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The application of mass spectrometry in pharmacokinetics studies

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1. Introduction

Pharmacokinetics is the study of the absorption, distribution, metabolism, and excretion (ADME) of drugs; the respective rates of which are of fundamental importance in determining their effect on the organism to which they are administered. Pharmacokinetic studies with mass spectrometry can provide quantitative information about a compound's half-life in the body and how quickly it is metabolized or excreted. These studies are also used to determine drug distribution, partitioning within an organism, and drug dosing régime. The latter is important in determining a drug's therapeutic range, which is a balance between the concentration required for positive therapeutic effect versus higher doses which may have toxicological effects. Finally, pharmacokinetics is used to investigate drug dosing with regards to administration factors of age, gender, ethnicity, concomitant drugs, or diseases.

Pharmacokinetic analysis involves the determination of the concentration of drugs and/or their metabolites in biological fluids, which may be present at very low concentrations. Quantitative analysis is important since a major consideration in these types of studies is to determine how much drug gets into the systemic circulation. The integration of drug concentration over time (the so-called Area-Under-The-Curve or AUC) is a measure of both how well the drug is cleared by an individual and how well the analytical system is performing. Therefore, reliable, sensitive and specific methods are required for these types of analyses.

With the increased pressure on researchers and pharmaceutical companies to discover and develop novel therapeutic compounds in a rapid manner, mass spectrometry now plays a central role in pharmacokinetics studies. The initial mass spectrometric technique used in pharmacokinetics, gas chromatography/mass spectrometry (GC/MS) was limited to molecules that were either volatile or could be made thermally stable by pre-column derivatization. Derivatization is often used to improve a molecule's ability to be vaporized and ionized with a GC/MS, yet it has significant disadvantages because many molecules are still not amenable to GC/MS analysis after derivatization and the sample handling associated with derivatization results in sample loss.

More recently, liquid chromatography coupled with soft ionization techniques such as electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) with triple quadrupole mass analysis (Fig. 1), have become the most common approaches in pharmacokinetic studies. This is because

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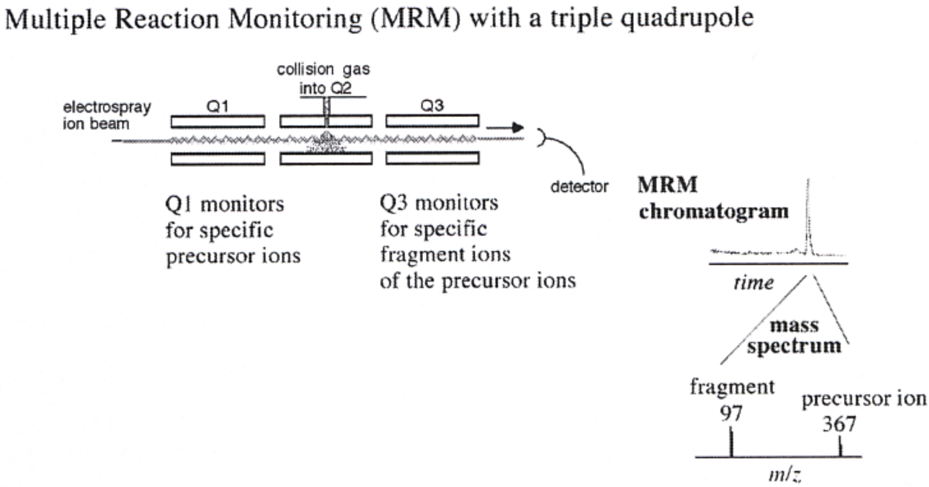

Fig. 1. Multiple reaction monitoring (MRM) is a valuable tool in the quantitation of drugs and their metabolites, this example is a sulfated steroid where m/z 367 is the precursor [sulfated steroid–H]$^-$ and the fragment ion m/z 97 is HSO_4^-.

LC/MS is capable of simultaneously generating molecular weight, structural, and accurate quantitative information [1–5] regarding the metabolism of a drug. This is highly significant given that recently approved drugs are more potent and therefore administered at concentrations that are difficult to detect with traditional techniques. Mass spectrometry has quite literally revolutionized bioanalytical analysis in the drug discovery and development process, reducing the method development time to a few days or less [6] and providing limits of quantitation of less than nanogram/ml from complex matrices such as plasma, serum, urine, and cellular media.

1.1. Sample collection and extraction

To measure levels of administered drugs and/or metabolites, biological samples such as urine and plasma are typically collected from human patients or laboratory animals. Interfering materials can be removed from these samples and the drug and/or its metabolites of interest concentrated prior to analysis using one of several techniques, including protein precipitation [7–9], solid phase extraction, liquid-liquid extraction, solid-liquid extraction or acid-base extraction. Liquid-liquid extractions and protein precipitation can be performed in 96-well plates using modern robotic liquid-handling systems, reducing sample preparation times by 3-fold [10]. Solid-liquid extraction using 96-well diatomaceous earth plates has the advantage of providing clean extracts, reduced sample preparation time, and increased sample throughput [11]. Once the compound(s) of interest have been extracted, quantitative measurements of the drug's lifetime in the biofluid can be established by LC/MS (Fig. 2).

2. High throughput screening techniques in pharmacokinetics

The introduction of biological high throughput screening in the 1980's [12], a consequence of both the introduction of laboratory automation and target cloning using recombinant DNA technology, was followed by the development of combinatorial libraries and high throughput parallel organic synthesis in the 1990s [13,14]. Improvements in the mass spectrometer's ionization source, its overall accuracy, and the implementation of computer-controlled automation have increased the efficiency of screening

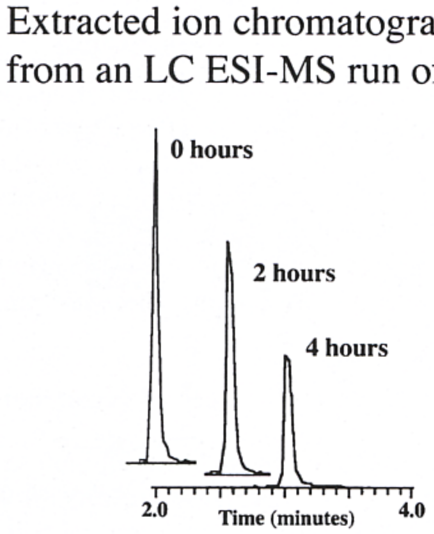

Fig. 2. LC/MS of an extracted drug at different time points after incubation or dosing.

potential drug molecules for potential efficacy [15]. Faster production of pure compound libraries, which feeds these high throughput screens, has increased the hit rate on biological targets. This corresponding increase in hits has introduced more lead compounds into the discovery process. Historically, ADME assays were performed during the development stage of drug discovery. Recently, more and more of these assays have become a routine part of a screening regimen, designed to advance only those compounds with a high likelihood of becoming orally delivered drugs. Indeed, many of the examples given in the following sections utilize high throughput techniques.

Over the past decade, rapid high throughput procedures have become increasingly important in the area of drug discovery and development. This is largely due to heightened pressure to reduce the time it takes a drug to become marketable. One such technique is flow injection analysis coupled with ESI or APCI, which eliminates the chromatographic column and therefore reduces the turnaround time for screening large numbers of samples. However, although fast, the absence of chromatographic separation in this method means that little can be gleaned regarding compound purity. In addition, signal suppression may arise due to interferences in the sample.

Given that problems may arise with solvent ionization and signal suppression of analytes, separation prior to mass analysis is usually necessary. For this reason LC/MS has become a relatively high throughput screening tool for potential drug candidates. Although the overall analyses are slower than with flow injection analysis, chromatographic separation is often required for both purity assessment as well as quantitative measurements, which is critical for certain assays. To increase throughput, the simultaneous use of multiple columns can help compensate for longer runtimes by allowing samples to be run in parallel. Multiple-column systems can also be coupled to a multiplexed ionization source (MUX$^{\text{TM}}$), which enables 4 or 8 streams (Fig. 3) to be sprayed into the mass spectrometer, performing the LC/MS analysis 4 to 8 times faster than with a single sprayer [16].

Cassette-accelerated screening of several drug compounds simultaneously using 96-well plate formats combined with LC/MS has also reduced sample preparation time and increased compound throughput [17]. Alternatively, ultrafast LC/MS methods can be applied. These involve gradient separations on narrow bore, short HPLC columns and high flow rates that are performed in less than 2 minutes [18–21]

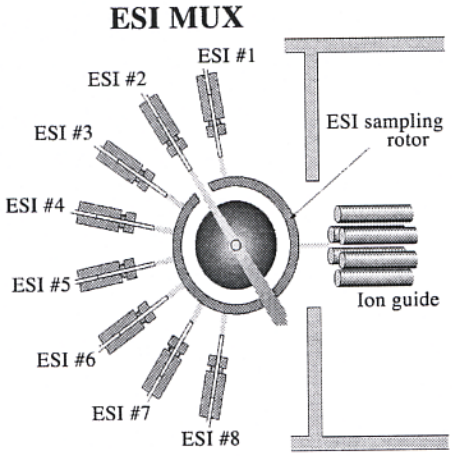

Fig. 3. An electrospray ionization (ESI) MUX setup for high throughput pharmacokinetics studies.

while still maintaining chromatographic integrity. These ultrafast techniques have the advantage of simplicity over multiplexing, for which both the equipment and method validation is more complicated [22].

3. Metabolite identification and quantitation

In the drug discovery process, early metabolite identification is needed for patient protection against toxicity and to determine if any metabolites are related to known drugs or have inherent activity against the therapeutic target or other targets. This, in turn, can accelerate the selection of potential clinical candidates and allow early rejection of candidates that would otherwise require critical resources to develop. For compounds that are screened for their metabolic stability, metabolite identification can yield important feedback information for chemists who can then avoid synthesizing structures that are rapidly turned over. The use of triple quadrupole and ion trap instruments for LC/MS/MS represents a powerful combination to investigate various biotransformations by allowing multiple reaction monitoring (MRM), neutral loss, precursor ion scanning, and MS^n for the identification of drug metabolites. Positive and negative mode single ion monitoring (SIM) can also be used to identify the intact drug and any anticipated metabolites. Commonly occurring metabolites are produced as a result of keto-formation (M+14), hydroxylation (M+16), dihydroxylation (M+32) and glucuronidation, (M+176) [23] among others. For quantitation of specific metabolites, to determine enzyme kinetics, and for MRM a triple quadrupole mass spectrometer is most commonly used (Fig. 1).

Capillary LC coupled to an ion trap mass spectrometer has also been used successfully for the rapid determination of drug metabolites in urine [24]. Due to the reduction in column diameter, a low sample volume of only 1 μl of untreated urine was required. Both molecular and structural information was obtained on just 5 pg of compound. In addition, when selected reaction monitoring target analysis was used, as little as 200 fg of compound was detected on the column.

4. LC-TOF/MS

The use of LC-API-MS/MS in metabolite recognition and structure elucidation studies often involves extensive method development. Due to the scarcity of supporting qualitative information [25], this can extend the time of the drug discovery process. The application of TOF to these studies can provide additional data to that obtained with quadrupole or quadrupole ion trap instruments, as full scan spectra can be collected with greater sensitivity and speed. TOF instruments retain, separate and detect a larger percentage of the ions (5–50%) that have been sampled in the high vacuum region than quadrupole instruments [26]. This is because quadrupole analyzers filter out more ions in an effort to obtain optimum signal-to-noise ratios. Quantitative and qualitative data can be concurrently obtained, as has been used to characterize several drug compounds simultaneously dosed to rats in a cassette format [6]. However, TOF instruments cannot yet match the signal-to-noise ratios achieved in tandem mass spectrometry experiments such as MRM, so are best used to add to the information content of the LC/MS experiment [6].

5. Supercritical Fluid Chromatography Mass Spectrometry (SFC-MS)

In recent years, supercritical fluid chromatography (SFC) has grown in popularity as a complementary technique to liquid chromatography for the separation of compound libraries. SFC is a normal phase chromatographic technique that has high chromatographic efficiency and selectivity and therefore allows for short run times (typically under 1 minute) [27]. The excellent mass transfer of SFC allows for higher flow rates (mobile phase linear velocities) and the use of longer columns without increased back-pressure. Another advantage is that since carbon dioxide is the preferred bulk eluent, organic solvent consumption and waste is reduced, which is particularly useful for large-scale operations. SFC has demonstrated high resolution, speed, and efficiency when screening pharmaceutical compound libraries [27–30]. Rapid, sensitive, selective, and reproducible SFC methods have been developed to measure, among others, plasma levels of (R)- and (S)-enantiomers of ketoprofen (using chiral stationary phases), a potent nonsteroidal anti-inflammatory drug [31] and sulfadoxine, a long-acting sulfonamide used to treat malaria [32].

6. Quantitation using internal or external standards

LC/MS is used quantitatively in pharmacokinetics when authentic standards of the drugs and/or their metabolites are available [33,34]. The most common calibration method used for pharmacokinetic studies is "internal standardization" whereby a precise quantity of reference material is "spiked" into a sample. A requirement needed for internal standards is that their physicochemical characteristics should be identical or similar to those of the analyte of interest during the measurement. Traditionally, stable isotope labeled compounds and structural homologs or analogs have been used as internal standards. When an internal standard is used, quantitation is typically based on a ratio of the analyte to the internal standard, multiplied by the known concentration of the internal standard [7] (Fig. 4). As expected, the more closely the final concentration of the reference material is to the analyte, the more reliable are the results. Strictly speaking, a reference standard is a material that is certified as such by a national or international governing body such as the United States Pharmacopoeia (USP). Therefore, there is a disconnection between the formal lexicon relating formal analytical methodology and what is commonly used in everyday laboratory activities. In this text the use of reference materials and standards will be used interchangeably.

This simple method of quantitation is acceptable if the linearity of the method, that is the response factor or intensity-of-signal per unit concentration, has been demonstrated to be constant over the concentration range of both the analyte and internal standard. While it is often challenging to arrive at an appropriate internal standard, the benefit of this standardization technique is that the internal standard

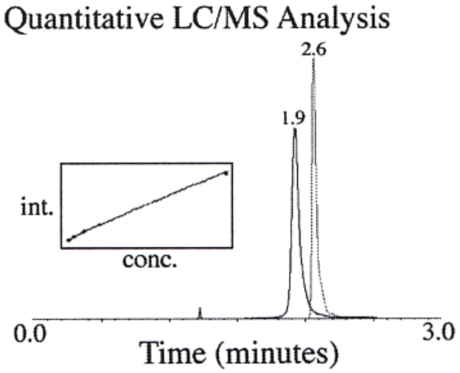

Fig. 4. Quantitation of lidocaine using LC/MS with an internal standard.

can be added to a sample early in the analysis and prior to sample preparation. Therefore, if the internal standard and analyte have similar characteristics, any loss of the analyte during sample preparation will be reflected by a concomitant loss of internal standard but the ratio of their concentrations will still reflect the original quantity of the analyte. The benefits also extend to the analytical system, where the absence of internal standard peaks during a run can lead to accelerated identification of injector errors or other system failures.

Calibration of a method using an external standard has the benefit of allowing use of authentic reference material such as a reference batch developed specifically for analysis of all batches of material generated during the clinical development of a drug. In this case, the reference material is used to generate an external calibration curve for the batch of samples. The frequency of recalibration of the system depends on the stability of the analytical methods, in this case an LC/MS/MS system, and can be as frequent as twice daily. In general, "system-suitability standards" or "QC/QA controls" are also interspersed in the sample batch during analysis. These controls serve as internal process controls that qualify the analytical testing throughout the entire process.

The use of external calibration is predicated on qualification of the method, demonstrating both linearity of the test method (or at least some other response that is highly reproducible) as well as high recovery of analyte during sample preparation. Poor sample recovery will lead to negative systematic errors which can cause significant bias in results, particularly when analyte mass balance (Area Under the Curve, AUC) measurements are made.

7. Mass spectrometry in pharmacokinetic studies

Pharmacokinetic studies are *in vivo* studies whereby an animal is dosed with a compound whose levels and/or its metabolites are measured at extended time intervals, such as 24 hours for caffeine (Fig. 5). These studies provide information about the bioavailability of a drug, or the concentration of a drug in the bloodstream from the time when it is dosed until the time it is either metabolized or cleared from the body. Pharmacokinetic studies are conducted using a variety of administration routes, such as oral, subcutaneous, intraperitoneal, direct injection into a vein, or by intramuscular injection. This data is then used in the drug discovery process, to enable the determination of toxic levels, a therapeutic range, a dosing regimen, and to establish proper animal models for development.

Pharmacokinetic studies are also performed in humans as a necessary part of drug development. It is most often associated with early phase clinical trials (Phase I) where a very small number of patients

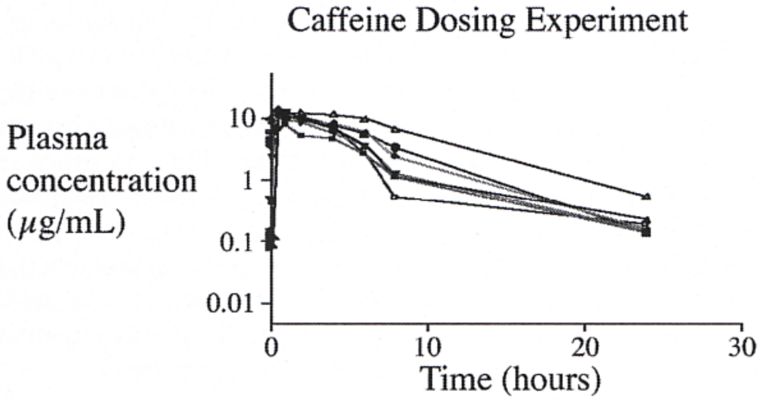
Caffeine Dosing Experiment

Fig. 5. To investigate the pharmacokinetics of caffeine in 7 rats, plasma levels were measured over 24 hours. The drug was dosed at the portal vein and the uptake, and then the gradual clearance of the drug over time can be seen.

are individually studied for drug tolerance. For example, in one study group 20 healthy male volunteers were dosed with one of two oral formulations of 20/12.5 mg tablets of enalapril/hydrochlorothiazide and multiple plasma samples were collected over the following 96 hours. Drug bioavailability was evaluated on the basis of plasma concentrations of enalapril and its main active metabolite, enalaprilat and hydrochlorothiazide by assaying plasma samples for these three compounds using a LC/MS [35].

8. Mass spectrometry in mass balance studies

When investigating the elimination of an administered drug, it is necessary to identify and account for the majority of the drug and its metabolites. This is achieved through mass balance studies, most frequently by administering the drug containing a radioactive isotope label such as ^{14}C or ^{3}H, followed by liquid scintillation counting as well as direct or indirect radioactive monitoring of materials subjected to HPLC fractionation. However, the possibility of high radiation exposure (when handling drugs with long half-lives) and the difficulty of tracing a radiolabeled physiological dose preclude the use of radiolabels in human volunteers. In addition, there are environmental issues relating to radioactive material disposal.

To circumvent these problems, a number of alternative mass spectrometry-based analytical methods have been devised, all with the intent of increasing the sensitivity of the method to reduce the level of radioactive material needed. For instance, isotope-labeling combined with, accelerator mass spectrometry (AMS), chemical reaction interface-mass spectrometry (CRIMS), and continuous flow-isotope ratio mass spectrometry (CF-IRMS) have emerged as alternatives to the more traditional techniques.

In the ultrasensitive technique of AMS, the drug is labeled with either ^{3}H or ^{13}C and the samples converted into solid forms amenable to AMS [36]. The wide dynamic range of AMS allows for a 12-fold-lower concentration limit of detection than liquid scintillation counting, with better accuracy and precision [37,38] and therefore a negligible radiological dose requirement for distribution and mass balance studies (<10 nCi) [38]. However, at present, there are limitations to this technique, including instrument size (typically 4.5 × 6 m) and lack of interface between the HPLC and AMS, resulting in the need to collect samples in an off-line fashion.

HPLC combined with chemical reaction interface-mass spectrometry (HPLC/CRIMS) can be used to detect stable isotope enriched macromolecules. Analytes are first eluted from an HPLC and then dissociated in a microwave reaction chamber. The dissociated analytes are oxidized using SO_2 and the resulting small molecules are detected using a mass spectrometer. The stable-isotope labeled analyte can be distinguished from the matrix carbon by monitoring for the enrichment of $^{13}CO_2$ [39].

Continuous flow-isotope ratio mass spectrometry has been used successfully for the analysis of $^{15}N^{13}C_2$ acetaminophen in urine, feces and bile from humans in mass balance studies [40]. Samples are dried and then converted into a gas in an elemental analyzer. The combustion products, in this case ^{15}N and $^{13}CO_2$, are transferred to a gas chromatograph isotope ratio-mass spectrometer via a continuous stream of helium.

Recently, the combination of LC/MS and chemiluminescent-nitrogen detection (CLND) has been used to study pharmacokinetic properties and the measurement of mass balance *in vivo* [41]. This method was used as an alternative to studies using a radiolabeled tracer, with the only requirement for quantitation being the presence of nitrogen in the parent compound and the metabolites.

9. Improvements in biomolecule characterization using mass spectrometry

Mass spectrometry also plays an important role in both the discovery and characterization of biotherapeutic agents. The high demand to discover new drugs and to identify new therapeutic targets has led researchers to focus on less abundant cellular proteins. This has been greatly facilitated by the significant advances of mass spectrometry in the field of proteomics. Mass spectrometric techniques that have been established in the drug discovery arena, including new combinations of mass analyzers, improved ion sources, and miniaturization of on-line sample methods, are now applied to all parts of drug discovery and development. The role of mass spectrometry in proteomics is constantly expanding. The most commonly used techniques in this area are LC/ESI-MS^2 and MALDI-TOF (Fig. 6), which can be used to characterize protein expression and to identify protein variants and post-translational modifications.

A major consideration and challenge in the development of biopharmaceuticals remains the determination of the distribution and elimination of these drugs. Previously, drugs had a natural, biological base and were either direct hormone replacements (such as insulin or human growth factor), vaccines, or antisera. The administration of these biologicals was often based on arbitrary units of activity, such as international units, which in turn were based on an international reference standard. There was little concern with distribution and metabolism with these biologically-based products. This has changed

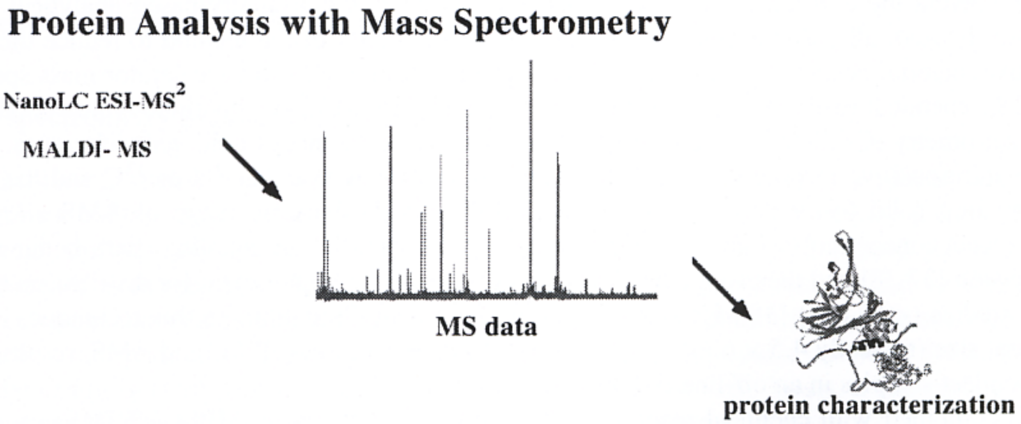

Fig. 6. Mass spectrometry in proteomics is facilitating the discovery of new protein drugs which is providing new challenges for pharmacokinetics.

with the evolution of biopharmaceuticals, as seen with the widespread development of monoclonal antibodies (native or conjugant), fusion proteins, recombinant enzymes, soluble receptors, etc. The latest trend in this area is the development of generic classes of biologicals; this requires defining and grouping these products based on their physical and chemical properties rather than on their apparent activity and manufacturing procedures. The use of LC/MS for both distribution and mass balance studies of these molecules will follow the same principles as those for traditional drugs. Marketing of these new drugs will have the additional challenges of accounting for heterogeneity of the drug candidate, unfamiliar metabolic transformations, and the need to determine what role immunological interaction will have on drug potency and elimination.

10. Summary

A major driving force in the development of mass spectrometry is its utility in the discovery and development of drugs in the pharmaceutical industry. With the increased demand for ADME data earlier in the drug discovery process, the sensitivity and versatility of mass spectrometry has proven it to be a reliable and invaluable tool in the pharmacology laboratory. Improved chromatographic techniques coupled with advances in mass analysis technology have meant that biodistribution and elimination studies can now be developed and implemented in a matter of days rather than weeks or months. Widespread use of electrospray ionization and atmospheric pressure chemical ionization have contributed to ease of use in acquisition of high quality quantitative data necessary for pharmacokinetic studies.

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