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1.1 The perspective

Haemolysis is the rupturing of erythrocytes (red blood cells) and the release of their contents (cytoplasm) into surrounding fluid (e.g., blood plasma). It can occur in two ways as In vivo and In vitro. In vivo it can be caused due to certain amount of medical treatment/therapy which includes many gram-positive bacteria, some parasites (e.g., malaria), some autoimmune disorders (e.g., hemolytic disease of the newborn), and some genetic disorders (e.g., sickle-cell disease or G6PD deficiency). In vitro haemolysis can be caused due to improper blood collection or processing of blood samples while specimen collection, or by bacterial action in cultured blood specimens. For example collection technique, contamination, and incorrect needle size, as well as improper tube mixing and incorrectly filled tubes are all frequent causes of haemolysis. In most of the cases during blood collection, it occurs due to difficulty in vein identification during vein puncture. Experience and proper technique are key for any phlebotomist or nurse to prevent haemolysis. In vitro haemolysis during specimen collection can cause inaccurate laboratory test results by contaminating the surrounding plasma with the contents of haemolyzed red blood cells. Another factor is the storage of samples which can store before centrifugation because at times proper storage requirement is must as per to stability when causes haemolysis. Above and all the mentioned factors precaution is required during the handling of blood sample to avoid haemolysis. The challenge to estimate the behaviour with drug estimation in such type of haemolysed matrix during bioanalysis is too much hard to understand the behaviour with drug in vivo and in vitro situation.

Identification of haemolysed samples:

![Image of haemolysed plasma sample scale](Figure 1: Haemolyzed plasma sample scale)
After blood sample collection, all the samples required to be centrifuged for extraction of plasma. On visually base when plasma samples appear as reddish colour as shown in the above picture identified as haemolysed samples or plasma samples (Figure 1). For the bioanalysis mild to severe grade consider as haemolized plasma sample [1].

Matrix effect:
During bioanalysis where the methods involved the extraction technique of the analyte of interest from a biological matrix (for example plasma, whole blood, serum and urine), the type of biological matrix can have an effect on the quantification of the analyte. Matrix effect occurs when matrix ions co elute with the analyte of interest and influence ionization such that there might be a suppression or enhancement of peak response and hence a significant effect on quantification. One special case of matrix effect is the haemolysis effect. It is commonly accepted that as long as the matrix effect is consistent, then its presence is acceptable. One approach to determine matrix effect is calculation of the matrix factor and another approach is quantifying the analyte in different sources of the matrix. However each approach is not without their limitation.

It was generally agreed that matrix effect and haemolysis effect should be considered since they can have a significant impact on the ruggedness of the method. The evaluation may be performed as part of method development or method validation.

An additional approach to determine the effect of the matrix on ionization suppression or enhancement is post column infusion. Post column infusion is a simultaneous injection of an extracted matrix sample with an infusion of high concentration analyte solution directly into the source. The resulting signal is observed which increases or decreases in response. Matrix effect can be different depending on the species being tested. When the same compound is being tested in different species, it was generally agreed that full validation should be performed for each species. Furthermore, to perform the matrix effect test; the matrix factor can be used as evaluative tool. However it was generally agreed that it is not representative of sample analysis. An alternative approach is to spike six different lots of matrix and determine their back calculated concentrations.

Drug bioanalysis, employed for the quantitative determination of drugs and their metabolites in biological fluids, plays a significant role in the evaluation and interpretation of bioequivalence, pharmacokinetic, and toxicokinetic studies. It is an integral part of characterization of drug from the time of its discovery and during various stages of drug development leading to its market authorization and thus development of sound bioanalytical method is of paramount importance. Drugs that are given in combination can produce effects that are greater than or less than the effect predicted from their individual potencies. Knowledge of drug levels in body fluids, such as whole
blood, plasma, serum and urine, allows the optimization of pharmacotherapy and provides a basis for studies of patient compliance, bioavailability, pharmacokinetics and the influences of co-medications. Selective and sensitive analytical method development becomes necessary during the quantitative and qualitative analysis of drugs and their metabolites that are purported to display pharmacological activity, determination of multiple drugs in combating a disease, biotransformation investigation, drug monitoring for therapeutic benefits and for invitro experiments [2-3].

Determination of drugs and their metabolites is difficult in biological matrix compared to in formulations. Biological matrix (e.g. blood, plasma, serum and urine) samples contain mostly water and other components like dissolved proteins, glucose, clotting factors, mineral ions, hormones and acids [4, 5]. These components may interfere at the time of quantification of analyte of interest if matrix free sample solution is not injected [6]. Drug absorption in body depends upon the properties of drugs and also some patient related factors, therefore, it is not always possible to avail high drug concentration in biological samples. Thus, efficient extraction procedures are imperative for successful bioanalysis of drug(s) and their metabolites. Methods generally used in the analysis of drugs and their metabolites are radioimmunoassay (RIA), capillary electrophoresis (CE), gas chromatography (GC), GC-mass spectrometry (GC-MS), high performance liquid chromatography (HPLC) with UV, fluorescence, refractive index and mass spectrometric detection (MS) [7-9]. LC-MS/MS has become an ideal and widely used method in the analysis of drugs and their metabolites due to its unmatched sensitivity, extraordinary selectivity and rapid rate of analysis [10]. Analytes that are easily separated by liquid chromatography can be detected even at lower concentration by MS/MS detection using different ionization techniques like electrospray (ESI), atmospheric chemical ionization (APCI) and atmospheric pressure photo ionization (APPI) [11-14].

High performance liquid chromatography is the premier technique for chemical and pharmaceutical analysis with an ability to separate, analyze, and/or purify virtually any sample. The principle of separation of analytes is based on differences in relative rates of migration through the column arising from different partition of the analytes in the stationary and the mobile phase. Reverse phase HPLC having hydrophobic stationary phase and polar mobile phase is generally used for the analysis of most of the compounds [15]. Sample preparation plays an important role in achieving desired selectivity and sensitivity. It is necessary to clean the biological sample as much as possible to get matrix free sample solution. An efficient extraction procedure need to be developed that can give quantitative and reproducible recovery. Sometimes,
concentrating the sample after extraction, derivatization at sample processing step or at chromatographic stage and adduct ion formation can enhance the sensitivity of the method. Thus, development of selective and sensitive analytical methods for the quantitative evaluation of drugs and their metabolites are critical for the successful conduct of preclinical and clinical pharmacology studies [16, 17]. These requirements are generally met with HPLC, especially if combined with an advanced detection technique such as mass spectrometry (MS). Now days, analysis time of biological samples can be decreased sharply using ultra performance liquid chromatography (UPLC), but the choice of an appropriate sample preparation method is essential for reliability and accuracy of the analysis as separation of analytes from other matrix components on column takes a short time.

1.2 Liquid chromatography (LC)

Chromatography, a physical method of separation in which the components/solutes to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction. It is an analytical tool widely employed for the separation, identification of chemical/pharmaceutical components in complex mixtures. The components must interact with the stationary phase to be retained and separated by it. The mobile phase may be a gas, liquid or a supercritical fluid which moves over or through the stationary phase, carrying the components along with it. Mass spectrometer is generally used for quantification of compounds in different biological matrices/complex mixtures. Liquid chromatography (LC) [18, 19] was the first type of chromatography to be discovered and, in the form of liquid-solid chromatography (LSC) this technique was originally used in the late 1890s by the Russian botanist, Tswett to separate and isolate various plant pigments. The colored bands he produced on the adsorbent bed evoked the term chromatography (color writing) for this type of separation. In the late 1930s and early 1940s Martin and Synge introduced a form of liquid-liquid chromatography by supporting the stationary phase, in this case water, on silica gel in the form of a packed bed and used it to separate some acetyl amino acids. They published their work in 1941 and in their paper recommended the replacement of the liquid mobile phase with a suitable gas which would accelerate the transfer between the two phases and provide more efficient separations. Thus, the concept of gas chromatography was born. In the same paper in 1941, Martin and Synge suggested the use of small particles and high pressures in LC to improve the separation which proved to be the critical factors that initiated the development of high performance liquid chromatography.
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The basic liquid chromatograph consists of six fundamental units. They are the mobile phase supply system, the pump and programmer, the sample injection valve, the column, the detector and finally a means of presenting and processing the results. A block diagram of the basic liquid chromatograph is shown in Figure 2.

![Block diagram of the basic liquid chromatograph](image)

Figure 2. Block diagram of the basic liquid chromatograph

Types of Liquid Chromatography

Liquid chromatography can be classified into four different types based on mechanism of separation.

a. Adsorption chromatography: This type of chromatography makes use of a solid stationary phase (silica gel or any other silica based packing) and a liquid or gaseous mobile phase. The solute gets adsorbed on the surface of the solid particles. Equilibration between the stationary phase and the mobile phase accounts for separation of different analytes.

b. Partition chromatography: The separation of analytes is afforded by differential partitioning between a liquid stationary phases coated on the surface of a solid support. The solute equilibrates between the stationary liquid and the mobile phase.

c. Ion-exchange chromatography: Ion exchange chromatography (or ion chromatography) is a process that allows the separation of ions and polar molecules based on their charge. This technique is used almost exclusively with ionic or ionizable samples. Solute ions of the opposite
charge are attracted to the stationary phase by electrostatic force. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time.

d. Size exclusion chromatography: Also called gel filtration or gel permeation chromatography, this technique separates molecules by size. The stationary phase is a porous gel with precisely controlled pore sizes through which the liquid mobile phase passes. The pores are small enough to exclude large solute molecules but not small ones. The sample is simply screened or filtered according to its solvated molecular size. Larger molecules are rapidly washed through the column; smaller molecules penetrate inside the pores of the packing particles and elute later. Unlike other forms of liquid chromatography, there is no attractive interaction between the stationary phase and the solute, only physical entrapment.

Adsorption chromatography on bare silica is an example of normal-phase chromatography, in which a polar stationary phase and a less polar solvent is used. Reversed-phase chromatography is more commonly used in which the stationary phase is non-polar or weakly polar and the mobile phase is more polar. Reversed-phase chromatography eliminates peak tailing because the stationary phase has few sites that can strongly adsorb a solute to cause tailing. Thus, majority of chromatographic applications are executed using reversed-phase chromatography.

Liquid chromatography can be conducted either under isocratic or gradient elution conditions. Isocratic elution is performed with a single solvent or a constant solvent mixture. If one solvent does not provide sufficiently rapid elution of all components, gradient is the preferred choice. Under gradient elution there is a continuous change of solvent composition to increase eluant strength.

LC Columns

Column is the heart of a chromatographic system, where compounds are retained and separated between the stationary phase and the mobile phase. Due to a wide variety of columns available, the challenge is to pick the right analytical column to analyze the sample correctly. The decision is based on several factors like column specifications, dimensions, particle and pore sizes, and chemistry of the bonded phase, all of which can affect the separation efficiency, inertness, durability, pH range, batch-to-batch reproducibility, resolution, solvent usage etc. There is also the complexity and quantity of the sample available and the desired cost and accuracy of analysis to be considered. Separation performance depends on both component retention and band broadening. Band broadening is, in general, a kinetic parameter, dependent on the adsorbent particle size, porosity, pore size, column size, shape, and packing performance. On the other hand, retention
does not depend on the above mentioned parameters, but it reflects molecular surface interaction and depends on the total adsorbent surface.

Consider a separation of a two component mixture dissolved in the eluant, where the component A has the same interaction with the adsorbent surface as an eluant, and component B has strong excessive interaction (Figure 3). Once injected into the column, these components will be forced through by eluant flow. Molecules of the component A will interact with the adsorbent surface and retard on it by the same way as an eluant molecules.

![Figure 3. A typical chromatogram for separation of components A and B](image)

Thus, as an average result, component A will move through the column with the same speed as an eluant. Molecules of the component B being adsorbed on the surface (due to their strong excessive interactions) will stay on it much longer. Thus, it will move through the column slower than the eluant flow.

**LC Mobile Phases**

The choice of phase system can be very complex, particularly if multicomponent mixtures are to be separated. In the first instance the type of stationary phase needs to be chosen and this choice must be based on the interactive character of the solutes to be separated. If the solutes are predominantly dispersive then the stationary phase must also be dispersive (a reversed phase) to promote dispersive interaction with the solutes and provide adequate retention and selectivity. If the solutes are strongly polar then a polarizable stationary phase (one containing aromatic rings or cyano groups) would be appropriate to separate the solutes by polar and induced polar interactions. If the solutes are weakly polar then a strong polar stationary phase would be required (such as silica gel) to separate the solute by polar interactions.

The mobile phase must be chosen to complement the stationary phase so that the selected interactions are concentrated in the stationary phase. Thus, a reversed phase having strong dispersive interactions would be used with a strongly polar mobile phase (e.g., mixtures of methanol and water, acetonitrile and water or tetrahydrofuran and water). In contrast, if the strongly polar silica gel is selected for the stationary phase then a strongly dispersive mobile phase would be appropriate (e.g., n-heptane, n-heptane/chloroform or n-heptane with a small quantity of n-propanol or ethanol). In general the mobile phase must be chosen so that the selected interactions strongly dominate in the stationary phase and are minimized in the mobile phase.
Mobile phase pH is a primary tool for controlling this selectivity through the change of the analyte ionization state.

**Analyte Ionization**

A simplistic rule for the retention in reversed-phase HPLC is that the more hydrophobic the component the more it is retained. By simply following this rule one can conclude that any organic ionizable component will have longer retention in its neutral form than in the ionized form. Ionization is pH dependent process; ionization of the analyte could be expressed as,

\[ AH \rightarrow A^- + H^+ \text{ for acidic components} \]
\[ B + H^+ \rightarrow BH^+ \text{ for basic components} \]

Equilibrium constants are usually written in one of the following forms:

\[ K_a = \frac{[A^-][H^+]}{[AH]} \]  

(1)

and using the definition for the pH, one can rewrite as

\[ pK_a = pH + \log \left( \frac{[AH]}{[A^-]} \right) \]  

(2)

Similar expression could be written for bases. As mentioned above, compound in its ionic form is more hydrophilic so it not only tends to have less interaction with hydrophobic stationary phase; it also tends to be more solvated with water molecules. This also causes significant decrease of the retention of ionic components. Since the pK_a is a characteristic constant of the specific analyte, from the above equation one can conclude that relative amounts of neutral and ionic forms of the analyte could be easily adjusted by varying the pH of mobile phase. Moreover, if the pH is about two units away from the component pK_a more than 99% of the analyte will be in either ionic or neutral form, depending upon the direction of the pH shift. Chromatographic resolution between two or more peaks depends upon three factors — column efficiency, selectivity and retention. With ionizable analytes (bases and acids), all of these factors change dramatically with pH. For example retention can be improved by changing the separation pH, so that analytes are separated in their non-ionized form. A change in mobile phase pH also improves column efficiency because the ionization of the analyte and the residual silanols can both be altered. This minimizes secondary interactions between analytes and the silica surface that cause poor peak shape. Achieving optimum resolution requires changing the mobile phase pH. The following method development strategy explains how this is done with superior column lifetime.
Figure 4. Three pH regions for HPLC separation of basic compounds. The inflection point of the curve corresponds to the component $pK_a$.

Low, mid and high pHs are the three general regions for chromatographic separations as defined in Figure 4. This figure highlights the benefits of performing separations of ionizable analytes in each pH region. Method development proceeds by investigating chromatographic separations at low pH and then higher pH until optimum results are achieved.

Effect of temperature

Temperature effects in HPLC are not as significant as in gas chromatography. Volatile solvents are not allowed to rise to higher temperatures too much, and the high temperature may influence the stability of the attached bonded ligands on the adsorbent surface. Therefore, the main temperature range is from ambient temperature to 60 or 70 °C. Picture below illustrates the influence of the column temperature on the HPLC retention (Figure 5).

Figure 5. Influence of column temperature on the HPLC retention

There are two other significant effects of separation under elevated temperature.
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→ Stabilization of the column under the elevated temperature usually leads to the stabilization of the retention times. The origin of this effect is not well understood yet. A possible explanation could be that the solvent viscosity decreases and more uniform stabilized temperature with absence of local temperature fluctuations due to the solvent friction may lead to a more uniform adsorption-desorption process.

→ Another effect is the increase in column efficiency. At elevated temperatures, viscosity of liquids decrease and the diffusion coefficient increases. From the Van Deemeter equation, the longitudinal diffusion term will increase, which will lead to decrease in the efficiency at very low flow rates (which is not important). The mass transfer term will decrease which will lead to the increase in the efficiency at the common flow rates. It also widens the flow rate range with optimum efficiency.

Retention parameters

The easiest way to find the chromatographic retention is to measure the time between the injection point and maximum of the detector response for the corresponding compound. This parameter is usually called "retention time". Retention time ($T_R$) is inversely proportional to the eluant flow rate. The product of retention time and eluant flow rate, so called "retention volume", is more of a global retention parameter. Retention volume ($V_R$) represents the volume of the eluant passed through the column while eluting a particular component. Retention volume is independent of the flow parameters for the particular run, but it depends on the geometrical parameters of the column. $V_R$ will be different for the same compound eluted on different columns packed with the same type of adsorbent.

1.3 Mass Spectrometry

Mass spectrometry (MS) has progressed to become a powerful analytical tool for both quantitative and qualitative applications. Over the past decade, mass spectrometry has undergone tremendous technological improvements allowing for its application to proteins, peptides, carbohydrates, DNA, drugs, and many other biologically relevant molecules. Due to ionization sources such as electrospray ionization and matrix-assisted laser desorption/ ionization (MALDI), mass spectrometry has become an irreplaceable tool in the biological sciences. The MS principle consists of ionizing chemical compounds to generate charged molecules or molecule fragments and measurement of their mass-to-charge ratio.

The compounds can be ionized by different techniques like electron ionization (EI), chemical ionization (CI), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photon ionization (APPI), nano spray ionization (NSI), matrix assisted laser
disorption ionization (MALDI), fast atom bombardment (FAB) and thermal ionization (TI). After ionization, ions are separated according to their mass-to-charge ratio by mass analyzer. Mass analysers have electric and/or magnetic field in vacuum. Time of flight, quadrupole, quadrupole ion trap, linear quadrupole ion trap, and fourior transform ion cyclotron resonance orbit trap are the different mass analysers working on their different characteristics. Quadrupole is the most compatible analyzer as they are reasonably priced and make good multi-purpose instruments [20]. Tandem mass spectrometry (MS/MS) is more useful technique compared to normal mass spectrometry as it has more than one analyzer that gives better selectivity while analyzing the compounds and so nowadays it is the most powerful technique used in quantitative determination of compounds from biological fluids. First the mass analyzer separates the ions according to their mass-to-charge ratio from other ions. The ions of interest enter into the collision cell where they are fragmented by an inert gas (He, N₂ or Ar) using collision activated dissociation process. The second mass analyzer allows those selective fragmented ions (product ions) to be detected by the detector. Mass analyzing and detection process is carried out in high vacuum.

1.4 HPLC- mass spectrometry

High performance liquid chromatography (HPLC) coupled with mass spectrometry is an extremely powerful and indispensable methodology practiced in virtually every stage of pharmaceutical discovery and development process, including biological target discovery, biological assay for high throughput screening, characterization of physiochemical properties of drug candidates, drug metabolism and pharmacokinetics. Compounds are separated on column in HPLC and then enter into mass spectrometer where they are first ionized in the source (parent ions). ESI, APCI and APPI are the ionization techniques mostly used for analysis of pharmaceutical compounds in biological fluids. Figure 6 represents major components of MS with pumps used to maintain vacuum in instrument [21, 22].
1.5 Sample preparation techniques

It is required to extract the drug from biological matrix before injecting it into LC-MS/MS. Also, the sample clean-up plays an important role as it results in proper quantification of drugs. Due to sample clean-up, ion suppression or enhancement caused by endogenous impurities from biological fluids can be minimized. Protein precipitation, liquid-liquid extraction and solid phase extraction are the extraction methods generally used to extract the drugs from biological matrix. The extraction method which gives higher and consistent recovery with minimum ion suppression/enhancement is used for analysis of incurred samples [23, 24].

i) Protein precipitation: it is a simplest procedure to remove proteins from biological matrix. The inorganic acid, organic acid or organic solvent such as perchloric acid (PCA), trichloroacetic acid (TCA), formic acid (FA), acetonitrile and methanol are used to precipitate proteins in biological matrix. The mixture is then centrifuged to remove denatured proteins. After centrifugation, clear supernatant is injected directly or after drying and reconstitution into LC-MS/MS. It is fast and cost effective extraction method but can give the sample with lots of matrix interferences that cause column clogging, ion suppression/enhancement and require frequent system clean-up [25].

ii) Liquid-liquid extraction: it is a method used to separate compounds based on their relative solubility in two different immiscible liquids, usually water and organic solvent. During extraction, the compound should be in unionized form and so pH adjustment of sample is necessary. Sometimes it is required to back extract the compounds or multiple extractions to remove interferences from the sample. It is a cost effective method compared to solid phase extraction, but is tedious and time consuming as it requires drying followed by reconstitution. LLE
is a simple and efficient method or the separation and concentration of relatively hydrophobic compounds. For some polar compounds, it is not possible to get matrix free clean sample using this extraction procedure [26, 27].

iii) Solid phase extraction: it is an extraction method that uses a solid phase and a liquid phase to isolate one or more analyte(s) from matrix samples. It is used to clean-up the sample before the chromatographic separation to quantitate the drug in the sample [28, 29]. With SPE, many of problems associated with liquid liquid extraction can be prevented, such as incomplete phase separations, less-than-quantitative recoveries, use of expensive, breakable specialty glassware, and disposal of large quantity of organic solvents. SPE methods are easy to perform, rapid and can be automated. Drugs can be extracted from small sample volumes with minimum use of solvents and reduced labour. In SPE, the sample is first loaded on a SPE cartridge, washed with suitable solvent to remove undesired components, followed by elution of desired analyte(s) into a collection tube. This method has a distinct advantage over protein precipitation as it affords clean and matrix free sample by washing out of undesired components. Different types of SPE cartridges are available to extract different types of drugs.

1.6 Bioanalytical method validation

Bioanalytical method validation employed for quantitative determination of drugs in biological matrices plays a significant role in the evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetics, and toxicokinetic study data. It can also be defined as the process of documenting that the method under consideration is suitable for its intended purpose. The method must generate reproducible and reliable data in order to permit valid interpretation of the studies they support. Essential bioanalytical method validation parameters are selectivity, matrix effect, precision and accuracy, dilution integrity and stabilities under different conditions. If a bioanalytical method is claimed to be for quantitative biomedical application, then it is important to ensure that a minimum package of validation experiments has been conducted and yields satisfactory results [30].

The guideline for industry by regulatory agencies like USFDA, EMA & ANVISA states that accuracy, precision, selectivity; sensitivity, reproducibility and stabilities under different conditions are the fundamental parameters of validation for a bioanalytical method validation. The validation must guarantee, through experimental studies, that the method meets the bioanalytical applications, ensuring the reliability of result. For this, it must present precision and accuracy, limit of quantification, selectivity, reproducibility, and recovery and stability data of the method [31, 32]. For a bioanalytical method to be considered valid, specific acceptance criteria should be
set in advance for accuracy and precision for the validation of the QC samples. Validations can be subdivided into the following three categories:

► Full validation

Full method validation is required when
- method is developed for the first time
- new drug entity
- a metabolite(s) added to an existing method
- change in anti coagulant
- change in matrix with species (human plasma to human serum)
- change in species with matrix (human plasma to rat plasma)
- change in detection system
- change in internal standard
- change in sample processing procedure

► Partial validation

Partial method validation need to be performed incase of
- method transfer between laboratories
- method transfer between instruments
- change in calibration concentration range
- change in sample volume
- change in software
- administration of concomitant medicament(s)
- change in matrix sample storage temperature

► Cross validation

Cross validation is a comparison of validation parameters when two or more bioanalytical methods are used to generate data within the same study or across different studies. An example of cross validation would be a situation when the original validated bioanalytical method serves as the reference and the revised bioanalytical method is the comparator.

1.7 Bioavailability and bioequivalence

Bioavailability and bioequivalence of drug products have emerged as critical issues in pharmacy and medicine during last three decades. Concern about lowering health care costs has resulted in a tremendous increase in use of generic drug products. With the increasing availability and use of generic drug products, health care professionals are confronted with an ever-larger array of multisource products from which they must select those that are therapeutically equivalent. This
phenomenal growth of generic pharmaceutical industries and the abundance of multisource products have prompted some questions among many health professionals and scientists regarding therapeutic equivalency of these products. 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. Drug concentrations usually cannot be measured directly at the site of action. Therefore, most bioavailability studies involve the determination of drug concentration in blood, plasma or urine. 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. 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.

Bioavailability of a drug from its dosage form depends upon 3 major factors:

i) Pharmacetic factors related to physicochemical properties of the drug (hydrophobicity, pKa, solubility) and characteristics of the dosage form

ii) Patient related factors

iii) Route of administration

The influence of nature of dosage form on drug's bioavailability is generally in the following order: solutions > suspensions > capsules > tablets > coated tablets > controlled release tablets. The decreasing bioavailability is related to the number of steps involved in the absorption process following administration. The influence of route of administration of dosage form is as follows: parenteral > oral > rectal > topical. Since drugs are generally administered to patients who are ill, it is important to consider the effects of the disease process on the bioavailability of the drugs. Disease states, particularly those involving the gastric intestinal tract, such as celiac disease, crohn's disease, achlorhydria, hypermotility syndromes, cardiovascular system, and liver may alter circulating drug levels after oral dosing. Gastric emptying rate, food and drug interaction may also affect absorption of drug.

Bioavailability of a drug can be assessed through the demonstration of a clinically significant effect. This method is expensive, complex, and time-consuming and requires large population. Another possible way to assess bioavailability is to quantify the pharmacological effect. This method is based on the assumption that a given intensity of response is associated with a particular
drug concentration of drug at the site of action. However, monitoring pharmacological data is quite
difficult, precision and reproducibility are difficult to establish, and also there are limited number
of pharmacological effect e.g. heart rate, body temperature, blood sugar level. So, blood
concentration-time profile method is generally used to assess bioavailability of drug. After
administration of drug, blood samples are collected over a period of time and are analyzed for drug
content. These studies are relatively easy to conduct and require a limited number of subjects. A
profile is constructed showing the concentration of drug in blood/plasma at specific time intervals.
Bioavailability can also be assessed using urinary excretion method but this method is less reliable
compared to blood/plasma concentration-time profile method. This method requires collection of
urine samples for a longer period of time to ensure complete recovery of drug from body. In
addition, subjects must be careful to completely void at each collection time and to avoid
accidentally discarding any samples. Also, renal excretion is a saturable process, the percentage of
the drug excreted unmetabolized in the urine may not reflect the rate and extent of the drug
absorption [33, 34].

Drug concentration in Blood/Plasma -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 7. 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).
Figure 7. Typical plasma concentration-time profile showing pharmacokinetic-pharmacodynamic parameters, obtained after oral administration of single dose of a drug

The peak represents the point of time when absorption rate equals elimination rate of drug. The portion of 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. The three important pharmacokinetic parameters that describe the plasma level-time curve and useful in assessing the bioavailabilities of a drug from its formulation are:

Peak Plasma concentration ($C_{\text{max}}$): The maximum concentration of drug in plasma is a function of both the rate and extent of absorption. $C_{\text{max}}$ will increase with an increase in the dose, as well as with an increase in the absorption rate. Peak concentration is often related to the intensity of pharmacologic response and it should be between minimum effective concentration and the maximum safe concentration.

Time of peak concentration ($T_{\text{max}}$): The time at which $C_{\text{max}}$ occurs. The $T_{\text{max}}$ reflects the rate of drug absorption, and decreases as the absorption rate increases. It is expressed in hours and is useful in estimating the rate of absorption. Onset time and onset of action are dependent upon $T_{\text{max}}$. The 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.
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Area under the curve (AUC): The area under the plasma concentration-time curve, is proportional to the total amount of the drug reaching the systemic circulation, and thus characterizes the extent of absorption. AUC is expressed in h.ng/ml (nanogram.hour/millilitre). It is also important for drugs that are administered repetitively for the treatment of chronic conditions like asthma or epilepsy.

Apart from all these three major parameters, elimination rate constant, $K_{el}$ and half-life, $T_{1/2}$ is assessed during bioavailability studies. Further, statistical analysis is done using either SAS or winnolin software. ANOVA is performed on log transformed pharmacokinetic parameters $C_{max}$, $AUC_{0-t}$, $AUC_{0-inf}$ and 90% confidence interval is constructed for the ratio of geometric least square mean of test and reference product, obtained from log transformed data. If the ratio of above mentioned parameter for test and reference fall within 80 to 125%, test product is considered bioequivalent to reference. These criteria may vary for different drugs and different regulatory guidelines [35, 36].

**1.8 Matrix selection criteria for method validation and routine analysis**

During the method development and validation, haemolysis effect exercise needs to be done for interference of matrix in plasma or serum. This exercise is done with different sources of matrix i.e. 6 different sources as per regulated bioanalysis.

For each analyte and the IS, the matrix factor (MF) should be calculated for each lot of matrix, by calculating the ratio of the peak area in the presence of matrix (measured by analysing blank matrix spiked after extraction with analyte), to the peak area in absence of matrix (pure solution of the analyte). The IS normalised MF should also be calculated by dividing the MF of the analyte by the MF of the IS. The CV of the IS-normalised MF calculated from the 6 lots of matrix should not be greater than 15 %. This determination should be done at a low and at a high level of concentration (maximum of 3 times the LLOQ and close to the ULOQ). If this approach cannot be used, for instance in the case of on-line sample preparation, the variability of the response from lot to lot should be assessed by analysing at least 6 lots of matrix, spiked at a low and at a high level of concentration (maximum of 3 times the LLOQ and close to the ULOQ).

**1.9 The Aim & Scope**

In the bio analysis where the matrix i.e. plasma is used for the analysis purpose for the quantification purpose, matrix effect related to haemolysed samples play critical role. Haemolysed plasma may have tendency of interference during spectrometry analysis. It is always seen that the plasma from blood samples from volunteers having some of them are haemolysed samples. To estimate the analyte in haemolyzed sample is very critical so the liquid
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cromatography with mass spectroscopy technique is much favourable. Analysis of drugs using liquid chromatography-mass spectrometry is extensively used in pharmaceutical industry especially for haemolysed plasma samples with proper extraction technique.

A survey of literatures reveals that only very few research work have been reported on the studies of haemolysis effect on drug estimation, hence the present investigation was under taken for the development of analytical methods of haemolysed samples based on LC-MS/MS detection.

The aim and scope of the investigation are:

i. To develop and validate high throughput, sensitive and rugged bioanalytical methods for haemolysed sample analyses based on LC-MS/MS detection.

ii. To separate the drugs from biological matrices by solid phase extraction (SPE), liquid liquid extraction (LLE) or protein precipitation (PPT) technique. The extraction procedure should employ smaller plasma volumes, with quantitative and precise recovery of the drugs.

iii. The developed method should have the following merits:

- High selectivity and sensitivity
- High throughput
- Less time consuming and less laborious extraction procedure
- Lower sample (biological fluid) volume requirement for processing
- Quantitative and precise recovery
- Rugged and robust enough for routine analysis

iv. To apply of these methods for bioequivalence/bioavailability studies of the drugs in healthy human volunteers and identifying the haemolyzed samples.

v. To identify the haemolyzed samples of plasma from volunteers.

vi. To compare the initial analyzed sample with repeating the same haemolyzed sample to prove method reproducibility as incurred sample reanalysis.

In the present study, the following important drugs have been studied for their bio analytical method development, method validation and their application to bioequivalence / bioavailability studies in human plasma with respect to determination of their haemolysis effect and the method reproducibility to challenge the method especially for haemolyzed samples and their behaviour due to the selected drugs having nature to create haemytic anemia.

✓ Amoxicillin & Clavulanic acid (Antibiotic)
✓ Erythromycin Ethylsuccinate (Antibiotic)
✓ Metformin (Anti diabetic)
✓ Mefenamic Acid (Non steroidal anti-inflammatory drugs NSAIDs)
1.10 Physicochemical properties, pharmacokinetic data and pharmacology of the selected drugs [37]

Amoxicillin

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Amoxicillin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physicochemical properties</td>
<td></td>
</tr>
<tr>
<td>IUPAC name</td>
<td>(2S,5R,6R)-6-{(2R)-2-amino-2-(4-hydroxyphenyl)-acetyl}amino}-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-24-carboxylic acid</td>
</tr>
<tr>
<td>Category</td>
<td>Beta-lactam antibiotics</td>
</tr>
<tr>
<td>Molecular formula</td>
<td>C_{16}H_{19}N_{3}O_{5}S</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>365.4 g/mol</td>
</tr>
<tr>
<td>CAS registry no.</td>
<td>26787-78-0</td>
</tr>
<tr>
<td>Melting point</td>
<td>194 °C</td>
</tr>
<tr>
<td>Water solubility</td>
<td>Slightly soluble</td>
</tr>
<tr>
<td>Pharmacokinetic data</td>
<td></td>
</tr>
<tr>
<td>Log P</td>
<td>0.87</td>
</tr>
<tr>
<td>pKa</td>
<td>3.23, 7.43</td>
</tr>
<tr>
<td>Protein binding</td>
<td>20%</td>
</tr>
<tr>
<td>Bioavailability</td>
<td>95%</td>
</tr>
<tr>
<td>Metabolism</td>
<td>Bio transformed in liver</td>
</tr>
<tr>
<td>Half life</td>
<td>61.3 Min</td>
</tr>
<tr>
<td>Excretion</td>
<td>renal</td>
</tr>
</tbody>
</table>

Pharmacology: Amoxicillin is an antibiotic useful for the treatment of a number of bacterial infections. It is a moderate-spectrum, bacteriolytic, β-lactam antibiotic in the amino penicillin family used to treat susceptible Gram-positive and Gram-negative bacteria. It is usually the drug of choice within the class because it is better-absorbed, following oral administration, than other β-lactam antibiotics. Amoxicillin is susceptible to degradation by β-lactamase-producing bacteria, which are resistant to a narrow spectrum of β-lactam antibiotics, such as penicillin. For this reason, it is often combined with clavulanic acid, a β-lactamase inhibitor. This increases effectiveness by reducing its susceptibility to β-lactamase resistance. This drug acts by inhibiting the synthesis of bacterial cell walls. It inhibits cross-linkage between the linear peptidoglycan polymer chains that make up a major component of the cell walls of both Gram-positive and Gram-negative bacteria.
Clavulanic Acid

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Clavulanic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physicochemical properties</td>
<td></td>
</tr>
<tr>
<td>IUPAC name</td>
<td>(2R,5R,Z)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-aza-bicyclo[3.2.0]heptane-2-carboxylic acid</td>
</tr>
<tr>
<td>Category</td>
<td>Beta-lactamase inhibitors</td>
</tr>
<tr>
<td>Molecular formula</td>
<td>C₈H₉NO₅</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>199.16g/mol</td>
</tr>
<tr>
<td>CAS registry no.</td>
<td>58001-44-8</td>
</tr>
<tr>
<td>Melting point</td>
<td>117.5-118 °C</td>
</tr>
<tr>
<td>Water solubility</td>
<td>Soluble</td>
</tr>
<tr>
<td>Pharmacokinetic data</td>
<td></td>
</tr>
<tr>
<td>logP</td>
<td>-0.919</td>
</tr>
<tr>
<td>pKa</td>
<td>2.7</td>
</tr>
<tr>
<td>Protein binding</td>
<td>22-30%</td>
</tr>
<tr>
<td>Bioavailability</td>
<td>Well Absorbed</td>
</tr>
<tr>
<td>Metabolism</td>
<td>Hepatic (Extensive)</td>
</tr>
<tr>
<td>Half life</td>
<td>1 h</td>
</tr>
<tr>
<td>Excretion</td>
<td>renal (30-40%)</td>
</tr>
</tbody>
</table>

Pharmacology: Clavulanic acid is a mechanism-based β-lactamase inhibitor combined with penicillin group antibiotics to overcome certain types of antibiotic resistance. It is used to overcome resistance in bacteria that secrete β-lactamase, which otherwise inactivates most penicillins. Clavulanic acid has negligible intrinsic antimicrobial activity, despite sharing the β-lactam ring that is characteristic of β-lactam antibiotics. However, the similarity in chemical structure allows the molecule to interact with the enzyme β-lactamase secreted by certain bacteria to confer resistance to β-lactam antibiotics.
**Erythromycin ethylsuccinate**

<table>
<thead>
<tr>
<th>Physicochemical properties</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>IUPAC name</td>
<td>( \text{N,N-dimethylimidodicarbonimidic diamide} )</td>
</tr>
<tr>
<td>Category</td>
<td>Antidiabetic</td>
</tr>
<tr>
<td>Molecular formula</td>
<td>( \text{C}_4\text{H}_9\text{N}_5 )</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>129.164 g/mol</td>
</tr>
<tr>
<td>CAS registry no.</td>
<td>657-24-9</td>
</tr>
<tr>
<td>Melting point</td>
<td>223 °C</td>
</tr>
<tr>
<td>Water solubility</td>
<td>Freely soluble</td>
</tr>
</tbody>
</table>

**Pharmacokinetic data**

| logP                      | -0.5                       |
| pKa                       | 8.8                        |
| Protein binding           | Negligible                 |
| Bioavailability           | 50 to 60% (fasting)        |
| Metabolism                | none                       |
| Half life                 | 6.2 h                      |
| Excretion                 | renal                      |

Pharmacology: Erythromycin belongs in a group of drugs called macrolide antibiotics. Macrolide antibiotics slow the growth of, or sometimes kill, sensitive bacteria by reducing the production of important proteins needed by the bacteria to survive. It is also used to treat such as bronchitis; diphtheria; Legionnaires' disease; pertussis (whooping cough); pneumonia; rheumatic fever; venereal disease (VD); and ear, intestine, lung, urinary tract, and skin infections. It is also used before some surgery or dental work to prevent infection. Antibiotics will not work for colds, flu, or other viral infections.
Pharmacology: Metformin is an antihyperglycemic agent, which improves glucose tolerance in patients with type II diabetes, lowering both basal and postprandial plasma glucose. Metformin is not chemically or pharmacologically related to any other classes of oral antihyperglycemic agents. Unlike sulfonylureas, metformin does not produce hypoglycemia in either patients with type II diabetes or normal subjects and does not cause hyperinsulinemia. With metformin therapy, insulin secretion remains unchanged while fasting insulin levels and daylong plasma insulin response may actually decrease.
Mefenamic acid

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Mefenamic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physicochemical properties</td>
<td></td>
</tr>
<tr>
<td>IUPAC name</td>
<td>2-(2,3-dimethylphenyl)aminobenzoic acid</td>
</tr>
<tr>
<td>Category</td>
<td>non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>Molecular formula</td>
<td>C₁₅H₁₅NO₂</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>241.285 g/mol</td>
</tr>
<tr>
<td>CAS registry no.</td>
<td>61-68-7</td>
</tr>
<tr>
<td>Melting point</td>
<td>230-231 °C</td>
</tr>
<tr>
<td>Water solubility</td>
<td>Insoluble</td>
</tr>
<tr>
<td>Pharmacokinetic data</td>
<td></td>
</tr>
<tr>
<td>logP</td>
<td>4.58</td>
</tr>
<tr>
<td>pKa</td>
<td>4.2</td>
</tr>
<tr>
<td>Protein binding</td>
<td>90%</td>
</tr>
<tr>
<td>Bioavailability</td>
<td>90%</td>
</tr>
<tr>
<td>Metabolism</td>
<td>Hepatic</td>
</tr>
<tr>
<td>Half life</td>
<td>2 h</td>
</tr>
<tr>
<td>Excretion</td>
<td>renal</td>
</tr>
</tbody>
</table>

![Mefenamic acid molecule](image)

Pharmacology: Mefenamic acid is a non-steroidal anti-inflammatory drug used to treat pain, including menstrual pain. It is typically prescribed for oral administration. Mefenamic acid decreases inflammation (swelling) and uterine contractions by a still-unknown mechanism. However, it is thought to be related to the inhibition of prostaglandin synthesis. There is also evidence that supports the use of Mefenamic acid for perimenstrual migraine headache prophylaxis, with treatment starting 2 days prior to the onset of flow or 1 day prior to the expected onset of the headache and continuing for the duration of menstruation.

Since hepatic metabolism plays a significant role in Mefenamic acid elimination, patients with known liver deficiency may be prescribed lower doses. Kidney deficiency may also cause accumulation of the drug and its metabolites in the excretory system. Therefore, patients suffering from renal conditions should not be prescribed Mefenamic acid.
Doxycycline hyclate

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Doxycycline</th>
</tr>
</thead>
</table>

Physicochemical properties

<table>
<thead>
<tr>
<th>IUPAC name</th>
<th>(4S,4aR,5S,5aR,6R,12aS)-4-(dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Category</td>
<td>Anti biotic</td>
</tr>
<tr>
<td>Molecular formula</td>
<td>C_{22}H_{24}N_{2}O_{8}</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>444.43 g/mol</td>
</tr>
<tr>
<td>CAS registry no.</td>
<td>564-25-0</td>
</tr>
<tr>
<td>Melting point</td>
<td>201 °C</td>
</tr>
<tr>
<td>Water solubility</td>
<td>6.30e-01 g/l</td>
</tr>
</tbody>
</table>

Pharmacokinetic data

| logP     | -3.4 |
| pKa      | -2.2, 7.75 |
| Protein binding | 90%   |
| Bioavailability | 100% |
| Metabolism    | Hepatic        |
| Half life     | 15 to 25 h     |
| Excretion     | Renal           |

Pharmacology: Doxycycline is an antibiotic useful for the treatment of a number of infections. It is in the tetracycline antibiotic class. In addition to the general indications for all members of the tetracycline antibiotics group, Doxycycline is frequently used to treat Lyme disease, chronic prostatitis, sinusitis, pelvic inflammatory disease, acne, rosacea and rickettsial infections. It is used in prophylaxis against malaria. It should not be used alone for initial treatment of malaria, even when the parasite is Doxycycline-sensitive, because the anti malarial effect of Doxycycline is delayed. This delay is related to its mechanism of action, which is to specifically impair the progeny of the apicoplast genes, resulting in their abnormal cell division. It can be used in a treatment plan in combination with other agents, such as quinine.
Rasagiline

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Rasagiline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physicochemical properties</td>
<td></td>
</tr>
<tr>
<td>UPAC name</td>
<td>(R)-N-(prop-2-ynyl)-2,3-dihydro-1H-inden-1-amine</td>
</tr>
<tr>
<td>Category</td>
<td>irreversible inhibitor of monoamine oxidase¹</td>
</tr>
<tr>
<td>Molecular formula</td>
<td>C₁₂H₁₃N</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>171.238 g/mol</td>
</tr>
<tr>
<td>CAS registry no.</td>
<td>1875-50-9</td>
</tr>
<tr>
<td>Melting point</td>
<td>157 °C</td>
</tr>
<tr>
<td>Water solubility</td>
<td>2.49e-02 g/l</td>
</tr>
<tr>
<td>Pharmacokinetic data</td>
<td></td>
</tr>
<tr>
<td>logP</td>
<td>2.3</td>
</tr>
<tr>
<td>pKa</td>
<td>8.69</td>
</tr>
<tr>
<td>Protein binding</td>
<td>88-94%</td>
</tr>
<tr>
<td>Bioavailability</td>
<td>36%</td>
</tr>
<tr>
<td>Metabolism</td>
<td>Hepatic</td>
</tr>
<tr>
<td>Half life</td>
<td>3 h</td>
</tr>
<tr>
<td>Excretion</td>
<td>renal &amp; fecal</td>
</tr>
</tbody>
</table>

Pharmacology: Rasagiline is an irreversible inhibitor of monoamine oxidase used as a monotherapy in early Parkinson's disease or as an adjunct therapy in more advanced cases. It is selective for MAO type B over type A by a factor of fourteen. Rasagiline is being investigated for the treatment of Restless Legs Syndrome. Because of its melanin binding properties, Rasagiline was investigated and found to decrease melanoma growth; it may be candidate for combination therapy for melanoma. Rasagiline is now also being investigated for the treatment of Alzheimer's disease.

1.10 Present investigation

Chapter 1 Identification and causality of haemolysis were discussed in this chapter in terms of in vitro and vivo condition along with the importance matrix effect in bio analysis with various techniques of HPLC and MSMS. Basics of a bio analytical method validation, bio avaibility and bio equivalence has been discussed. The aim and scope as well as information on the Physicochemical properties, pharmacokinetic data and pharmacology of the selected drugs have been refered.
Chapter 2 describes bioanalytical methodology adopted in this research work related to parameters like selectivity, carry over, linearity, accuracy, precision, recovery, stability, dilution integrity, and matrix effect. Protocol for method validation and its acceptance criteria have been annotated for better clarification as per international guidelines. The condition of selected drugs application for bioequivalence study is detailed for more focus and clarity. The development and validation of bioanalytical assay methods suitable for quantification of the drugs with respect to hemolysis effect (matrix effect) in human plasma is discussed in Chapter 3 to Chapter 8. An attempt is made to develop superior and efficient bioanalytical methods compared to existing methods of the literature with respect to hemolysis effect. To quantify drugs in biological matrix, not only chromatography but efficient extraction procedure and high selectivity are also desirable. Also, the developed methods should fulfill various regulatory requirements. Initially, a thorough 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 others were assessed. Systematic validation as per USFDA guidelines is done for all the methods. The parameters investigated include selectivity, sensitivity, carry over effect, linearity, accuracy and precision, absolute and relative recovery, absolute and relative matrix effect (specially for hemolysis), 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. Identified hemolyzed samples have been selected from subjects and analyzed using the developed method. Initial results have been compared with repeat result as per guidance provided “Incurred sample reanalysis” from USFDA. Bioequivalence studies for all the molecules have been conducted as per national guideline, USFDA and schedule Y.

Chapter 3 describes the hemolysis effect on amoxicillin and clavulanic acid (Antibiotic drug of beta lactam series) which is generally used to treat respiratory track infection.

Chapter 4 describes the hemolysis effect on erythromycin ethylsuccinate (Antibiotic drug of the macrolide group) which is generally used to treat bronchitis.

Chapter 5 describes the hemolysis effect on metformin (Antidiabetic drug of new class niterones compound) which is generally used to treat type 2 diabetes.

Chapter 6 describes the hemolysis effect on mefenamic acid (Non-steroidal anti-inflammatory drug, member of fenamate group) which is generally used to severe pain.

Chapter 7 describes the hemolysis effect on doxycycline (Antibiotic drug, class of tetracycline antibiotic) which is generally used for several infections.
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Chapter 8 describes the haemolysis effect on rasagiline (Anti Parkinson agent, irreversible inhibitor of monoamine oxidase) which is generally used for monotherapy in early Parkinson's disease or as an adjunct therapy in more advanced cases.
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1.12 References


[3] Nicola C Hugh, Navgeet Bajaj, Juan Fan, Ernest YK wong, Bioanalysis (2009) 1 (6), 1057-1066


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[37] i. Drug bank: http://redpoll.pharmacy.uberta.ca/drugbank