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Determination of pharmaceuticaly important substances using High Performance Liquid Chromatography with Mass Spectrometry detection

D o k t o r s k á d i s e r t a č n í p r á c e

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This investigation was carried out at analitical chemistry department of faculty of science in Prague and bioannalytical department of CEPHA s.r.o. in Plzen 2002 – 2007. I claim that I worked on my own and mentioned all of the referneces.

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Contents

1 INTRODUCTION

High performance liquid chromatography (HPLC) is the most common method used in commercial analytical laboratories. HPLC in combination with Mass Spectrometry (MS) is frequently used for the determination of drugs in human plasma and it belongs to group of methods that enable new and modern modifications. That is why following new trends, which broaden the field of application of these methods, is so important.

HPLC with MS detection (HPLC-MS) satisfy demand for automation of determination of samples from complex matrixes, which lead to elimination of human error, decrease of expenditure of analytical process and finally handling potentially dangerous samples by laboratory staff.

Highly sensitive methods that do not require time consuming sample pre-treatment are desired for pharmacokinetic (PK) studies. Double MS (MS/MS) detection provides drugs identification and high sensitivity for quantitative determination. It effectively eliminates interferences from endogenous impurities and guarantees method selectivity at the same time.

Pharmacokinetic (PK) studies determine pharmacokinetic characteristics of particular drug that are then used to assess the bioequivalence of determined drug versus other chosen drug of the same nature. The PK parameters are calculated from the plasma concentrations of the drug determined by a validated method.

HPLC-MS/MS technique allows both sample extraction and chromatography to be simplified so that productivity of routine determinations can be considerably enhanced. By its principle it fulfils most of the requirements for analytic methods use for evaluation of PK parameters of drugs in human plasma.

2 METHOD DEVELOPMENT AND VALIDATION

2.1 INTRODUCTION

Analytical methods for determination biologically active substances evolve from the same theoretical principles and are using the same instrumentation. Methods for clinical pharmacology are usually developed for one drug and its metabolites when necessary, which are chemically similar substances. These methods require very low detection limits as well as high selectivity.

Pharmacology can be defined as a science that investigates interactions between substances and organism. The aim is to find out if, and under which conditions, it is possible to use the substance as a drug. Clinical pharmacology deals with impact of substances on the human body and its goal is to find optimal utilization of drugs in human therapeutics. An important sector of clinical pharmacology, which is concerned with results of analytical determination, is pharmacokinetics. It is engaged in qualitative classification of the motion of drugs within an organism, which depends on the particular drug, administration of the drug and interaction with the organism. Pharmacokinetics tracks the determined drug in human fluids and tissues with the help of mathematical models. The result is specification of many basic pharmacokinetic parameters (for example AUC – area under the plasma concentration curve; C_{max} – maximum plasma concentration; t_{max} – time point of maximum plasma concentration; $t_{1/2}$ – half-life of drug elimination during the terminal phase; k_e – terminal elimination rate constant) [1-4].

Analytical methods and pharmacokinetics participate in search of new drugs, their therapeutic and toxicological affect and study of their metabolism in organism. Results of pharmacokinetic studies provide valuable information about drug dosage and speed of its metabolism [5].

There is an increase in demand for PK studies and analytical methods as a consequence of the large number of new drugs and their generics that comes on the market each year. Currently there are three groups of methods that can be used for the determination of drugs in organic material, defined according to their principle: microbiological, immunochemical and physical-chemical methods.

The goal of this work is to develop HPLC- MS/MS methods for determination of drugs or simultaneous determination drugs and their metabolites in human plasma obtained in a PK study, and to use the results for evaluating PK parameters of the investigated drug. For that reason only physical-chemical methods and HPLC will be mentioned in chapters blow as well as method validation.

2.2 HPLC

High Performance Liquid Chromatography (HPLC) is a mode of chromatography; the most widely used analytical technique. Chromatographic processes can be defined as a separation techniques involving mass-transfer between stationary and mobile phase [6].

HPLC utilizes a liquid mobile phase to separate the components of a mixture. These components (or analytes) are first dissolved in a solvent, and then forced to flow through a chromatographic column under a high pressure. In the column, the mixture is resolved into its components. The amount of resolution is important, and is dependent upon the extent of interaction between the solute components and the stationary phase. The stationary phase is defined as the immobile packing material in the column. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems and it has the ability to easily separate a wide variety of chemical mixtures.

Liquid Chromatography was first discovered in 1903 by M.S.Tswett, who used a chalk column to separate the pigments of green leaves. Only in 1960's the more and more emphasis was placed on the development of liquid chromatography [7].

There are many ways to classify liquid column chromatography. If this classification is based on the nature of the stationary phase and the separation process, three modes can be specified [8].

In adsorption chromatography the stationary phase is an adsorbent (like silica gel or any other silica based packings) and the separation is based on repeated adsorption-desorption steps [8].

In ion exchange chromatography the stationary bed has an ionically charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionizable samples. The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time [9,10].

In size-exclusion chromatography the column is filled with material, which has precisely controlled pore sizes, and 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 porous of the packing particles and elute later. Mainly for historical reasons, this technique is also called gel filtration or gel permeation chromatography although, today, the stationary phase is not restricted to a gel [11,12].

Concerning the adsorption chromatography, two modes are defined depending on the relative polarity of the two phases: normal and reversed-phase chromatography. In normal phase chromatography, the stationary bed is strongly polar in nature (e.g., silica gel), and the mobile phase is nonpolar. Polar samples are thus retained on the polar surface of the column packing longer than less polar materials. Reversed-phase chromatography is the inverse of this. The stationary bed is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. Here the more nonpolar the material is, the longer it will be retained.

Above-mentioned types cover almost 90% of all chromatographic applications. Eluent polarity plays the highest role in all types of HPLC. There are two elution types: isocratic and gradient elution. In the isocratic elution constant eluent composition is pumped through the column during the whole analysis. In the gradient elution, eluent composition (and strength) is steadily changed during the run.

In general, HPLC is a dynamic adsorption process. Analyte molecules, while moving through the porous packing bead, tend to interact with the surface adsorption sites. Depending on the HPLC mode, the different types of the adsorption forces may be included in the retention process. Hydrophobic (non-specific) interactions are the main ones in reversed-phase chromatography separations. Dipole-dipole (polar) interactions are dominant in normal-phase chromatography mode. Ionic interactions are responsible for the retention in ion-exchange chromatography.

All these interactions are competitive. Analyte molecules are compete with the eluent molecules for the adsorption sites. So, the stronger analyte molecules interact with the surface, and the weaker the eluent interaction, the longer analyte will be retained on the surface [13].

2.2.1 HPLC Adsorbents [13-15]

HPLC adsorbents (often called "stationary phases") are the materials that, after being packed in the column, actually retain (adsorb) analytes and produce a separation. "Stationary phase" describes that part of the chromatographic system that is in equilibrium with the mobile phase. In general, HPLC system consists of two phases: liquid (mobile) and solid (stationary). In a few cases analyte molecules can penetrate into the stationary phase: liquid-liquid chromatography, and chromatography on the soft gels (agarose). In all others, (the great majority of HPLC applications) solid stationary phase is rigid and unpermeable for the analyte molecules. Only the surface of the stationary phase actually participates in the chromatographic retention process. So, chromatography is the dynamic interface (adsorption) phenomena. We have to treat a stationary phases as an adsorbent and consider its surface properties.

The dominating type of HPLC adsorbents is silica gel. A very broad variation of functionality of bonded species could be attached to the silica surface. The most popular one organofunctional groups, **(R)**. **R** may carry substituents of various functionality such as alcoholic or phenolic hydroxyl, amine, phenyl, carbonyl, nitril etc. The functional group **R** can be bonded to the surface silicon atoms in the following ways:

Si-R: R is directly bonded to the surface silicon atoms. The elimination of the original hydroxyl groups can be achieved by chlorination of the surface with subsequent treatment of the chlorinated surface with organometallic compounds. This type of surface modification is usually very laborious and does not give a stable reproducible surface modification.

Si-O-R: This bond is an ester bond and is easily formed by the reaction between an alcohol and surface hydroxyl groups.

Si-O-Si-R: This structure is obtained by treatment of a hydroxylated silica surface with organosilanes, R_nSiX_{4-n} where X is the reactive groups such as halogen, ethoxy and methoxy. This type of bonded ligands is the most widely used in HPLC.

Polymeric and monomeric bonding: Reaction of monochlorosilane with hydroxilated silica surface leads to the attachment of only one alkylsilane ligand to one silanol group. The type of bonded layer that involves the formation of a monomolecular organic layer on the silica surface is called "monomeric bonding". Polymeric phases can be prepared by reacting silica with di- or trichlorosilane in the presence of trace amount of water, which leads to the formation of tree-like structure on one reacted silanol group of the silica surface.

C1, C4, C8, and C18: Silica based adsorbents modified with trimethylchlorosilane (C1) and buthyldimethylchlorosilane (C4) have a few applications in HPLC, mainly for protein separation or purification. These adsorbents show significant polar interactions, although they do not have specific interactions caused by acidic silanols. Octyl (C8) and octadecyl (C18) modified adsorbents are the most popular. Almost 80% of all HPLC separations have been developed with these adsorbents.

Phenyl: Propylphenylsilane ligands attached to the silica gel show weak dipole - induced dipole interactions with polar analytes. Usually this type of bonded phase is used for group separations of complex mixtures. Amino-compounds show some specific interactions with phenyl modified adsorbent.

CN: A cyano modified surface is very slightly polar. Columns with this phase are useful for fast separations of mixtures consisting of very different components. These mixtures might show very broad range of retention times than on the usual columns.

Cyano-columns could be used on both normal- and reversed-phase modes of HPLC.

NH2: Amino-phase is a weak anion-exchanger. This type of column is mainly used in normal-phase mode, especially for selective retention of aromatic compounds.

Diol: Diols are slightly polar adsorbent for normal-phase separations. These are useful for separation of complex mixtures of compounds with different polarity, and which usually shows a strong retention on unmodified silica.

Chiral phases : Chiral stationary phases are the most common first approach for enantiomer separation. To be able to separate racemic mixture of stereoisomers , the chiral phase has to form a diastereomeric complex with one of the isomers, or has to have another type of stereospecific interactions. Detailed mechanism of chiral

recognition is not well understood yet. A large number of different bonded stationary phases are commercially available. Basically we can distinguish four major types of chiral bonded phases [16,17]:

Donor-acceptor (Pirkle) type- Those bonded ligands should have three possible points of interactions with analyte molecules. These interactions can include hydrogen bonding, dipole-dipole interactions, or charge transfer. Obviously, these ligands have to be a stereoisomeric. Due to the type of interactions, these adsorbents can be used with normal phase eluents.

Chiral cavity phases - Cavity type phases such as cyclodextrins bound to the surface of silica through a spacer are act like a cone shape cavity open at both ends with a relatively hydrophobic interior. Chiral separation will occur if one of the analyte stereoisomers fits exactly into the cone and its functional groups can interact with the hydroxyl groups at the wide openings of the cavity. These types of chiral adsorbents are used in the reversed-phase HPLC mode.

Helical polymer phases - Some helical polymers such as cellulose esters whose chirality comes from helicity also can be used for enantiomer separations. It is thought that the mechanism of separation on these columns involves a combination of attractive interactions and inclusion of the analyte into a chiral cavity.

Ligand exchange columns - Silica surface with bonded chiral moiety, such as proline, usually treated with copper salts. This treatment leads to the formation of the reversible complex with copper. Chiral analytes participate in the ligand exchange process. Depending on the type of enantiomer (R or S) formed, the complex may be more or less stable leading to the chiral separation.

2.2.2 Detection in HPLC [18,19]

Detectors equipped with the flow-through cell were a major breakthrough in the development of modern liquid chromatography. The group of Tiselius, in Sweden in 1940, first applied such detection by continuously measuring the refractive index of the column effluent. Current LC detectors have wide linear dynamic range normally allowing both analytical and preparative scale runs on the same instrument. They have high sensitivity often allowing the detection in nanograms of material, and the better

models are very flexible, allowing rapid conversion from one mobile phase to another and from one mode to another.

Almost all LC detectors are the on-stream monitors. The only relatively successful off-line detector is FTIR spiral disk monitor, which require sample transfer on the germanium disk and the following scanning in FTIR instrument. HPLC detectors always used under continuous flow conditions and the sample is always dissolved in the eluent during detection. Actual sample is only present in a ng quantity in the detector, but in trace analysis, this quantity could be femtogram and even to the single molecule.

The mobile phase is a factor, which must always be considered. In the last decade there has been a significant progression in the development of LC/MS interfacing systems. MS as an on-line HPLC detector is said to be the most sensitive, selective and in the same time the most universal detector.

Refractive index detector

The refractive index (RI) detector is the only universal detector in HPLC. The detection principle involves measuring the change in refractive index of the column effluent passing through the flow-cell. The greater the RI difference between sample and mobile phase, the larger the imbalance will become. Thus, the sensitivity will be increased for the higher difference in RI between sample and mobile phase. On the other hand, in complex mixtures, sample components may cover a wide range of refractive index values and some may closely match that of the mobile phase, becoming invisible to the detector. RI detection is a pure differential instrument, and any changes in the eluent composition require the rebalancing of the detector. This factor is severely limiting RI detector application due to the analyses requiring the gradient elution, where the mobile phase composition is changed during the analysis to effect the separation.

Ultraviolet/visible spectroscopic detectors

Any chemical compound can interact with an electromagnetic field. A beam of the electromagnetic radiation is passed through the detector flow-cell and will experience some change in its intensity. Measurement of these changes is the basis of most optical HPLC detectors.

Radiation absorbance depends on the radiation wavelength and the functional groups of the chemical compound. An electromagnetic field, depending on its energy (frequency), can interact with electrons causing their excitation and transfer onto a higher energy level, or it can excite molecular bonds causing their vibration or rotation of the functional group. An increase in the intensity of the beam, which corresponds to energy, will lead to an increase in possible transitions while it passes through the flow-cell. According to the Lambert-Bear law absorbance of the radiation is proportional to the compound concentration in the cell and the length of the cell.

The electromagnetic spectrum is traditionally divided into several regions:

infrared (IR)	2,500 - 50,000 nm
near infrared	800 - 2,500 nm
visible	400 - 800 nm
ultraviolet (UV)	190 - 400 nm

Three major regions (IR, visible, and UV) are used in the spectroscopy. In liquid chromatography, IR spectrophotometers have found only limited use. There are few transparent polar liquids, which can be used as the mobile phase. On the other hand, spectrophotometers working in the range (200 - 600 nm) are used widely as LC detectors.

UV and visible region of the electromagnetic radiation corresponds to the excitation of the relatively low energy electrons such as pi-electrons, or non-paired electrons of some functional groups. For example, n-alkanes can absorb in the UV region below 180 nm. σ-electrons require high energy radiation to get excited and to show absorption of the radiation. But any compounds that have benzene ring will show absorbance at 205-225 and 245-265 nm. The last corresponds to the excitation of conjugated p-electrons of the benzene ring.

UV/VIS detectors can analyze the majority of organic compounds. Almost 70% of published HPLC analyses were performed with UV/VIS detectors. This fact, plus the relative ease of its operation, makes the UV detector the most useful and the most widely used LC detector.

Table. Molar Absorptivity (*e*) Values of Various Compound Types at Specified Wavelengths.

Name	Chromophore	Wavelength [nm]	Molar extinction, □
acetylide	-C=C	175-180	6,000
Aldehyde	-CHO	210	1,500
amine	$-NH_2$	195	2,800
azo	-N=N-	285-400	3-25
bromide	-Br	208	300
carboxyl	-COOH	200-210	50 - 70
disulphide	-S-S-	194	5,500
ester	-COOR	205	50
ether	-O-	185	1,000
ketone	>C=O	195	1,000
nitrate	$-ONO_2$	270	12
nitrile	-C=N	160	-
nitrite	-ONO	220 - 230	1000-2000
nitro	$-NO_2$	210	strong

Diode-array detectors

A special feature of some variable wavelength UV detectors is the ability to perform spectroscopic scanning and precise absorbance readings at a variety of wavelengths while the peak is passing though the flow cell. Diode array adds a new dimension to the analytical capability of liquid chromatography because it permits qualitative information to be obtained beyond simple identification by retention time.

There are two major advantages of diode array detection. In the first, it allows for the best wavelength(s) to be selected for actual analysis. This is particularly important when no information is available on molar absorptivities at different wavelengths.

The second major advantage is related to the problem of peak purity. Often, the peak shape in itself cannot reveal the presence of two (or even more) components. In such a case, absorbance rationing at several wavelengths is particularly helpful in deciding whether the peak represents a single compound or, is in fact, a composite peak.

In absorbance rationing, the absorbance is measured at two or more wavelengths and ratios are calculated for two selected wavelengths. Simultaneous measurement at several wavelengths allows calculation of the absorbance ratio. The ratios at chosen

wavelength are continuously monitored during the analysis: if the compound under the peak is pure, the response will be a square wave function (rectangle). If the response is not rectangle, the peak is not pure.

Fluorescence detectors

Fluorescence detectors are probably the most sensitive among the existing modern HPLC detectors. It is possible to detect even a presence of a single analyte molecule in the flow cell. Typically, fluorescence sensitivity is 10 -1000 times higher than that of the UV detector for strong UV absorbing materials. Fluorescence detectors are very specific and selective among the others optical detectors. This is normally used as an advantage in the measurement of specific fluorescent species in samples.

When compounds have specific functional groups that are excited by shorter wavelength energy and emit higher wavelength radiation, called fluorescence. Usually, the emission is measured at right angles to the excitation.

Roughly about 15% of all compounds have a natural fluorescence. The presence of conjugated pi-electrons especially in the aromatic components gives the most intense fluorescent activity. Also, aliphatic and alicyclic compounds with carbonyl groups and compounds with highly conjugated double bonds fluoresce, but usually to a lesser degree. Most unsubstituted aromatic hydrocarbons fluoresce, with quantum yield increasing with the number of rings, their degree of condensation and their structural rigidity.

Fluorescence intensity depends on both the excitation and emission wavelength, allowing selective detection of some components while suppressing the emission of others.

The detection of any component depends significantly on the chosen wavelength and if one component can be detected at 280 nm ex and 340 nm em while another could be missed. Most of the modern detectors allow fast switch of the excitation and emission wavelength, which offer the possibility to detect all component in the mixture.

Electrochemical detector

The electrochemical detector is also a popular liquid chromatographic detector. It should be considered by the chromatographer because of the additional selectivity and sensitivity for some compounds.

This detector is based on the measurements of the current resulting from oxidation/reduction reaction of the analyte at a suitable electrode. Since the level of the current is directly proportional to the analyte concentration, this detector could be used for quantification.

The eluent should contain electrolyte and be electrically conductive. Most of the analytes to be successfully detected require a pH adjustments.

The areas of application of electrochemical detection are not large, but the compounds for which it does apply, represent some of the most important drug, pollutant and natural product classes. For these, the specificity, and sensitivity make it very useful for monitoring these compounds in complex matrices such as body fluids and natural products. Sensitivity for compounds such as phenol, catecholamines, nitrosamines, and organic acids are in the picomole (nanogram) range.

The purity of the eluent is very important, because the presence of oxygen, metal contamination and halides may cause significant background current and therefore, noise and drift in the base line.

2.3 *MS detection* [20-32]

HPLC science has always been moving to more specific and more sensitive detection modes. MS detector satisfies a demand for sensitive and selective determination while using very little sample pre-treatment is needed. Interfacing a HPLC system with a mass spectrometer is not trivial. The difficulty is to transform a solute into a gas phase ion. The challenge is to get rid of the solvent while maintaining adequate vacuum level in the mass spectrometer, and to generate the gas phase ions.

Since the early seventies, a number of approaches have been used. LC/MS became really popular with the introduction of the thermospray interface and the particle beam interface. The next big improvement was the introduction of the electrospray and APCI techniques. By far, the majority of applications are done with electrospray and APCI ionisation. New techniques like APPI (atmospheric photo ionisation) are appearing, but are not yet largely used.

Basically, any information gathered from a mass spectrometer comes from analysis of gas-phase ions. There are three main components of mass spectrometer: an ionization source, a mass analyzer and a detector.

2.3.1 Ionization sources

Electrospray Ionization (ESI)

Electrospray and APCI are both API (atmospheric pressure ionisation) techniques. Ionisation takes place at atmospheric pressure and both are considered to be soft ionisation method. The mass spectrum provides mainly the molecular weight information, unless fragmentation techniques are used. The possible fragmentation techniques are in source CID (collision induced dissociation), CID in the collision cell of a tandem type instrument, and fragmentation in an ion trap. This is very different from the spectra obtained with EI (electron impact ionisation).

The HPLC line is connected to the electrospray probe, which consists of a metallic capillary surrounded by a nitrogen flow. A voltage is applied between the probe tip and the sampling cone. In most instruments, the voltage is applied on the capillary, while the sampling cone is held at low voltage. First step is to create a spray. At very low flow rate (a few µl/mn) the difference in potential is sufficient to create the spray. At higher flow rate, a nitrogen flow is necessary to maintain a stable spray.

The API sources include a heating device, in order to speed up solvent evaporation.

A mandatory condition with electrospray is that the compound of interest must be ionized in solution.

If it is not compatible with the HPLC conditions (i.e. in case of normal phase chromatography), it is possible to use post column addition to get the appropriate conditions.

In the electrical field, at the tip of the capillary, the surface of the droplets containing the ionized compound will get charged, either positively or negatively, depending on the voltage polarity. Due to the solvent evaporation, the size of the droplet reduces, and, consequently, the density of charges at the droplet surface increases. The

repulsion forces between the charges increase until there is an explosion of the droplet. This process repeats until analyte ions evaporate from the droplet.

Multiply charged ions can be obtained depending on the chemical structure of the analyte. This is why ESI is the technique of choice for analyzing proteins and other biopolymers on quadrupole or ion trap analyzers.

Atmospheric Pressure Chemical Ionization (APCI)

The HPLC line is connected to the APCI probe, which consists normally of a glass capillary surrounded with a nitrogen flow, used for mobilization. Part of the APCI probe, or close to the probe tip is a heating device and an additional gas flow, to instantaneously volatilize the solvent and sample. Close to the probe, there is a metallic needle, which is at potential of a few kilovolts. This is the "corona discharge electrode". The "corona effect" term describes the partial discharge around a conductor placed at a high potential. This leads to ionization and electrical breakdown of the atmosphere immediately surrounding the conductor. This effect is known as corona discharge. St. Elmo's fire is an example of a naturally occurring corona. In the case of an APCI source, the atmosphere surrounding the corona electrode consists mainly in the vapour generated from the HPLC eluent, nitrogen, and the analyte molecules.

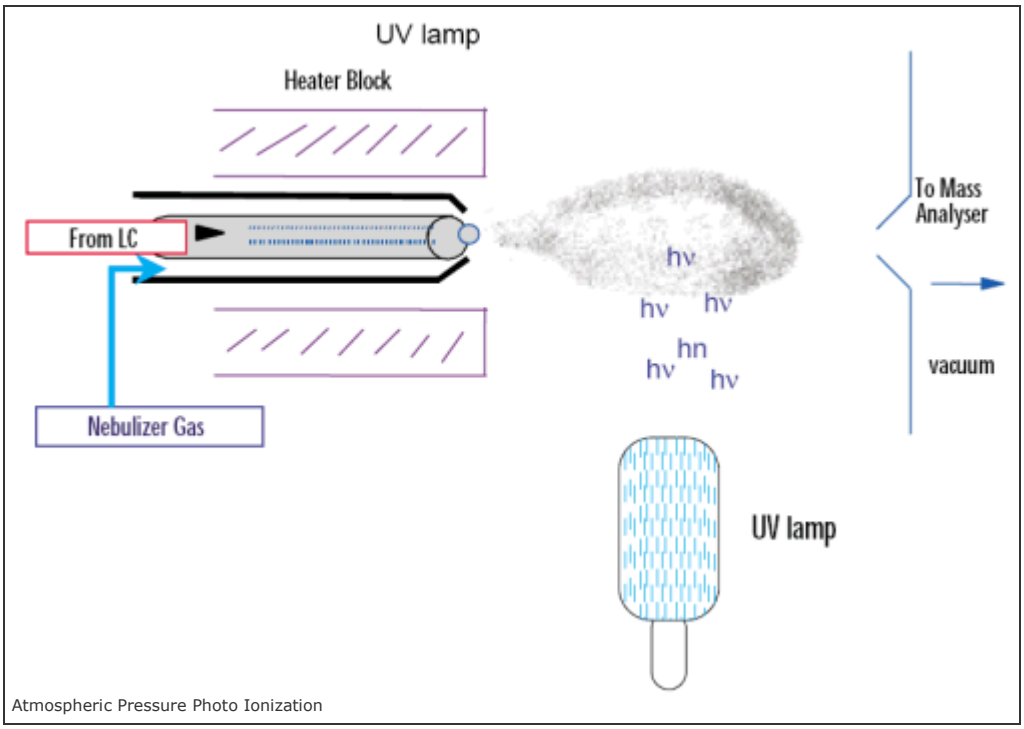
The eluent vapors are ionized by the corona effect, and react chemically with the analyte molecules in the gas phase.

The following conditions are required for APCI to work - the analyte must be volatile and thermally stable, the mobile phase must be suitable for gas phase acid-base reactions, for working in positive mode, the proton affinity of the analyte must be higher than the proton affinity of the eluent (in other words, the analyte can catch a proton from the protonated solvent) and for working in negative mode, the gas phase acidity of the analyte must be lower than the gas phase acidity of the eluent (in other words, the analyte can give a proton to the deprotonated solvent).

Atmospheric Pressure Photo Ionization

Atmospheric pressure photo ionisation is a newly introduced technique. It was presented at ASMS 2000 by Andries Bruins from the University of Groningen, Netherlands. The principle is to use photons to ionise gas phase molecules.

The source is a modified APCI source, with the corona electrode replaced by an UV lamp.



APPI is said to allow the ionisation of compounds that cannot be ionised with APCI or ESI, to be compatible with flow rates down to 100 µl/mn, and to be quantitative. Obviously, it is too early and there is not enough published work to estimate the range of applications.

Both APCI and ESI are soft ionisation techniques. Thus, the MS spectra obtained with API ionisation consist mainly of the "molecular" ions, unless fragmentation techniques are applied. Spectra often contains adduct ions (an ion formed by interaction of two species, usually an ion and a molecule, and often within the ion source, to form an ion containing all the constituent atoms of one species as well as an additional atom or atoms) and cluster ions (an ion formed by the combination of two or more molecules of a chemical species, often in association with a second species). Adducts and cationised or anionised molecules are often observed with API techniques. Those ions can be formed accidentally (for instance the presence of sodium in the mobile phase) or intentionally, for more specific or sensitive detection.

Spectrum can also be multicharged with electrospray ionisation, multiply charged ions are obtained for proteins, peptides oligonucleotides, and in general, for any

molecule which presents multiple ionization sites. Various levels of charge are present simultaneously, giving a spectrum with multiple peaks. The mass of the molecule can be obtained by calculation from the multiply charged spectrum. This can be done manually for small peptides, but de-convolution software options, available from instrument suppliers, are very useful for large molecules.

2.3.2 Analyzers

The "heart" of the mass spectrometer is the scanning mass analyser. This element separates the gas phase ions. A scanning mass analyzer is analogous to the equipment used in optical spectroscopy for analyzing the colour content of visible light. In optical spectroscopy, visible light composed of individual colours (different wavelengths of light) that are present at different intensities. A prism separates the light into its different wavelengths, and a slit is used to select which wavelength reaches the detector. The different wavelengths are then swept (scanned) across the detector slit and the light intensity is recorded as a function of time (wavelength). In scanning mass spectrometry, a mixture of ions having different mass-to-charge ratios and different relative abundances are used. Electromagnetic fields separate the ions according to their mass-to-charge ratios, and a slit is used to select which mass-to-charge ratio reaches the detector. The different mass-to-charge ratios are then scanned across the detector slit and the ion current is recorded as a function of time (mass). Both magnetic sector and quadrupole mass spectrometers can be "jumped" from one target mass to another instead of scanning over a given mass range. This *selected ion monitoring (SIM)* method is used to improve sensitivity for quantitative analysis and target compound identification by monitoring only the masses of interest for specific compounds. In contrast to scanning analyses, no time is wasted making measurements of the baseline noise between peaks or measuring ions that are not relevant to the analysis. However, this is only useful when the target masses are known in advance.

Magnetic Sector Mass Spectrometers

The analogy between scanning mass spectrometry and scanning optical spectroscopy is most apparent for magnetic sector mass spectrometers. From the 1950s through to the 1980s, instruments with sector analyzers were one of the predominant

types of mass spectrometer. All sector instruments include a magnetic sector and some also have an electric sector.

In a magnetic deflection mass spectrometer, ions leaving the ion source are accelerated to a high velocity. The ions then pass through a magnetic sector in which the magnetic field is applied in a direction perpendicular to the direction of ion motion.

A magnetic sector alone will separate ions according to their mass-to-charge ratio. Ions accelerated from the ionization source are curved by magnetic field and adopt a constant radius around the center of the field. The magnetic sector disperses ions on bases of their momentum at the same time as focusing ions that have the same momentum but diverging angles as they pass through the slit – direction focusing. To improve performance most sector mass spectrometers include an electric sector before or after magnetic sector. An electric sector disperses ions on the basis of their kinetic energy to charge ratio. Using double-focusing instruments, mass resolution in tens of thousands can be readily obtained.

Time-of-Flight Mass Analyzers

Conceptually, the simplest mass analyzer is probably time of flight (TOF) analyzer [33]. A TOF separates ions based on their velocity. Theoretically the ions are formed at the same time and place in the ion source and then accelerated through a fix potencial (1-20kV) in to TOF drift tube. As all the ions with the same charge obtain the same kinetic energy after acceleration, the lower m/z ions achieve higher velocities than the higher m/z ions. In fact, ion velocities are inversely related to the square root of m/z. After the ions are accelerated, they travel through a fixed distance before striking the detector. Thus, by measuring the time it takes to reach the detector after the ion is formed, the m/z can be determined.

TOF is the most commonly used analyzer for Matrix Assisted Laser Desorption Ionisation (MALDI) experiments. The best performance is achieved on more sophisticated TOF instruments that include reflection. The function of reflection with an electrostatic mirror that is the reflectron, is to compensate for small differences in velocities of ions with the same m/z. Mass range of TOF analyzers is theoretically unlimited.

Quadrupole Mass Spectrometers

Over the years, quadrupole has probably been the most widely used analyzer. This popularity is mainly a result of the relatively low cost of these instruments and their ease of automation. Quadrupole also use much lower voltages to accelerate the ion from the source of analyzer (2-50V versus kV) and are physically much smaller than sector analyzers or most TOF instruments.

Mass separation in quadrupole is a result of ion motion in a dynamic (radio frequency or RF) electric field and is dependent directly on the m/z of the ion. Whereas the kinetic energy is of the ion is crucial parameter in the sector and TOF instruments, it is not in quadrupole. Mass analysis is a function of RF voltages and direct current (DC) voltages applied to four rods, which are typically cylindrical in geometry. Combined DC and RF potentials on the quadrupole rods can be set to pass only a selected mass-to-charge ratio. All other ions do not have a stable trajectory through the quadrupole mass analyzer and will collide with the quadrupole rods, never reaching the detector. As a result of time varying nature of the RF voltage, the equations of motion are second order differential equation, as opposed to the more simple equation for sector and TOF instruments.

The mass accuracy of quadrupole is generally in the hundreds of ppm. The mass resolution is a function of the ratio of the RF and DC voltages and is often varied such that unit resolution is obtained over the whole mass range. As with most instruments, the sensitivity decreases as the resolution increases. Although the quadrupole had been the instrument of choice for combination with separation techniques; the qaudrupole ion trap has become an important competitor [34-39]. The one area in which the quadrupole still excel relative to the other instruments is in quantification.

Quadrupole ion trap

The quadrupole ion trap is a close relative of the quadrupole mass analyzer. Whereas a quadrupole has electric fields in two dimensions and ion move perpendicular of the fields, the ion trap has the electrical field in all three dimensions, which can result in ions being trapped in the field. In a quadrupole, the ions that are detected to obtain the mass spectrum have a stable trajectory through quadrupole, whereas to obtain a mass spectrum with an ion trap the ion trajectories must be made unstable. Ions are made unstable in a mass selective manner by increasing RF voltage that is applied to the

device. This mass selective instability mode of operation was developed in the 1980s[40].

Ion traps have similar resolution to quadrupoles. However it can be improved substationally by using slower RF voltage scan rates [41,42]. Unlike the quadrupole there is not there is no drastic fall off of sensitivity with ion traps at higher m/z. The mass accuracy of ion traps is similar to quadrupoles (hundreds of ppm).

2.3.3 Tandem mass spectrometry

Whereas the ionization techniques and mass analyzers developed in the mid-1980s are crucial to the application of MS to biological compounds, the technique tandem mass spectrometry (MS/MS) is equally important factor in the contribution to the field [43]. As the name implies, MS/MS involves two stages of MS. In the first stage of MS/MS, ions of desired m/z are isolated from the rest of the ions emanating from the ion source. These isolated ions (termed parent or precursor ions) are than induced to undergo a chemical reaction that changes their mass (m) or charge (z). Typically, the reactions involve some type of process to increase the internal energy of the ions leading to dissociation. The ions resulting from the various chemical reactions are termed product ions, and these are analyzed in the second stage of MS/MS.

With some instruments, it is possible to repeat the process, leading to what is termed an MS^n experiment, where n is equal to the number of stages of MS performed. MS/MS is particularly useful when analyzing complex mixtures.

2.4 Biological sample pretreatment

The quality of sample preparation is a key factor in determining the success of analysis. Analysis of pharmaceutically important compounds in biological matrixes has driven forward the development of sample clean up. In clinical pharmacology drugs are usually determined from a biological sample - blood, plasma, serum and urine.

Most samples need to be pretreated before analysis. The various sample pretreatment methods were designed to meet the requirements for drug analysis for complex matrices. Solid particles can be removed using centrifuge or filtration; some proteins are easy to precipitate. Extraction helps to remove endogenous impurities and it concentrates the sample, which is important for sensitivity and selectivity of determination.

2.4.1 Deproteination of biological sample

Deproteination of biological samples is used to remove proteins from the sample that can interfere with analyte and can damage column or detector. The deproteination procedure has to be chosen with respect to the determined drug that can be bonded to some proteins.

Deproteination that does not affect bond of the drug with protein are based on membrane processes –dialysis and ultra filtration. The size of pores of semi permeable membrane defined the selectivity of procedure.

Precipitation of biological sample on the other hand cancels the bond of the drug with protein. Precipitations can reversible or irreversible depending on precipitation solution used.

2.4.2 Liquid-liquid extraction (LLE)

Liquid-liquid extraction is one of the oldest methods. It allows cleaning up and concentration of the biological sample at the same time. Extraction takes out most of the endogenous impurities that interfere during determination. It is a method to separate

compounds based on their solution preferences for two different immiscible liquids. A distribution ratio is often quoted as a measure of how well extracted a species is. The distribution ratio (D) is equal to ratio of the concentration of an analyte in the immiscible two phases. Depending on the system, the distribution ratio can be a function of temperature, the concentration of chemical species in the system or a large number of other parameters. The separation factor is one distribution ratio divided by another, it is a measure of the ability of the system to separate two analytes. Efficient extraction is dependent on a system where the analyte should have high distribution ratio and endogenous impurities a low one. The factors that have an influence on the selectivity of the particular extraction circuit are choice of organic solvent, the pH aqueous phase, volume of both phases and length of extraction.

2.4.3 Solid phase extraction (SPE)

SPE is a sample preparation technique based upon principles similar to those of HPLC. It is used for the selective absorption of analytes or interferences from simple to complex matrices. It is used for sample cleanup and analyte concentration preceding HPLC, gas chromatography (GC), ion chromatography, and other separation techniques. It has replaced many of the classical liquid–liquid extraction (LLE) techniques. Relative to LLE, SPE uses much less solvent, improves sample throughput, provides more tunable selectivity by appropriate choice of stationary phase, is more readily automated, and avoids the formation of emulsions. In many cases, SPE provides cleaner extracts and higher, more reproducible recoveries.

In its simplest form, SPE uses a packing material such as bonded silica (40–50 μm) or polymeric media packed into a plastic, medical-grade syringe barrel. Similar to an HPLC column, porous polymeric or metallic frits contain the packing in the cartridge format. Other popular SPE formats are disks, pipette tips, and 96-well plates. The stationary phases typically used in SPE cartridges are reversed-phase (C8, C18), ion-exchange (strong anion and strong cation exchange), or normal-phase (silica, cyano, amino) packings. Usually, there are four steps in SPE operation: conditioning, sample addition, washing, and elution. The conditioning step solvates the bonded phase so that it can readily accept the liquid sample load, the washing step removes interferences, and the elution step involves the use of a strong solvent to elute the analyte of interest in a

small volume for direct injection into chromatographic column. Sometimes, the eluent is blown down by solvent evaporation to further concentrate the analyte or to allow redissolution of the analyte in a solvent more compatible with the subsequent chromatographic technique.

More and more applications are going to on-line or automated SPE. Compared with manual methods, automated SPE is less labor intensive, relieves the tedium of manual operation, requires less sample handling providing better recovery, is more reproducible, is performed in a closed system (less chance of sample oxidation or solvent evaporation), and allows smaller samples to be transferred and accommodated more easily. There are several approaches used for automation. The SPE phase can be packed into a stainless steel column and used repeatedly via column switching. Some SPE phases are packed into special high-pressure cartridges and used in an automated sampling device. Other forms of automation involve SPE cartridges, 96-well SPE plates, disks, or pipette tips that are handled by robotic or *xyz* liquid-handling devices. In these approaches, the sample is sometimes exposed to the atmosphere during handling.

Restricted Access Media (RAM)

Restricted access media are a special class of SPE sorbents used for the direct injection of biological fluids such as plasma, serum, or blood. They are used most often for the analysis of small drugs, their impurities, and metabolites. The technology was discussed earlier by Boos and Randolphi [44,45] and reviewed recently by Cassiano and colleagues [46]. Over the years, there have been many variations of the sorbents described as internal-surface reversed-phases, shielded hydrophobic phases, semipermeable surfaces, dual-zone phases, and mixed functional phases.

The most popular RAM phases are the dual-mode porous packings that are characterized by an outer hydrophilic layer and an inner surface porosity with a hydrophobic bonded phase. The outer hydrophilic surface has minimal interaction with proteins combined with small pores of the packing that exclude them, cause the proteins to be eluted unretained. While small drugs and drug metabolites pass into the pores and are retained by hydrophobic interactions with alkyl bonded phases. Despite being described as "nonfouling" phases, RAM has had a reputation for eventual fouling with repeated injection of straight biological fluids. However, if the pH and organic solvent

composition of the mobile phase are not optimized, protein precipitation can occur in the RAM causing fouling, so some care must be exercised in their use.

The RAM phases can be used in the single-column mode or with multidimensional LC–LC. In the single-column mode, conditions are selected to first exclude plasma proteins, then running gradient elution to elute and separate the drug compounds. Although this approach has worked satisfactorily, due to increased chances of fouling the RAM due to lack of reequilibration after gradient elution or due to inadequate selectivity of the hydrophobic stationary phase inside the pores, multidimensional LC–LC approaches have proven to be more popular. Here an additional reversed-phase column is plumbed into the system via a 6- or 10-port switching valve, isocratic conditions are used to inject the plasma onto the RAM, and an additional gradient pumping system is employed. In this approach, the trapped drug molecules from the RAM column are backflushed into a longer reversed-phase column, and gradient elution is performed to separate the impurities and metabolites. When multidimensional approaches are employed, the RAM column is backflushed and regenerated after each analysis. Long-term stability with repeated usage of the RAM column has been reported for soy isoflavones in rat serum [47]. In addition, the reversed-phase column also has a longer life because plasma proteins are not injected onto this second column but are vented to waste.

Molecular Imprinted Polymers (MIPs)

The molecular imprinted polymers are among the most selective phases used in SPE. The technique is sometimes referred to as molecularly imprinted SPE. Molecular imprinting is a technique that has been used in areas where selective recognition is required for complex separations or sample cleanup. Review articles [48-51] and a recent book [52] provide detailed information on the use and potential of MIPs in sample preparation and drug discovery.

A molecular imprinted polymer is a highly stable polymer that possesses recognition sites that are adapted to the three-dimensional shape and functionalities of an analyte of interest. The most common approach through noncovalent imprinting involves the use of a print molecule (template) that is chemically coupled with one of the building blocks of a polymer. After polymerization, the resulting bond must be cleaved to obtain a free selective binding site (receptor). The selective interactions

between the template and the monomers are based upon hydrogen bonding, ionic, or hydrophobic interactions. The most often used monomers are based upon methacrylic acid or methacrylates. The basic idea of MIPs is the "lock-and-key" concept, where a selective receptor or cavity on the surface of a polymer perfectly fits the template analyte that was used to prepare the MIP. The receptor site is complementary to the template in terms of its size, shape, and functionality. The concept is similar to immunoaffinity SPE phases, but obtaining and linking a suitable antibody for these sorbents can be very time consuming.

With aqueous mobile phases, MIPs can display reversed-phase and ion-exchange interaction because selective polar interactions are impaired. The MIP phases show the greatest selectivity when the experimental conditions are chosen that generate the selective interactions that are usually obtained in organic solvents used for the MIP synthesis. This approach allows the MIP to be used for trapping analytes from aqueous solution by hydrophobic or ionic interactions then washed with a solvent that breaks selective binding of matrix components, and finally washed with an organic solvent that disrupts the strong bonds between the analyte and the MIP polymer matrix. A disadvantage of the MIP approach to SPE is the fact that each sorbent must be custom made. The analyst determines the specificity of the MIP by choosing the appropriate template molecule. The MIP can be synthesized in the laboratory using published procedures or the template molecule can be sent to a specialty laboratory that will prepare it. Because of the relatively long process involved in making MIPs for SPE, one can justify it only if the application will be required frequently or if there is no other way to perform sample cleanup.

Besides molecularly imprinted SPE, MIPs are being exploited in many applications that include "tailor-made" separation materials, such as antibody–receptor binding site mimics, enzyme mimics in catalysis, and recognition elements in biosensors. There have been numerous applications of molecularly imprinted SPE for the successful isolation of target analytes in various matrices. Most of these applications resulted from "homemade" MIPs but point out the potential of these very selective phases in sample preparation experiments. Popular areas of applications have been in pharmaceuticals, particularly drugs in biological fluids, in food chemistry for pesticides, and in environmental chemistry for trace analysis. Schirmer and Meisel [53] synthesized an MIP for the selective SPE of chloramphenicol from honey. By

adjustment of the methanol-water ratio in the wash step, matrix components from the honey could be removed and chloramphenicol could be nearly quantitatively extracted and the cleanup reduced matrix effects in their LC–MS-MS analysis. Andersson and colleagues [54] investigated the extraction of local anesthetics from human plasma using an MIP with a structural analog to their target molecules. They compared their MIP phases with a control (nonimprinted) polymer phase prepared without the template being present. To cut down on nonspecific sorption, some elaborate washing steps and additives were required during the extraction and elution conditions. They were able to demonstrate strong selective interactions between the analgesics and the MIP but were not able to eliminate template bleed totally despite using harsh extraction conditions that caused some polymer degradation. Martin and colleagues [55] synthesized a propranolol-specific MIP and also found that they had to work hard on obtaining the optimum conditions for binding the propranolol. They also observed nonspecific interactions between molecules structurally similar and dissimilar to the template molecule. The investigation concluded that such molecular imprinted phases do have advantages over conventional SPE. Finally, Chapuis and colleagues [56] studied retention mechanisms of analytes in molecularly imprinted SPE. They investigated the extractions of triazines from grape juice and soil and found a high degree of selectivity for the target molecules compared to other compounds that possessed the same polarity and molecular size as the triazines. Molecular modeling provided a better understanding of the retention mechanism involved and was a useful guide for reducing nonspecific interactions.

One application area that has promise is the synthesis of chiral MIPs for the separation of enantiomers. Ever since the early work of Curti and Columbo [57], reported in 1952, on enriching racemic camphorsulfonic and mandelic acids using "tailored" silica gel adsorbents, there has been interest in the preparation of chiral MIPs. Here, the polymer is synthesized in the presence of one racemate and then used to separate a racemic mixture. Such packing would be useful for the quick separation of racemic mixtures in the pharmaceutical industry and could possibly be scaled up for large-scale processes. A number of successes in the chiral field have been achieved.

Immunoaffinity Extraction of Small Molecules

Similar to MIPs, immunoaffinity phases are based upon molecular recognition but use chemically attached mono- or polyclonal antibodies rather than surface cavities. Undoubtedly, the immunoaffinity phases are the most selective because they are designed primarily around biological antigen–antibody interactions that provide high selectivity and high affinity. These sorbents enable the selective extraction and concentration of individual compounds or classes of compounds from matrices, often in a single step. Antibodies for large biomolecules are readily available and have been used for many years in immunology and medical research and in the immunoextraction of enzymes, hormones and other biospecies. However, antibodies for small molecules are more difficult to obtain so the development of small molecule immunoaffinity extraction is more recent and less developed [58–62].

As long as an antibody can be prepared, the numbers of immunoaffinity extraction sorbents can be almost unlimited. However, there is a great deal of time involved in making an antibody, purifying it, and bonding it to a solid support. So it only makes sense to develop immunoaffinity phases with widespread use based upon popular groups of compounds that need to be isolated for further sample workup. Despite these limitations, there are several commercial products based upon immunoaffinity principles that have become available. Class-specific immunosorbents are available for a variety of pharmaceutical, food, and environmental applications.

2.5 Method validation

One of the most critical factors in developing pharmaceutical drug substances and drug products is ensuring that the HPLC analytical method that are used to analyze the products generate meaningful data. The US Food and Drug Administration (FDA), United States pharmacopoeia (USP) and International Conference on Harmonization (ICH) have each recognized the importance of this to the drug development process and have separately increased validation requirements in resent years.

Analytical test method validation is completed to ensure that an analytical methodology is accurate, reproducible and robust over the specified range that an analyte will be analyzed. Method validation provides an assurance of reliability during normal use, and can be defined as the process of providing document evidence that the method does what is intended to do. For pharmaceutical HPLC methods validation, a guideline from FDA [63, 64], USP [65] and ICH [66, 67] provides a framework for performing such a validation. Method validation has received considerable attention in literature [68-73] and regulatory agencies. The FDA has proposed adding a section on method validation to the current Good Manufacturing Practice (cGMP) regulations [74]. This would require the manufacturer to establish and document the accuracy, sensitivity, specificity, reproducibility and any other attribute necessary to validate test method. Unfortunately, there is no single source or final guideline on analytical method validation. Validation is customized by choosing necessary tests and acceptance criteria for a given method. The comprehensiveness of this kind of validation is based upon the type of method and its requirements.

2.5.1 Bioanalytical method validation and pre-study validation

Validation of methods that are use in our bioanalytical laboratory are founded on FDA requirements for Bioanlytical Method Validation [75].

Selective and sensitive analytical methods for quantitative evaluation of drugs and their metabolites are critical for the successful conduct of preclinical and/or biopharmaceutical and clinical pharmacological studies. Bioanalytical method

validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, or urine, is reliable and reproducible for the intended use. The fundamental parameters for validation include accuracy, precision, selectivity, sensitivity, reproducibility, and stability. Validation involves documenting, through the use of specific laboratory investigation, the performance characteristics of the method that are suitable and reliable for the intended analytical applications. The acceptability of analytical data corresponds directly to the criteria used to validate the method.

The analytical laboratory conducting clinical pharmacology, bioavailability and bioequvivalence studies should closely adhere to FDA's Good Laboratory Practices (GLPs) and to sound principles of quality assurance throughout the testing process. The analytical laboratory should have a written set of standard operating procedures (SOPs) to ensure a complete system of quality assurance. The SOPs should cover all aspects of analysis from the time the sample is collected and reaches the laboratory until the results of analysis are reported. They also should include record keeping, security and chain of sample custody (accountability systems that ensure integrity of test articles), sample preparation, and analytical tools, such as methods, reagents, equipment, instrumentation, and procedures for quality control and verification of results.

Analysis of drugs and their metabolites in a biological matrix is invariably carried out using samples spiked with calibration standards and quality control (QC) samples. The quality of the reference standard used to prepare spiked samples can affect study data. For this reason, an authenticated analytical reference standard should be used to prepare solutions of known concentrations. If possible, the reference standard should be identical to the analyte. When this is not possible, an established chemical form (free base or acid, salt or ester) of known purity can be used as a surrogate. Three types of reference standards are usually used: (1) certified reference standards (e.g., USP compendial standards); (2) commercially supplied reference standards obtained from a reputable commercial source; and/or (3) other materials of documented purity custom-synthesized by an analytical laboratory or other noncommercial establishment. The source and lot number, certificates of analyses when available, and/or internally or externally generated evidence of identity and purity should be furnished for each reference standard. All reference materials should be checked prior to use to determine if there are significant interfering chromatographic peaks at the retention time of the

analyte and/or the internal standard, using the analytical procedure to be used in the study.

Pre-study validation should include analytical method development and documentation.

Validation should be performed for each biological matrix and for each chemical species to be measured in the biological matrix. In addition, the stability of quality control samples and the analyte in spiked samples should be determined. Typical performance parameters that should be assessed during pre-study validation include (1) specificity, (2) calibration curve and its linearity, (3) precision, accuracy, recovery, (4) quality control samples, (5) stability of analyte in spiked samples, and (6) acceptance criteria.

2.5.2 Specificity

Specificity is the ability of an analytical method to differentiate and quantitate the analyte in the presence of other constituents in the sample and refers directly to the ability of the method to produce a response for a single analyte (Karnes 1991). For specificity, analyses of blank samples of the appropriate biological matrix (plasma, urine, or other matrix) should be obtained from six individuals under controlled conditions, with reference to time of day, food ingestion, and other factors considered important in the intended study. Each blank sample should be tested for interference using the proposed extraction procedure and chromatographic or spectroscopic conditions. The results should be compared to those obtained with an aqueous solution of the analyte at a concentration near the limit of quantitation (LOQ).

Any blank sample with significant interference at the retention time of the drug, metabolites, or internal standard should be rejected. If more than 10% of the blank samples exhibit significant interference at these retention times, additional matrix blank samples should be tested. If more than 10% of this subsequent group of blank samples still shows interference, the method should be changed to eliminate the interferences.
Potential interfering substances in a biological matrix include endogenous matrix components, metabolites, decomposition products, and, in the actual study, concomitant medication. Potential interference from nicotine and common ‘over the counter’ drugs

and metabolites, such as caffeine, aspirin, acetaminophen, and ibuprofen should be routinely tested. If the method is intended to quantitate more than one analyte, each analyte should be injected separately to determine its retention time and to ensure that impurities from one analyte do not have the same retention time as another analyte.

2.5.3 Calibration Curve

Calibration is the relationship between instrument response and known concentrations of the analyte. A calibration (standard) curve should be generated for each analyte in the sample. A sufficient number of standards should be employed to adequately define the relationship between concentration and response. A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking with known concentrations of the analyte. Precautions should be taken to avoid precipitation while spiking the biological matrix. The number of standards used in constructing a calibration curve will be a function of the anticipated range of analytical values and the nature of the analyte/response relationship. Concentrations of standards should be chosen on the basis of the concentration range expected in a particular study. A calibration curve should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard), and five to eight non-zero samples covering the expected range, including lower LOQ. Blank and standard zero samples should not be used in the calibration function, but should only serve to evaluate interference. Additional factors in developing a calibration curve relate to LOQ and linearity.

1. Limit of Quantitation (LOQ)

The lowest standard on the calibration curve should be accepted as the limit of quantitation if the following conditions are met:

No interference present in blanks at the retention time of the analyte at this concentration, or typical response at this concentration at least five times greater than any interference in blanks at the retention time of the analyte.

Analyte peak (response) is identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80-120%.

2. Linearity

The simplest workable regression equation should be used with minimal or no weighting. Selection of weighting and use of a complex regression equation should be justified. Four factors should be met in developing a calibration curve:

20% deviation of the LOQ from nominal concentration.

15% deviation of standards other than LOQ from nominal concentration.

At least four out of six non-zero standards meeting the above criteria, including the LOQ and the calibration standard at the highest concentration.

0.95 or greater correlation coefficient (r).

2.5.4 Precision, Accuracy, and Recovery

The ***precision*** of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The precision determined at each concentration level should not exceed 15% coefficient of variation (CV) except for the LOQ where it should not exceed 20% CV. Precision is further subdivided into within-day, intra-batch precision or reproducibility, which assesses precision during a single analytical run, and between-day, inter-batch precision or reproducibility, which measures precision with time and may involve different analysts, equipment, reagents, and laboratories.

The ***accuracy*** of an analytical method describes the closeness of test results obtained by the method to the true value of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. A minimum of five determinations per concentration should be conducted for a minimum of three concentrations in the range of expected concentrations. The mean value should be within 15% of the actual value except at LOQ, where it should not deviate by more than 20%. The deviation from the true value serves as the measure of accuracy.

The ***recovery*** of an analyte in an assay is the detector response obtained from an amount of the analyte added to and recovered from the biological matrix, compared to the detector response obtained for the pure authentic standard (Brooks 1985, Mehta 1989).

Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Although recoveries close to 100% are desirable, the extent of recovery of an analyte and/or the internal standard may be as low as 50 to 60% if the recovery is precise, accurate, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery.

2.5.5 Quality Control Samples

Pre-study validation of an analytical method should be carried out using at least three batches of biological matrix, where each batch is collected from a different source. Each batch should contain a calibration curve constructed using a blank sample, zero sample, and five to eight non-zero standards, low QC samples, medium QC samples, high QC samples, a matrix blank sample, and a reference standard. Quality control samples at concentrations noted below should be made from a stock solution separate from that used to prepare the standards.

Low QC sample**:** 3 x LOQ

Medium QC sample**:** Approximately midway between the high and low QC concentrations

High QC sample**:** 75 to 90% of highest calibration standard

The accuracy of preparation of calibration and QC samples should be checked with the first batch. The data from replicate analyses of QC samples and duplicate analyses of reference standards should be used to obtain the intra-day (within batch) precision, interday (between batch) precision, accuracy, and recovery.

To obtain within-batch data, the mean, standard deviation, and coefficient of variation (CV) of each QC concentration in each batch should be calculated. The global (overall) mean, standard deviation, and CV for each QC concentration from the three

batches should be calculated to obtain between-batch data. Precision is indicated by the %CVs. Accuracy percentage is determined by dividing the mean concentration of a QC by its nominal concentration, and multiplying by 100.

2.5.6 Stability

Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system. The stability of an analyte in a particular matrix and container system is relevant only to that matrix and container system and should not be extrapolated to other matrices and container systems. Stability procedures should evaluate the stability of the analytes in biological fluids after long-term (frozen at the intended storage temperature and conditions) and short- term (bench top, room temperature and conditions) storage, and after going through freeze and thaw cycles and the analytical process. The procedure should also include an evaluation of analyte stability in stock solution.

All stability determinations should use a set of standard samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free biological matrix. Stock solutions of the analyte for stability evaluation should be prepared in an appropriate solvent at concentrations defined in the method SOP. Further information about validation for these factors appears in the following five sections.

1. Freeze and Thaw Stability

Testing for freeze and thaw analyte stability should be determined during three freeze and thaw cycles. At least three aliquots at each of the low and high concentrations should be stored at the intended storage temperature, for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples should be transferred back to the original freezer and kept refrozen for 12 to 24 hours. The cycle of thawing and freezing should be repeated two more times, then analyzed on the third cycle. If an analyte is unstable at -20°C, the stability sample should be frozen at -70°C during the three freeze and thaw cycles.

2. Short-Term Room Temperature Stability

Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature from 4 to 24 hours (based on the expected duration that samples will be maintained at room temperature in the intended study) and analyzed.

3. Long-Term Stability

The storage time in long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis. Long-term stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions as the study samples. A suggested storage temperature for the majority of drugs and metabolites in a biological matrix is -20°C, but lower temperatures (e.g., -70°C) may be recommended to prevent the degradation problems observed at higher temperatures.

The volume of samples should be sufficient for analysis on three occasions. The concentrations of all the stability samples should be compared to the mean of back calculated values for the standards at the appropriate concentrations from the first day of long-term stability testing.

4. Stock Solution Stability

The stability of stock solutions of drug and the internal standard should be evaluated at room temperature for at least 6 hours. The stability samples should then be refrigerated or frozen for 7 to 14 days or other relevant period. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions.

5. Autosampler Stability

The stability of processed samples in the autosampler should be determined at the autosampler temperature that will be used during analysis, which is usually room temperature, but may sometimes be a lower temperature (e.g., when a refrigerated autosampler is used). Stability should be assessed over the anticipated run time for the batch size to be used in studies. The stability of both the drug and the internal standard should be evaluated in validation samples under these conditions by determining concentrations on the basis of original calibration standards.

2.5.7 Matrix Effect

It may be important to consider the variability of a matrix due to the physiological nature of the sample. In the case of HPLC-MS/MS based procedures, appropriate steps should be taken to ensure the minimum of matrix effects throughout the application of the method, especially if the nature of matrix changes from the matrix used during method validation.

In recent years HPLC-MS/MS has been demonstrated to be a powerful technique for the quantitative determination of drugs and their metabolites in biological fluids. The methodology achieved its preferred status because it has been perceived that MS/MS detection is highly selective and thus effectively eliminates interference by endogenous impurities. Even without any clean up or extraction of samples and very little or no chromatographic separation, endogenous impurities from biofluids were not detected, and the only MS/MS signal observed in control biofluids originated from desired analyte. However, the common and early perception that utilization of HPLC-MS/MS practically guarantees selectivity is being challenged by number of reported examples [76-78] of lack of selectivity due to ion suppression or enhancement caused by sample matrix, interferences with metabolites or 'cross-talk' effect.

The matrix effect phenomenon was originally described by Kebarle and Tang [79] who showed that electro spray response of organic bases decreased with increase in concentration of other organic bases. In the context of quantitative bioanalysis of drugs, the matrix effect was recently studied by P.J. Larger [80]. The mechanism and the origin of the matrix effect is not fully understood [79, 81], but may originate from competition between an analyte and co eluting, undetected matrix components reacting with primary ions formed in HPLC-MS/MS interface. Depending on environment in which the ionization and ion evaporation process take place, this competition may effectively decrease (ion suppression) or increase (ion enhancement) the efficiency of formation of desired analyte ion present at the same concentration in the interface. Primary ions react in the interface with analyte molecules in a very complex series of charge transfer and ion transfer reactions. The rate and efficiency of these reactions are highly dependent on relative ionization energies, proton affinities, or both of the molecules present in the interface at any given time. The efficiency of formation of the desired ions must be very much matrix-dependent due to competition between the

molecule of interest and a number of other undetected but co eluting molecules present in the system that are capable of reacting with primary ions. This effect may reduce or increase the intensity of analyte ions and affect the reproducibility and accuracy of the assay. [82]

The current regulatory requirements include the need for the assessment and elimination of matrix effect in the bioanalytical methods, but the experimental procedures to assess the matrix effect are not detailed.

In recent investigation to evaluate matrix effect the publication written by Matzsuzewski [82] was used as a guide. Three sets of seven calibration standards and a blank with internal standard were prepared for evaluation of recovery and ionization suppression or enhancement. Set 1 was prepared to evaluate the MS/MS response of working standard solutions injected in the mobile phase. Working standard solutions were diluted to reach the concentration expected in plasma samples. Set 2 consisted of eight plasma samples spiked with each working standard solutions *after* extraction. Plasma samples spiked *before* extraction and S0 were processed and analyzed to obtain Set 3. Three replications of each set were used for determination of recovery and matrix effects. Internal standard was not added to standards. The matrix effect (ME)—the possibility of ionization suppression or enhancement and recovery (RE) were evaluated by comparing the results of analysis of three sets of samples as follows:

ME (%) = B/A *100

RE (%) = C/B *100

A-mean peak area for single conc. of Set1

B-mean peak area for single conc. of Set2

C-mean peak area for single conc. of Set3

2.5.8 Acceptance Criteria

An analytical method is considered fully validated when it meets the following criteria:

Precision: The between-batch CVs for low, medium, and high concentrations of QC should be 15%, and 20% for the LOQ QC, using a minimum of three batches.

Accuracy: The between-batch mean value should be within ±15% of the nominal value at low, medium, and high QC concentrations and should not deviate by more than ±20% at the LOQ.

Sensitivity: The lowest standard should be accepted as the limit of quantitation of the method if the between-batch CV at the LOQ is 20%.

Specificity: The responses of interfering peaks at the retention time of the analyte should be less than 20% of the response of an LOQ standard. Responses of interfering peaks at the retention time of the internal standard should be 5% of the response of the concentration of the internal standard to be used in studies.

Stability: Long-term, short-term, freeze and thaw, stock solution, and autosampler stability data should meet the criteria specified in the SOP.

3 DEVELOPMENT AND VALIDATION OF ANALYTICAL METHOD FOR DETERMINATION OF DRUGS IN BIOLOGICAL FLUIDS

3.1 Determination of atorvastatin and 2-hydroxyatorvastatin in human plasma

Cholesterol-reducing statin drugs are the most frequently prescribed agents for reducing morbidity and mortality rates related to coronary heart disease. Atorvastatin (ATV), also referred to as atorvastatin acid, is a competitive inhibitor of 3-hydroxy-3-methylglutarylcoenzyme-A (HMG-CoA) reductase. By inhibiting HMG-CoA reductase ATV reduces plasma cholesterol and lipoprotein levels.

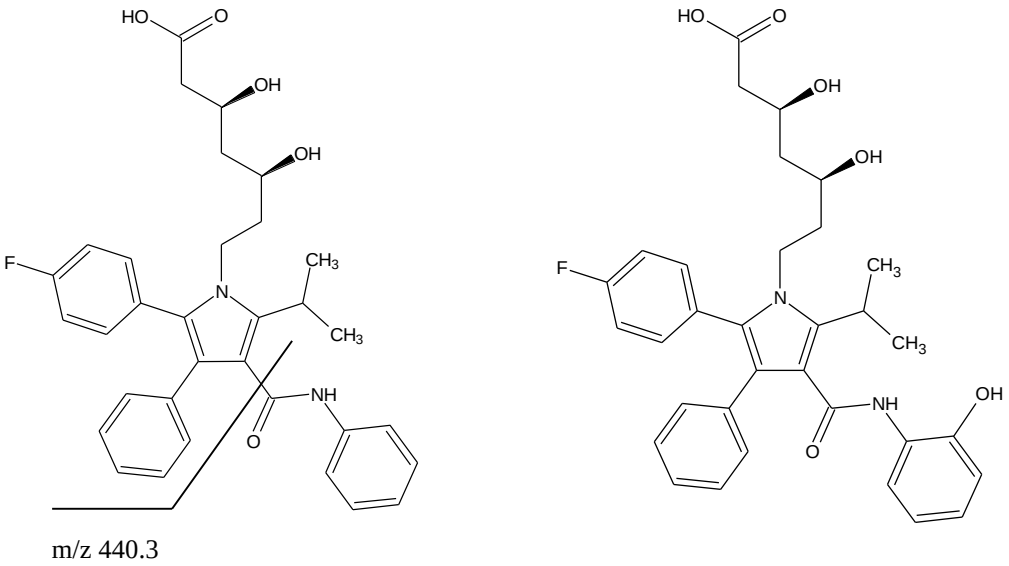
Dose-dependent reductions in total cholesterol, low-density lipoprotein (LDL) cholesterol and triglyceride levels have been observed after administration of ATV to patients with hypercholesterolemia and to patients with hypertriglyceridemia [83–85]. The usual starting dose is 10 mg ATV daily [84, 85]. The dose should be adjusted in accordance with plasma lipid levels and can be increased to up to 80 mg daily [84, 85]. ATV is rapidly absorbed after oral administration and maximum plasma concentrations are reached within 1 to 3 h. More than 98% of ATV is protein-bound in plasma [83–87]. ATV is transformed into two active metabolites—2-hydroxyATV and 4-hydroxyATV [83–85, 87, 88]. The concentrations of 4-hydroxy ATV acid are much lower than those of 2-hydroxyATVacid, and are too low for calculation of the pharmacokinetics [59].

Several methods have been reported for determination of ATV and HATV. HPLC with UV detection has been successfully used to determine ATV in bulk drug and tablets [88]. Square-wave adsorptive-stripping voltametry has been used for determination of ATV in human plasma but the LOQ was not low enough to enable pharmacokinetic study [89]. Van Pelt et al. published an unusual but easy method for determination of ATV and five of its metabolites using a four-column parallel chromatography system with MS/MS detection [89]. In another paper [91] a complex

HPLC–MS method is reported for determination of ATV and HATV in serum. Bullen et al. have reported an HPLC–MS/MS method for determination of ATV and HATV in human, dog, and rat plasma. This method, however, requires an inconvenient and complicated sample-preparation step, entailing double extraction, before the analysis [92]. Furthermore, none of these methods are suitable for concentrations of ATV and HATV found in human plasma after administration of a therapeutic dose.

According to the literature, ATV and HATV concentrations between 0.1 and 20 ng mL^{-1} in human plasma could be expected after administration of 80-mg doses of ATV, which was the dose tested.

A validated, highly sensitive, and selective isocratic HPLC method was developed for quantitative determination of the major statin drug atorvastatin (ATV) and its metabolite 2-hydroxyatorvastatin (HATV). Detection was performed with an electrospray ionization triple-quadrupole mass spectrometer equipped with an ESI interface operating in positive-ionization mode. Multiple reaction monitoring (MRM) was used for MS/MS detection. The calibration plot was linear in the concentration range 0.10–40.00 ng mL^{-1} for both ATV and HATV. Inter-day and intra-day precision and accuracy of the proposed method were characterized by measurement of relative standard deviation (RSD) and percentage deviation, respectively; Inter-day and intra-day precision and accuracy were less than 8% for both analytes. The limit of quantitation was 0.02 ng mL−1 for ATV and 0.07 ng mL^{-1} for HATV. There were no interferences from endogenous plasma components or from other sources, and no "cross-talk" effect was observed in plasma samples. ATV, HATV, and the internal standard (CLI) were well separated and their peaks were narrow and symmetric. The simple sample preparation procedure and short retention time enable determination of more than 250 samples per day. The method has been successfully used in a pharmacokinetic study approved by the appropriate ethics committee and performed on 44 healthy subjects after obtaining their written informed consent. More than two thousand samples were assayed by use of the proposed method.



m/z 440.3

a **b**

Picture 2 : Stucture of ATV a HATV and their probable fragmentation

3.2 Determination of azithromycin in human plasma

Azithromycin (AZI) is a macrolide antibiotic derived from erythromycin. AZI differs structurally from erythromycin by insertion of a methyl-substituted nitrogen at position 9a in the lactone ring to create a 15-member macrolide. This modification results in significantly improved antibacterial potency against Gram-negative bacteria compared with erythromycin [93, 94]. AZI causes the death of the bacteria or inhibits their growth, depending on the organism, its sensitivity to AZI, and the concentration of AZI in the infected tissue.

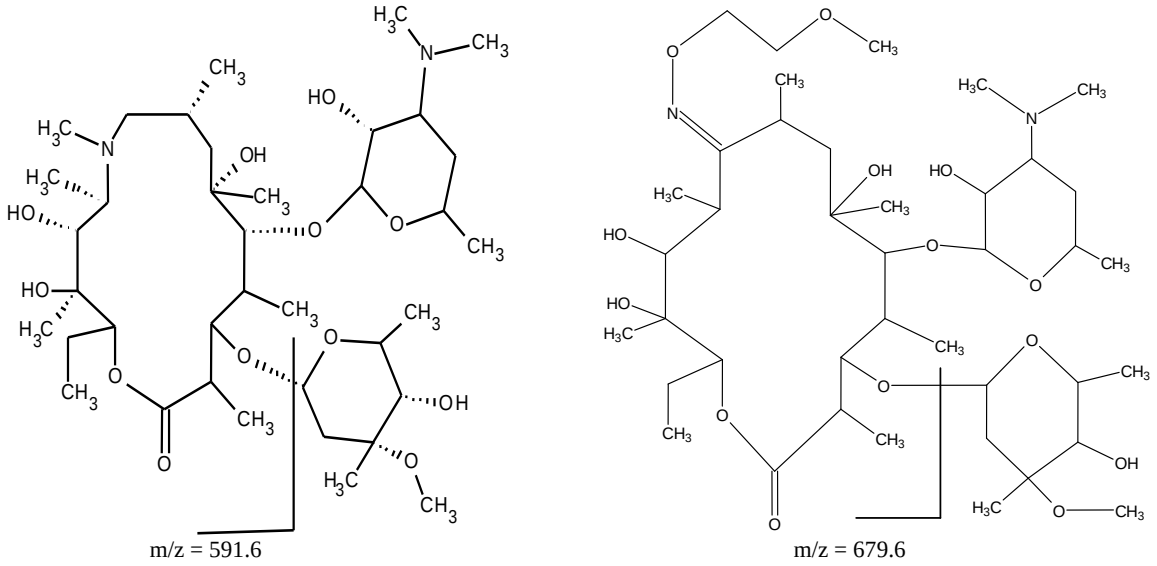
Several high-performance liquid chromatographic methods have been reported for determination of AZI in human plasma. Most of these methods require electrochemical detection [95–99] to ensure sufficient sensitivity. UV detection is of low sensitivity for determination of macrolides in body fluids [100]. Torano and Guchelaar devised a method for determination of macrolides in human serum using fluorescence detection [101]. This method, however, requires complex sample pretreatment involving pre-column derivatization of the analyte with 9-fluorenylmethyloxycarbonyl chloride. Song and Wang [102] developed a sensitive method for determination of AZI using a flow-injection technique (FIA). Although this method is more sensitive than existing methods, it requires apparatus uncommon for routine analysis and commercial utilization.

HPLC–MS/MS is currently regarded as the method of choice for quantitative determination of drugs. Because MS/MS detection is sensitive and effectively eliminates interference from endogenous impurities, it usually guarantees method selectivity and sample extraction and chromatography can be simplified [103, 104].

According to the literature, plasma concentrations between 0.3 and 500 ng mL^{-1} could be expected in human plasma after administration 250 mg AZI [105–107], which was the dose tested.

A method for determination of AZI in human Na2EDTA plasma covering the concentration range 2.55–551.43 ng mL−1, using 0.5 mL plasma, has been developed and validated. Human plasma containing AZI and internal standard was ultrafiltered through Centrifree Micropartition devices and the concentration of AZI was determined by isocratic HPLC–MS/MS. Multiple reaction monitoring mode (MRM) was used for MS/MS detection. Inter-day and Intra-day precision and accuracy of the

proposed method were characterized by R.S.D and percentage deviation, respectively; both were less than 8%. Limit of quantification was 2.55 ng mL^{-1}. No interferences from endogenous plasma components or other sources were found and no "crosstalk" effect was observed in plasma samples. AZI and internal standard Roxithromycin (ROX) were well separated and the peaks were narrow and symmetric. The simple preparation procedure and short retention times enable determination of more than 180 samples per day. The analytical method has proved to be useful for the investigation of the pharmacokinetic characteristics of AZI in human plasma in a pharmacokinetic study. More than two thousand samples were assayed by use of this method.



A B

Picture 3 : Stucture of AZI a ROX and their probable fragmentation

3.3 Determination of Quetiapine in human plasma

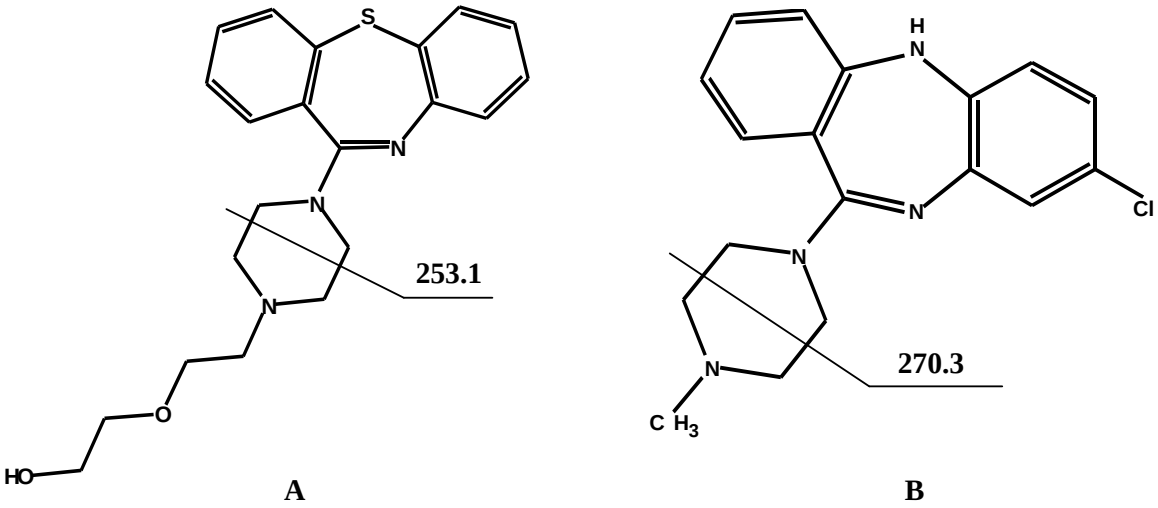
Quetiapine(2-[2-(4-Dibenzo[b,f][1,4]thiazepin-11-ylpiperazinyl)ethoxy]ethanol-fumarate (2:1 salt)) is an atypical antipsychotic drug with a unique receptor-binding profile belonging to a new chemical class, the dibenzothiazepine derivatives [108-111]. Quetiapine is used in the treatment of schizophrenia or manic episodes associated with bipolar disorder. The advantages of the therapeutic profile of Quetiapine have led to increasing use in clinical practice, which encourages the development of new pharmaceutical preparations.

Quetiapine (QUE) Inter-day and intra-day precision and accuracy is metabolized by the liver and eleven confirmed metabolites of Quetiapine have been identified. QUE appears to be the major circulating species in plasma. According to the literature, a QUE concentration between 1.5 and 350 ng/mL [112,113] in human plasma could be expected after the administration of a 100 mg QUE dose.

Several HPLC methods for the determination of QUE have been reported. Most of these require ultraviolet detection [114-116], as QUE is not electro active. However none of these methods is sensitive enough for determination of the expected drug levels and some of them are time-consuming and require complex sample pretreatment or long run times [117]. Some gas chromatography- mass spectrometry (GC-MS) methods have also been employed, however here QUE needs to be derivatized before analysis [118,119].

Recently, two HPLC-MS methods have been published for determination of QUE [117, 120]. The first paper [90] compares HPLC methods with ultraviolet and MS/MS detection. Although the sample preparation is fully automated, the run time is 35 min, thus the method allows determination of only 40 samples a day, which is not enough for routine analysis and commercial utilization in pharmacokinetic studies. The next paper [120] describes HPLC-MS method for simultaneous determination of Clozapine, Olanzapine, Risperidone, and QUE in plasma. Nevertheless, this method requires two-step extraction and LOQ is too high for our purpose.

A validated, highly sensitive and selective HPLC-MS/MS method was developed for the quantitative determination of QUE in human Na_2EDTA plasma with MS detection. Clozapine (CLO) was employed as an internal standard. Samples were extracted using solid phase extraction (SPE) Oasis HLB cartridges and the concentration of quetiapine was determined by isocratic HPLC-MS/MS. The SRM mode was used for MS/MS detection. The method was validated over a concentration range of 1.0 –382.2 ng/mL. Inter- and Intraday precision and accuracy of the proposed method were characterized by relative standard deviation (RSD) and the percentage of deviation, respectively; both were lower than 8%. No interferences from endogenous plasma components or other sources were found and no "cross-talk" was observed in plasma samples. A simple preparation procedure and short retention time could allow determination of more than 250 samples per day. The analytical method has been proved useful for the investigation of the characteristics of QUE in human plasma in pharmacokinetic studies.



Picture 4 : Stucture of QUE a CLO and their probable fragmentation

3.4 Determination of simvastatin and simvastatin hydroxy acid in human plasma

Cholesterol lowering statin drugs are the most frequently prescribed agents for reducing morbidity and mortality rates related to coronary heart disease. Simvastatin (SIM) is widely used in the treatment of various types of hypercholesterolaemias. SIM—a methylated analogue of lovastatin—is synthesized from a fermentation product of Aspergillus terreus. The parent drug is a pharmacologically inactive lactone (prodrug form), which is absorbed from the stomach, extracted by the liver, and largely converted to several active metabolites. The most notable is simvastatin hydroxy acid (SIMA) [121–125]. The active metabolites tend to concentrate in the liver, a major site of cholesterol endogenous synthesis. The inhibition of HMG-CoA reductase results in a moderate reduction in cholesterol synthesis and, more importantly, in an increase of low-density lipoprotein (LDL) receptors located on the cell membranes of the liver [126]. In humans, a linear increase in the inhibitory activity of simvastatin occurs in the dose range from 5 to 120 mg daily [124,127].

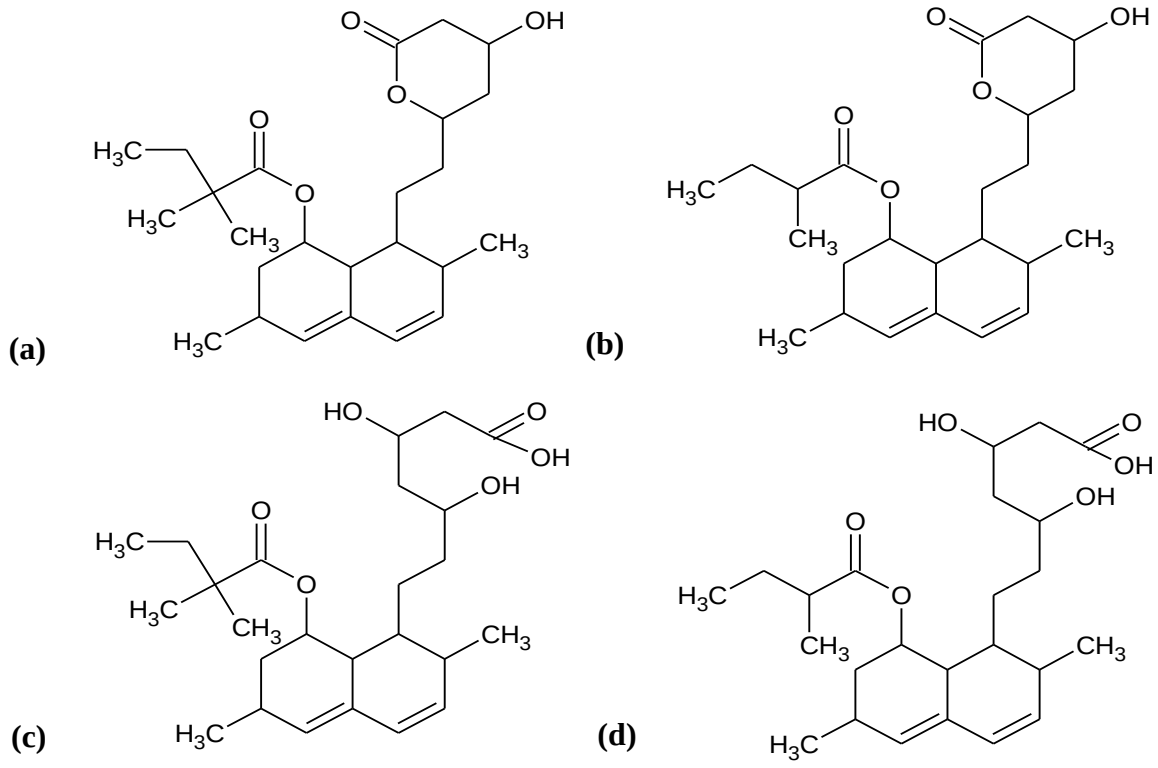
Gas chromatography–mass spectrometry (GC–MS) methods are highly sensitive and selective enough to determine the therapeutic plasma levels of both SIM and SIMA [128,129], but the operation and clean up procedure prior to analysis is complicated. Several HPLC methods for SIM and SIMA determination have been reported. Liquid chromatography–UV detection (LC–UV) methods are simpler than those in the GC–MS but they are not sensitive enough for determining the drug levels in plasma at therapeutic dose [130,131]. Although LC with fluorescence detection is a highly sensitive method, the samples need complex inconvenient derivatization before their analysis [132].

Recently, several HPLC–MS/MS methods have been published for the determination of SIM. The first paper [133] describes an HPLC method with MS/MS detection for the determination of SIM in aqueous samples with LOQ 1 ng/mL, which is too high for the purpose of the pharmacokinetic study (PK study). The next paper [134] presents an HPLC–MS method for the determination of SIM in plasma with suitable sensitivity, however, none of these papers take into consideration the

metabolites of SIM. SIM together with its metabolite were quantified using the method published by Jemal et al. [135]. The authors coupled on-line SPE with LC–MS/MS and presented a fairly sensitive method with a limit of quantitation of 0.5 ng/mL. However, on-line SPE is not suitable for routine use where hundreds of samples may need to be analyzed.

According to the literature, SIM and SIMA concentrations between 0.2 and 15.0 ng/mL in human plasma could be expected after the administration of an 80 mg SIM dose, which was the dose tested. Confident monitoring of the concentration levels published in the literature would require a limit of quantification of 0.10 ng/mL.

A validated, highly sensitive and selective isocratic HPLC method was developed for the quantitative determination of the major statin drug simvastatin (SIM) and its metabolite simvastatin hydroxy acid (SIMA). Detection was performed on an electrospray ionization triple quadrupole mass spectrometer equipped with an ESI interface operated in positive and negative ionization mode. The multiple reaction-monitoring mode (MRM) was used to provide MS/MS detection. The linearity for the calibration curve in the concentration range of 0.10–16.00 ng/mL for SIM and 0.10–16.00 ng/mL for SIMA is presented. Inter- and intra-day precision and accuracy of the proposed method were characterized by relative standard deviation (RSD) and percentage deviation, respectively; with both lower than 7% for all analytes. The limit of quantitation was 0.03 ng/mL for SIM and 0.02 ng/mL for SIMA. No interferences from endogenous plasma components or other sources were found and no “cross-talk” effect was observed in plasma samples. SIM, SIMA and their internal standards Lovastatin and Lovastatin acid (LOV and LOVA) were well separated and their peaks were narrow and symmetric. Simple preparation procedure and short retention time allow determination of more than 250 samples per day. The analytical method has proved to be useful for the investigation of the pharmacokinetic characteristics of SIM and SIMA in human plasma and was successfully applied to a PK study that was approved by the appropriate Ethics Committee and performed in 72 healthy subjects after obtaining their written informed consent. More than 3000 samples were assayed using the proposed method.



Picture 5 : Stucture of SIM (a), LOV (b), SIMA (c), LOVA (d) and their probable fragmentation

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Attachment:

1. B. Barrett, V. Bořek-Dohalský, P. Fejt, S. Vaingátová, J. Huclová, B. Němec, I. Jelínek. Validated HPLC–MS–MS method for determination of azithromycin in human plasma. Anal. Bioanal. Chem. 383 (2005), 210-223.
2. B. Barrett, J. Huclová, V. Bořek-Dohalský, B. Němec, I. Jelínek. Validated HPLC–MS/MS method for simultaneous determination of simvastatin and simvastatin hydroxy acid in human plasma. J. Pharm. Biomed. Anal. 41 (2006) 517-526.
3. V. Bořek-Dohalský, J. Huclová, B. Barrett, B. Němec, I. Ulč, I. Jelínek. Validated HPLC–MS/MS method for simultaneous determination of atorvastatin and 2-hydroxyatorvastatin in human plasma – pharmacokinetic study. Anal. Bioanal. Chem. 386 (2006) 275-285.
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