. SPE uses a small volume of a chromatographic stationary phase material to isolate desired analytes from a sample. This technique allows both removal of interfering biological matrix components and enhancing the concentrations of analytes for LC-MS/MS analysis. SPE is a straightforward method that uses a sorbent of 50–200 mg in a cartridge to separate required analytes from a complex matrix. In general, the biological samples are loaded onto SPE cartridges and the biological matrices are then washed out of the cartridges. The retained analytes are often eluted using small aliquots of an organic solvent so that the desired analytes are concentrated enough for analysis without further evaporation and concentration procedures [57, 63]. A comparison of PP, LLE and SPE is presented in Table 4.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>PP</th>
<th>LLE</th>
<th>SPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab labor</td>
<td>Less</td>
<td>More</td>
<td>More</td>
</tr>
<tr>
<td>Selectivity</td>
<td>Bad</td>
<td>Good</td>
<td>Very good</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>Good</td>
<td>Good</td>
<td>Good</td>
</tr>
<tr>
<td>Ion suppression</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Automation</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Analyte suitability</td>
<td>Hydrophilic</td>
<td>Lipophilic</td>
<td>Hydro- and Lipophilic</td>
</tr>
<tr>
<td>Cost</td>
<td>Low</td>
<td>High</td>
<td>High</td>
</tr>
</tbody>
</table>

1.7 Chromatographic separation and mass spectrometric detection

The recent development of liquid chromatography and mass spectrometry instrumentation has led to reduced analysis times, improved selectivity and increased throughput in drug bioanalysis. Today, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has evolved into a vital technology used to perform routine experiments in numerous clinical and pharmaceutical laboratories. It is known that the drug discovery process requires high throughput screening methods and thus LC-MS/MS has become a very important tool in pharmaceutical industry. The use of LC-MS/MS starts in the drug discovery stage and continues until or after drug manufacturing. LC-MS/MS has revolutionized the strategies and expectations of modern drug discovery [57, 64].

LC is a physical separation technique conducted in the liquid phase in which a sample is separated into its constituent components (or analytes) by distributing between the mobile phase (a continuous flowing liquid) and a stationary phase (sorbents packed inside a column). Modern LC uses high pressure to force solvent
through closed columns containing very fine particles that give high-resolution separations. The column is the heart of the system. The efficiency of a packed column increases as the size of the stationary phase particles decreases. The combination of LC and MS is an ideal merger of separation and detection. The principle of MS is in production of ions from analyzed compounds that are separated or filtered on the basis of their mass-to-charge ratio (m/z). Various procedures are used to form gas phase ions from molecules, depending on the physical state of the analyte. Choices are available as to the types of ions produced (positively and negatively charged, radical cations, protonated molecules, etc.) and the degree to which these ions are internally excited. Internally excited molecular ions dissociate to produce fragment ions, which may reveal details of molecular structure. On the other hand, an intact molecular ion provides information on molecular weights. The major ionization techniques used for organic or biological compounds can be grouped into four categories: electron ionization (EI), chemical ionization (CI), desorption ionization (DI) including matrix-assisted laser desorption ionization (MALDI) and spray ionization (SI) like thermospray (TS), electrospray (ES) and atmospheric pressure chemical ionization (APCI) techniques. Among these ionization techniques ESI offers many advantages over other techniques, including the ability to analyze low- and high-mass compounds, excellent quantitative capabilities and reproducibility, high sensitivity, simple sample preparation, amenability to automation, soft ionization, and the absence of a matrix. In its simplest form, ESI can be quite effective even without separation, especially when combined with tandem mass spectrometry and is thus employed in the present work [65, 66].

Ions can be separated on the basis of their mass-to-charge ratios using electric or magnetic fields arranged so as to spread them in time or space. Among many others, five distinct mass analyzers are: sector magnetic fields, time-of-flight analyzers, quadrupole mass filters, quadrupole ion traps and ion cyclotron resonance. In some of these analyzers, physical separation of ions in space is achieved, in others the mass dependent frequency of ion motion is examined and in still others ion velocity is measured using timing circuitry. Most applications for quantitative bioanalysis use tandem mass spectrometry (MS/MS) to characterize individual compounds in a complex mixture or to identify a compound’s structure. These goals are achieved by separating the ionization step from the fragmentation process and thus controlling the degree of fragmentation. Mass analysis is performed twice in a tandem instrument to identify both the parent and product ion. This can be done in
two distinct ways, (i) by separating the mass analysis operations in space or (ii) by separating them in time. Separation in space can be achieved by coupling two mass analyzers. For example, a sector magnet can be coupled to a quadrupole mass filter. Parent ions are selected by the sector magnet, and are thus separated from all other ions generated from the sample. These selected ions are activated by a collision process, and the resulting set of product ions is subjected to mass analysis with the quadrupole mass filter. Fragmentation is achieved by raising the internal energy of the ions by collision-induced dissociation (CID). This process involves passing the energetic beam of parent ions through a cell containing a collision gas, such as He, N$_2$ or Ar. The most important separation-in-space tandem mass spectrometer is the triple quadrupole as shown in Figure 10. In this tandem analyzer system, an intermediate quadrupole (Q2) is used to confine ions to the axis in the presence of a collision gas. This quadrupole is not operated in a mass analysis mode but is set to transmit all ions. The products of CID are passed into the third quadrupole (the second mass analyzer) for mass analysis [65, 66].

1.8 Bioanalytical method validation

Method validation is a necessary process to demonstrate that an analytical method is suitable for its intended use, thus, that it can offer accurate, precise and reproducible results. These reliable results are essential for bioavailability,
bioequivalence, pharmacokinetic, pharmacodynamics or toxicological studies where analytes must be quantified in biological matrices such as urine or plasma. Method validation is a part of any GLP study and it is performed to ensure the quality of the analytical method. Bioanalytical method validation includes processes, parameters, and data treatments. There are different types of validation; (i) first is the full validation which is done for a newly developed bioanalytical method. The second is partial validation and it is run when modifications of already validated bioanalytical method are done (for example: new detection system, change in sample matrix or calibration range). The third is cross-validation and it is a comparison of validation parameters when two or more bioanalytical methods are used to generate data within the same study. In addition cross-validation is considered when data generated using different analytical techniques (e.g., LC-MS/MS vs. ELISA), requires modifications in validated method (another LC column, mobile phase, etc.).

Full validation involves linearity (standard curve), accuracy, precision [repeatability (within-day) and ruggedness (between-day)], selectivity (specificity), reproducibility, sensitivity, recovery, dilution integrity, stability. In addition matrix effect and carry-over are also investigated [57, 67].

1.9 Quantitative drug analysis, bioavailability, bioequivalence & pharmacokinetics: A synergistic partnership

The lead optimization/selection, confirmation and testing process for new drug candidate is well-defined as a series of activities. Broadly, these can be split into discovery, lead optimization and preclinical development, through to clinical evaluation (Phase I to IV). Each stage places different requirements on the bioanalytical assay to provide specific information. The use of LC-MS/MS assays provides the specificity, flexibility and sensitivity to enable fast and effective decision-making at each stage. Development in bioanalytical technology and the application of pharmacokinetic (PK) principles have created a synergistic partnership that plays a vital, influential role in the discovery and development of new medicines. The origins of this partnership can be traced back some 30 years, and an understanding of the way in which the application of these two scientific disciplines has evolved is helpful in ensuring optimal use of current analytical technology [68].

PK was applied mainly as a descriptive science, essentially defining what happened to the test drug when it was administered to an animal in toxicology or to
a healthy human subject in phase I clinical pharmacology. Drug safety and tolerability, plus the determination of PK parameters like plasma clearance, volumes of distribution, elimination half-life and bioavailability were and still are the objectives of phase I studies. Over time, it has become evident that poor PK profiles in new drug candidates accounted for clinical failure and termination of the development of many compounds. In phase I, a poor PK profile would usually be characterized by low and/or highly variable oral bioavailability or short elimination half-life. During later stages of clinical development, when the drug is evaluated in the intended patient population (Phase II and III of drug development), the incidence of adverse side effects attributable to drug metabolism or drug-drug interactions also led to termination of potential new medicines [68].

In all of these situations, a clear understanding of the plasma clearance mechanisms of the test drug can provide an early warning of potential problems with the compounds. Furthermore, such an understanding could potentially help to avoid the progression of a poor drug candidate into clinical development studies and thus assist in selecting an alternative compound with a superior PK profile. This has expanded the application of PK and quantitative bioanalysis into lead-candidate optimization during drug discovery [68].

Further, at patent expiration of a brand drug, generic versions that demonstrate bioequivalence to the innovator’s product may be marketed via the Abbreviated New Drug Application (ANDA) process. In order to demonstrate the bioequivalence of two proprietary preparations of the same drug molecule, studies must be conducted to show an equivalent rate and extent of bioavailability of the two products.

The therapeutic effectiveness of a drug depends upon the ability of the dosage form to deliver the medicament to its site of action at a rate and amount sufficient to elicit the desired pharmacologic response. This attribute of the dosage form is referred to as physiologic availability, biologic availability or simply bioavailability. For most drugs, the pharmacologic response can be related directly to the plasma levels. Thus, the term bioavailability is defined as the rate and extent (amount) of absorption of unchanged drug from its dosage form. Absolute bioavailability is the fraction of drug effectively absorbed after extravascular administration, when compared to the administration of the same drug intravenously. Relative bioavailability or bioequivalence between drugs, administered by the same extra vascular route, may be evaluated by comparing pharmacokinetic
parameters related to bioavailability, i.e., to the quantity absorbed and to the rate of the absorption process [69, 70]. Figure 11 is a schematic representation of processes comprising the pharmacokinetics of a drug.

Bioequivalent drugs are pharmaceutical equivalents (same pharmaceutical formulation and quantity of the same active ingredient) that, when given in the same molar dose, in the same condition, does not present significant statistical differences regarding bioavailability. The rate or rapidity with which a drug is absorbed is an important consideration when a rapid onset of action is desired as in the treatment of acute conditions such as asthma attack, pain, etc. A slower absorption rate is however desired when the aim is to prolong the duration of action or to avoid the adverse effects. On the other hand, extent of absorption is of special significance in the treatment of chronic conditions like hypertension, epilepsy, etc.

**Plasma Drug Concentration-Time Profile**

A direct relationship exists between the concentration of drug at the biophase (site of action) and the concentration of drug in plasma. A typical plasma drug concentration-time curve obtained after a single oral dose of a drug and showing various pharmacokinetic and pharmacodynamic parameters is depicted in Figure 11.

![Figure 11. A typical plasma concentration-time profile showing pharmacokinetic-pharmacodynamic parameters](image-url)
Such a profile can be obtained by measuring the concentration of drug in plasma samples taken at various intervals of time after administration of a dosage form and plotting the concentration of drug in plasma (Y-axis) versus the corresponding time at which the plasma sample was collected (X-axis). The five important pharmacokinetic parameters that characterize the plasma level-time curve which is useful in assessing the bioavailability of a drug from its formulation are:

**Peak Plasma concentration ($C_{\text{max}}$):** The point of maximum concentration of drug in plasma is called as the peak and the concentration of drug at peak is known as peak plasma concentration. It is also called as peak height concentration and maximum drug concentration. The peak level depends upon the administered dose and rate of absorption and elimination. The peak represents the point of time when absorption rate equals elimination rate of drug. The portion of the curve to the left of peak represents absorption phase i.e. when the rate of absorption is greater than the rate of elimination. The section of curve to the right of peak generally represents elimination phase i.e. when the rate of elimination exceeds rate of absorption. Peak concentration is often related to the intensity of pharmacologic response and should ideally be above minimum effective concentration (MEC) but less than the maximum safe concentration (MSC).

**Time of peak concentration ($T_{\text{max}}$):** The time for drug to reach peak concentration in plasma (after extravascular administration) is called as the time of peak concentration. It is expressed in hours and is useful in estimating the rate of absorption. Onset time and onset of action are dependent upon $T_{\text{max}}$. This parameter is of particular importance in assessing the efficacy of drugs used to treat acute conditions like pain and insomnia which can be treated by a single dose.

**Area under the Curve (AUC):** It represents the total integrated area under the plasma level-time profile and expresses the total amount of drug that comes into the systemic circulation after its administration. AUC is expressed in $\mu$g/mL $\times$ h. It is the most important parameter in evaluating the bioavailability of a drug from its dosage form as it represents the extent of absorption. AUC is also important for drugs that are administered repetitively for the treatment of chronic conditions like asthma or epilepsy.

**Elimination half-life ($t_{1/2}$) and rate constant ($K_{\text{el}}$):** The time required for the concentration of the drug to reach half of its original value is termed as elimination half-life. Elimination rate constant can be defined as the rate at which a drug is removed from the body.
Incurred sample reanalysis

Recently, the ‘incurred’ or study sample reanalysis (ISR) has become mandatory for bioanalytical methods used to support the drug development process. Viswanathan et al. [71] have suggested that an evaluation of the reproducibility in the analysis of incurred samples be performed on each species used for Good Laboratory Practices (GLP) toxicology assessments, as well as an appropriate evaluation of incurred sample reproducibility from clinical studies. Incurred or study samples can vary in their composition when compared with the standards and quality control samples used to validate the method and analyze these samples. During the 3rd American Association of Pharmaceutical Scientists (AAPS)/Food and Drug Administration (FDA) Bioanalytical Workshop, it was suggested that the reproducibility in the analysis of incurred samples be evaluated in addition to the usual pre-study validation activities performed. Although every attempt is made to formulate standards and QCs to be as similar to the study samples being analyzed as possible, “incurred” or study samples can differ in a variety of ways. These differences are dependent in part on whether the analyte(s) in question are small molecules or macromolecules. Moreover, it becomes even more important when metabolites are measured, as they may convert in vitro to their parent drug molecule [72]. In addition, the philosophy of ISR will ensure continuous review and monitoring of results through scientific procedures. This will help to adopt improved practices in bioanalysis, while safeguarding the optimal use of time, labour and laboratory resources [73, 74].

1.10 Brief summary of the present research

The present work discusses the best available practices in liquid chromatography when performing LC-MS/MS quantitative bioanalysis. Chromatography plays a pivotal role for the establishment of a bioanalytical LC-MS/MS method to support the intended studies. The life cycle of a bioanalytical method evolves, and new findings may require additional method enhancements. In the early development stages of a drug candidate, due to the high attrition rate of the drugs and typically smaller number of samples, some of the features of chromatography such as high-speed analysis, automation and multiplexing of multiple instruments, which are important considerations when analyzing large sets of clinical samples to achieve optimal throughput, may be less important. With the
new challenges encountered in drug discovery and drug development, new strategies are put in place to achieve high-throughput analysis, using serial and parallel approaches.

Therefore, the development and validation of bioanalytical assay methods suitable for quantitation of the selected drugs in FDCs and/or along with their active metabolite(s) in human plasma is discussed in this work. Relevant literature sources were consulted to understand the different parameters that must be included in method development and validation, to identify what constitutes a good assay method and to know the international regulations pertaining to bioanalytical methodology that determine whether a developed assay method is acceptable or not. Further, literature search was done to collect information on assay methods reported for the selected drugs (discussed in respective chapters). The different aspects of these assay methods viz. extraction, instrumentation, total turn-around time and sensitivity, selectivity etc. were assessed. Thus, an objective was set to develop selective, sensitive and rapid LC-MS/MS assay methods that have short and simple extraction procedures, consume small amounts of solvent and biological fluid for extraction and rapid compared to previously established methods. Systematic validation as per USFDA guidelines was done for all the methods. The parameters investigated include selectivity, sensitivity, cross specificity, carry over effect, linearity, accuracy and precision, absolute and relative recovery, absolute and relative matrix effect, stability in plasma and dilution integrity. The application of these methods for bioequivalence study is conducted with test and reference formulation of the selected drugs on healthy human subjects. The pharmacokinetic parameters investigated include $C_{\text{max}}$, $T_{\text{max}}$, $t_{1/2}$, AUC and $K_{\text{el}}$. These studies were conducted strictly in accordance with guidelines laid down by International Conference on Harmonization and USFDA.

### 1.11 Bibliography


