10

Microdosing Assessment to Evaluate Pharmacokinetics and Drug Metabolism Using Liquid Chromatography-Tandem Mass Spectrometry Technology

Jinsong Ni and Josh Rowe
Department of Drug Safety Evaluation, Allergan, Inc., Irvine, CA, USA

1. Introduction

Drug development is an expensive, complicated and time-consuming process. According to current estimates, a new drug approval, on average, takes about 10 years and costs around US$1.0 billion. For all approved drugs, an estimated 30% could make a return on the investment. In addition, large pharmaceutical companies will collectively lose about US$70 billion of revenue over the next five years because of patent expiration (Adams & Vu Brantner, 2010). As a result, there is tremendous sense of urgency for the pharmaceutical industry to develop new tools to accelerate the drug development process and to reduce attrition rate on drug candidates. Microdosing is one of these tools.

A "microdose" is defined as a dose less than 1/100 of the test substance calculated to yield a pharmacologic effect, with a maximum dose of 100 μg (Food and Drug Administration, 2006). The concept of microdosing to accelerate drug development was first introduced in 2004 by the Europe Medicines Agency in the position paper on non-clinical safety studies to support clinical trials with a single microdose (Europe Medicines Agency, 2004). The Food and Drug Administration in 2006 issued a guidance document on exploratory Investigative New Drug detailing the regulatory process for microdosing clinical studies (Food and Drug Administration, 2006). In 2008, the Ministry of Health, Labor and Welfare in Japan also issued a guidance on microdose clinical studies as the means to understand the bioavailability and pharmacokinetic profiles of test compounds in human, to evaluate the metabolic profiles of test compounds in human or to obtain the information on the tissue distribution of test compounds in human by using molecular imaging technology (Ministry of Health, Labor and Welfare, 2008). Since the dose is sub-pharmacological, the potential for adverse side effect to a human subject in the clinical study is considered to be minimal. As a result, only an abridged non-clinical package is required to support a microdosing clinical study. This makes the microdosing concept attractive when a speedy decision on drug candidate selection around pharmacokinetics and drug metabolism is critical, particularly when clear decisions cannot be made with in vivo animal and in vitro pre-clinical pharmacokinetic data.

2. Microdosing strategy on human pharmacokinetics, metabolism and drug development

There are many reasons drugs can fail in clinical trials. Although drug attrition due to unfavorable absorption, distribution, metabolism and excretion properties in humans has dropped from 40% in 1991 to 10% in 2000, while drug attrition for efficacy, toxicity and safety has increased to 20-30% during the same period (Frank & Hargreaves, 2003), one could argue that the reason for the insufficient pharmacological effect in vivo might be related to the low concentrations at the target tissues. In addition, accumulation of the drug or its metabolites in organ tissues might lead to unwanted adverse effects in humans (Sugiyama & Yamashita, 2011). Therefore, issues related to lack of efficacy or safety of drug candidates may be attributed not only to the pharmacodynamics, but also to the pharmacokinetics and metabolism of the compound.

Typically, during the pre-clinical stage, a number of *in vitro* models and *in vivo* pharmacokinetic and drug metabolism studies are conducted in experimental animals such as rats, dogs and monkeys. The allometric scaling approach, or physiologically based pharmacokinetic models, have often been used to predict human pharmacokinetics. However, large genetic and species differences in drug metabolism, particularly for drugs with high first-pass metabolism, extra-hepatic metabolism, significant polymorphic metabolism, or that are transporter substrates sometimes make prediction of human pharmacokinetics difficult. As a result, unfavorable pharmacokinetic and metabolism properties such as low oral bioavailability, high clearance, short half-life and extensive drug distribution could lead to unexpected adverse effects or lack of efficacy in clinic trials. Therefore, in these circumstances, and where there is conflicting animal data that make predicting human pharmacokinetics and metabolism difficult, microdosing in the clinic could be useful to quickly obtain such information.

Conceptually, microdosing clinical studies could help (1) choose a drug from a series of candidates with the best human pharmacokinetic and metabolism properties for further development; (2) evaluate if sufficient exposure could be achieved at proposed clinical doses to test pharmacological activity; (3) provide valuable information for formulation optimization; and (4) estimate the amount of active pharmaceutical ingredient to support clinical drug development (Ings, 2009; Garner, 2010). The underlying fundamental assumption, however, for the success of the microdosing concept is that pharmacokinetics are linear from microdose to therapeutic dose in the clinic. In order to accurately characterize microdosing pharmacokinetics and drug metabolism, a highly sensitive and selective bioanalytical method is vital.

3. Analytical challenges: Advantages and disadvantages of liquid chromatography-tandem mass spectrometry, accelerator mass spectrometry and positron emission tomography to support microdosing studies

Microdosing studies for pharmacokinetics and drug metabolism investigations rely on analytical techniques with adequate sensitivity. Liquid chromatography-tandem mass spectrometry (LC-MS/MS), accelerator mass spectrometry (AMS) and positron emission tomography (PET) are currently three major analytical tools to study microdosing

pharmacokinetics and drug metabolism, and each technique has its advantages and disadvantages.

AMS employs an instrument for measuring long-lived radionuclides that occur naturally in our environment. It uses a particle accelerator in conjunction with ion sources, large magnets, and detectors to separate out interferences and count single radionucleotide atoms in the presence of $1x10^{15}$ stable atoms. Because of the powerful magnet employed, AMS typically displays excellent sensitivity with the lower limit of quantitation at femtogram or attogram per mL levels (Lappin *et al.*, 2006). Despite its ultra-low sensitivity, AMS has many limitations. It requires the synthesis of ^{14}C-radiolabeled drug, which can be costly and time-consuming (Wilding & Bell, 2005) and necessitates extra precautions during sample handling and preparation to prevent contamination by extraneous sources of ^{14}C. In addition, AMS measures total ^{14}C radioactivity, that is, drug plus metabolites. In order to accurately measure parent drug concentrations, the parent drug in plasma or blood extracts must first be separated by high performance liquid chromatography (HPLC) with fraction-collection followed by subsequent analysis using AMS (Sandhu *et al.*, 2004). At present, unlike LC-MS/MS, there is no direct interface between HPLC and AMS. Furthermore, AMS methodology requires biological samples to be graphitized prior to analysis, which involves a time-consuming process of sample oxidation followed by reduction. These procedures result in low throughput, large instrument space and high operating cost (Lappin & Garner, 2005).

PET is a relatively new imaging technique that, due to its high sensitivity, has the potential to support microdosing studies. In pharmacokinetic studies using PET imaging technology, a drug labeled with a positron-emitting radiotracer, such as ^{11}C, is administered. Three dimensional images showing the distribution of the radiolabel with spatial resolution of 2-5 mm are then produced. In dynamic PET, the images can be acquired rapidly and the time-course can be followed with temporal resolution of a few seconds. Typically, the radiotracers employed have very high specific activity, which allows for doses of 10 μg or less, consistent with the microdosing concept (Lappin *et.al.*, 2009). However, the short half-life of positron emitting radionucleotides typically limits the duration of these studies and prevents accurate assessment of pharmacokinetics beyond the initial distribution phase. The main advantage of PET compared with other analytical techniques is the ability to quantitatively image drug distribution in the clinic under a microdosing paradigm, gaining insight into concentrations of drug in specific tissues of interest. Another advantage of PET is that it is non-invasive. Although PET is mainly used to study pharmacokinetics of compounds in the target tissues, it could also be used to analyze blood or plasma samples. In this practice, an HPLC with radiodetection is used to separate parent drug from the metabolites, thereby gaining information on the quantities of both parent drug and metabolites. This procedure, however, could add considerably to the complexity of the experiments and can be challenging due to the short half-life of the radionucleotides. Other disadvantages of PET are that the instrument is expensive and only available at certain locations that have the specialized hot chemistry facilities, an on-site cyclotron and a positron emission tomography camera.

LC-MS/MS is widely available in the pharmaceutical industry and academic institutions as a powerful analytical tool to measure drug concentrations. It is easy to use and highly automated. Mass Spectrometry can also be directly linked to a HPLC system to separate

parent drug from the metabolites. In addition, LC-MS/MS has the functionality to characterize drug metabolites. LC-MS/MS is relatively inexpensive compared to AMS or PET, and occupies much smaller footprint in the laboratory setting. At present, however, LC-MS/MS can only achieve lower limits of quantitation at picogram or femtogram per mL level, an order of magnitude less sensitive compared to AMS technique. Nevertheless, LC-MS/MS has gained considerable attention in the recent years as an analytical technique to study microdosing pharmacokinetics and drug metabolism.

4. Brief description on liquid chromatography-tandem mass spectrometry and sample preparation techniques

Since its widespread introduction more than 20 years ago, LC-MS/MS has made an enormous impact on biomedical research, particularly on the study of drug metabolism and pharmacokinetics (Kamel & Prakash, 2006) in the pharmaceutical industry (Lee, 2005). It has been the preferred technique for bioanalysis of small molecules in biological fluids for more than 10 years (Marzo & Dal Bo, 2007). Although considered as a mature technology, rapid and exciting advances continue to occur that promise even greater performance. The inherent sensitivity, selectivity, robustness and speed of LC-MS/MS make it an attractive technique for supporting microdosing studies even though sensitivity is still somewhat of a challenge at the extremely low doses. Advances in mass spectrometry technology, chromatography and sample preparation have made bioanalytical assays with sensitivities at the low pg/mL range more common, if not yet routine. As the technology continues to advance, improvements in sensitivity are likely to continue.

Several excellent books are available that cover LC-MS/MS in general (Niessen, 2006) and application to analysis of small molecule pharmaceuticals in biological matrices (Korfermacher, 2004), and numerous review articles (Xu *et al.*, 2007) cover recent developments for the reader interested in a comprehensive review of LC-MS/MS technology. An excellent review on sample preparation, which is a key factor in bioanalysis, is also available (Wells, 2003). The objective of this brief introduction to LC-MS/MS is to provide an understanding of the technology, as well as its promise and limitations, that would assist a researcher interested in microdosing, but not necessarily familiar with analytical chemistry, with emphasis on aspects and recent developments relevant to microdosing studies.

4.1 Overall liquid chromatography-mass spectrometry analysis

LC-MS/MS is a joining of two techniques: HPLC and mass spectrometry (MS). A schematic diagram of a LC-MS/MS system is shown in Figure 1. In an HPLC system, the components of the sample are separated on the basis of physical properties by distributing into two immiscible phases, the stationary phase (contained in a column) and the mobile phase (which flows through the column). The effluent from the HPLC column is directed to the ionization source of the mass spectrometer, where the analyte(s) is converted into gas phase ions. These ions are then introduced in several stages to the high vacuum region of the mass analyzer, where the ions are separated by mass to charge ratio and measured by the detector. In most applications related to bioanalysis, tandem mass spectrometers are utilized.

4.2 Sample pretreatment

Prior to analysis by LC-MS/MS, complex samples such as plasma are typically pretreated to remove proteins and other potentially interfering materials. Table 1 lists the most common sample preparation techniques along with the key advantages and disadvantages.

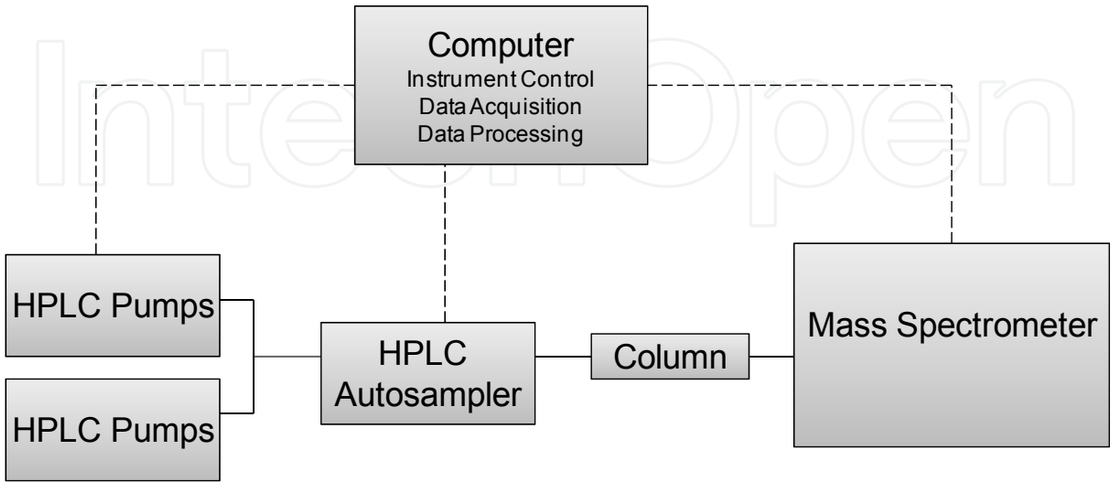


Fig. 1. Schematic diagram of a liquid chromatography-mass spectrometry system.

Typical bioanalytical assays involve preparing calibration standard and control samples, then pre-treating the samples prior to injection and analysis by LC-MS/MS. Thus, the technique can be divided into three parts: sample pretreatment, HPLC and MS/MS.

Technique	Pro	Con
Protein precipitation	• Little or no method development needed • Good recovery of wide variety of analytes (i.e. metabolites)	• Matrix ion suppression
Liquid-liquid extraction	• Provides clean extract • Concentrates sample to improve sensitivity	• Recovery of polar analytes (i.e. metabolites) may be poor • Less amenable to automation and high throughput
Solid phase extraction	• Provides clean extract • Concentrates sample to improve sensitivity • Amenable to high throughput • Large variety of SPE sorbents	• Extensive method development may be needed to optimize • Recovery of metabolites may be poor
Online sample preparation (turbulent flow, monolithic)	• No sample preparation needed • Amenable to automation and high throughput • High sensitivity can be achieved	• More complex valve switching system is needed • Extensive method development needed to achieve high sensitivity • Higher carry-over

Table 1. Summary of pros and cons of selected bioanalytical sample preparation techniques.

Solid phase extraction and liquid-liquid extraction are the two most common techniques applied to microdosing studies, since these techniques allow for concentration of the sample to help achieve high sensitivity. Adequate sample clean-up to remove background interferences and to reduce matrix ion suppression is critical for achieving highly sensitive and robust bioanalytical assays. Note that if analysis of metabolites is desired, a less specific sample preparation procedure (i.e. protein precipitation) may be necessary to ensure recovery of the metabolites.

4.3 High performance liquid chromatography

Selected techniques and advances in high performance liquid chromatography used in bioanalysis, along with key advantages and limitations, are shown in Table 2.

Technique	Pro	Con
Reverse phase liquid chromatography	• Most common mode to connect mass spectrometry • Predictable retention of metabolites • Larger variety of stationary phases available	• Difficult to retain highly polar analytes
Normal phase liquid chromatography	• Optimal mode for chiral separations	• Not amenable to electrospray • Unpredictable retention of metabolites
Ion pairing liquid chromatography	• Provides retention for very polar analytes	• Ion suppression
Very or ultra high pressure liquid chromatography	• Higher chromatographic efficiency improves sensitivity and speed	• Special columns and pumps needed
Fused core particle technology columns	• Higher chromatographic efficiency improves sensitivity and speed	• Ultra high pressure liquid chromatography - like performance without special pumps
Hydrophilic interaction liquid chromatography	• Provides retention and improves sensitivity for very polar analytes	• Unpredictable retention of metabolites
Two dimensional - high performance liquid chromatography	• Cleaner background improves sensitivity	• Special equipment and extensive method development needed

Table 2. Selected advances and techniques of HPLC along with a summary of the pros and cons of each.

Reverse-phase liquid chromatography, wherethe stationary phase is a non-polar material such as C8 or C18 and the mobile phase is a mixture of polar solvents, is by far the most common configuration. Normal phase, ion pairing (Gao *et al.*, 2005), ion exchange and chiral chromatography (Chen *et al.*, 2005) are less common modes used in bioanalysis. Key technology developments within reverse-phase HPLC that improve sensitivity include ultra-high-pressure liquid chromatography (Guillarme *et al.*, 2010) and fused-core particle columns (Song *et al.*, 2009), which improve the efficiency and speed of liquid

chromatographic separations. Improving chromatographic efficiency increases sensitivity in two ways; by producing sharper, more concentrated peaks and by separating matrix components that could cause matrix ion suppression.

4.4 Mass spectrometry

4.4.1 Ionization source

The development of atmospheric pressure ionization, in particular electrospray and atmospheric pressure chemical ionization, was the key development that made the union of liquid chromatography and mass spectrometry successful. In electrospray (Figure 2), the mobile phase effluent is nebulized and a charge of 3-5 kV is applied to the spray needle. In the spray zone, small charged droplets are formed and as the solvent evaporates, the excess charge in the droplets becomes more concentrated and, at some point, the Coulomb repulsion overcomes the competing force of surface tension and causes the droplets to disintegrate and gas phase ions of the analyte(s) are produced. The exact mechanisms of how ions are produced from charged droplets are complex and still a matter of intense research and debate, and several reviews summarize practical implications of recent findings (Cech, 2002; Cole, 2000). Electrospray is capable of ionizing almost any polar analyte molecule, and works especially well with weakly basic or acidic compounds. For less polar or non-polar analytes, atmospheric pressure chemical ionization is often used.

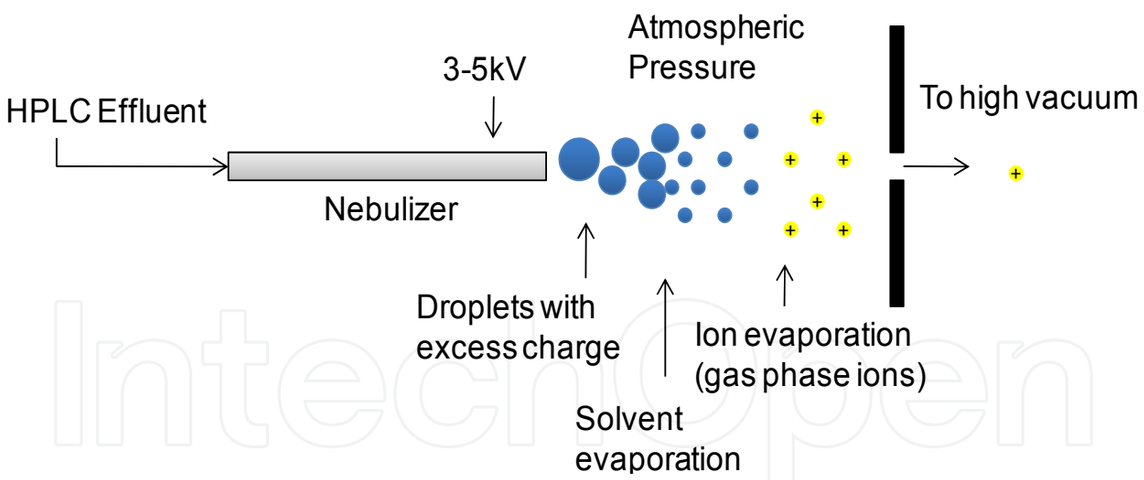


Fig. 2. Electrospray ionization.

In atmospheric pressure chemical ionization (APCI), the mobile phase effluent is almost completely evaporated in a heated quartz tube and a corona discharge reacts with gas molecules from evaporation of the various mobile phase components, which undergo a series of gas phase ion-molecule reactions, especially proton transfer reactions, that eventually result in the production of gas phase ions of the analyte(s). Unlike electrospray, in atmospheric APCI, ionization occurs in the gas phase, which could explain why atmospheric pressure chemical ionization is less susceptible to matrix ion suppression effects.

Both electrospray and atmospheric pressure chemical ionization are "soft" ionization methods, which typically result in protonated molecular ions, $[MH]^+$, in positive mode or deprotonated molecular ions, $[M-H]^-$, in negative mode. In either case, the composition of the mobile phase has a profound influence on ionization (Kostiainen *et al.*, 2009). The choice of composition of the mobile phase is therefore a compromise between its effects on the chromatography and the effects on ionization in the mass spectrometer. The ionization efficiency, and therefore assay sensitivity, is also highly compound dependent.

4.4.2 Mass analyzer types and configurations

There are many different types of mass spectrometers. In a tandem mass spectrometer, two mass analyzers are used to provide an additional dimension of selectivity, where the first mass analyzer selects ions of only the desired mass to charge ratio, which are fragmented and the resulting fragment ions analyzed by the second mass analyzer. Tandem mass spectrometers improve the selectivity and sensitivity for quantitative assays, and greatly expand the capabilities for gaining qualitative information of unknown metabolites.

4.4.2.1 Triple quadropole mass spectrometers for quantitative bioanalysis

LC-MS/MS utilizing a triple quadrupole mass spectrometer operated in multiple reaction monitoring mode is currently the method of choice for quantitative bioanalysis of small molecules. A schematic diagram of a triple quadrupole mass spectrometer is shown in Figure 3.

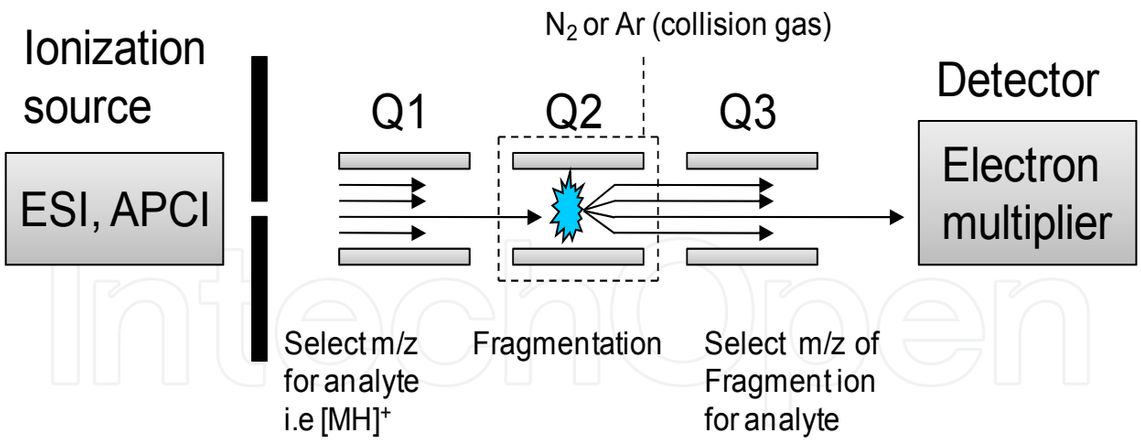


Fig. 3. Schematic diagram of a triple quadrupole mass spectrometer.

The first quadrupole acts as a mass filter to select only ions of a specific mass to charge ratio,typically of the $[MH]^+$ or $[M-H]^-$ ions of the analyte, to enter into the second quadrupole. The second quadrupole is the collision cell, where collision with a gas (N_2 or Ar) causes the ions to fragment through a process known as collision activated dissociation. The resulting fragment ions are transmitted to third quadrupole, where only the fragment ions of the desired mass to charge ratio are allowed to pass and impinge on the detector

(electron multiplier). The two levels of selectivity in the multiple reaction monitoring experiment, combined with the chromatographic separation, provided a very high level of selectivity and are critical to achieving high sensitivity.

4.4.2.2 Mass spectrometers for qualitative analysis

Despite the current predominance of triple quadrupole mass spectrometers in quantitative bioanalysis, other instrument types show promise and may prove to be powerful tools for use in microdosing studies. Several mass spectrometer configurations are available that, in addition to quantifying parent drug and known metabolites, offer the ability to gain information about metabolite pathways even without a priori knowledge of metabolism. These instruments vary widely in their configurations, principles of operation, but can provide structural information on metabolites. Several examples are briefly discussed below.

High resolution mass spectrometers, including time-of-flight (Williamson *et al.*, 2007; Williamson *et al.*, 2008), orbitrap instruments (Zhang *et al.*, 2009; Bateman *et al.*, 2009) and linear ion trap-fourier transform ion cyclotron mass spectrometers (Yamane *et al.*, 2009) provide high selectivity and are able to characterize metabolites.

Ion trap and hybrid triple quadrupole-ion trap mass spectrometers are low resolution instruments that could provide the ability to simultaneously measure and characterize metabolites along with quantitative bioanalysis. The hybrid linear ion trap–triple quadrupole mass spectrometer, or Quadrupole-Trap, by configuring third quadrupole to function either as a quadrupole mass filter or a linear ion trap, combines the features of a triple quadrupole instrument with the features of an ion trap instrument (King & Fernandez-Metzler, 2006). The quadrupole-trap instruments can therefore provide the same sensitivity as a triple quadrupole mass spectrometer and also provide simultaneous qualitative metabolite characterization data, which has allowed these instruments to be used to support microdosing studies.

5. Application of liquid chromatography-tandem mass spectrometry to support microdosing pharmacokinetic studies

LC-MS/MS has been successfully used to investigate the pharmacokinetic linearity of drugs in animals as well as in clinical trials. Balani et al. first reported the evaluation of microdosing to assess pharmacokinetic linearity of fluconazole, tolbutamide and an investigational compound MLNX in rats using LC-MS/MS (Balani *et al.*, 2006). In this study, fluconazole was orally administrated at 0.001, 0.005, 0.05 and 5 mg/kg; tolbutamide at 0.001, 0.002, 0.01, 0.1 and 1 mg/kg; and MLNX at 0.01, 0.1, 1, 10 mg/kg to rats. Because of the low plasma clearance, low volume of distribution, and high oral bioavailability for these compounds, the plasma concentrations in rats declined slowly and were easily quantifiable in 24 hour postdose plasma samples. Thus, the LC-MS/MS sensitivity of 0.1 to 1 nM was adequate to support microdosing studies for these compounds in rats. Both fluconazole and tolbutamide showed linear pharmacokinetics throughout the entire dose range and MLNX showed linear pharmacokinetics between 0.1 and 1 mg/kg, but not to 10 mg/kg.

A more comprehensive study involving five drugs of antipyrine, metoprolol, carbamazepine, digoxin and atenolol from three different classes of the Biopharmaceutical Classifications Systems and with the diverse chemical structures were used as model compounds to evaluate the feasibility and sensitivity requirements of LC-MS/MS as an analytical tool to support microdosing studies (Ni *et al.*, 2008). These five drugs were individually administered orally to rats at 0.167, 1.67, 16.7, 167 or 1670 μg/kg doses, where 1.67 μg/kg was equivalent to the maximal microdose of 100 μg in 60 kg human. The 10,000 fold dose range from 0.167 μg/kg to 1670 μg/kg was designed to evaluate the linearity of pharmacokinetics. Using 100 μl plasma sample aliquots, the lower limits of quantitation for antipyrine (10 pg/ml), carbamazepine (1 pg/ml), metoprolol (5 pg/ml), atenolol (20 pg/ml) and digoxin (5 pg/ml) were achieved. Proportional pharmacokinetics were obtained from 0.167 to 1670 μg/kg for antipyrine and carbamazepine and from 1.67 to 1670 μg/kg for atenolol and digoxin, while metoprolol, which is known to undergo extensive metabolism in rats, exhibited non-proportional pharmacokinetics.

LC-MS/MS technology has also been successfully utilized in support of microdosing clinical studies. A validated assay using LC-MS/MS methodology was developed to support quantitative analysis of fexofenadine in human plasma for microdose and pharmacologic dose clinical trials (Yamane *et al.*, 2007). Calibration standards for microdosing study were prepared in the range from 10 to 1000 pg/ml while calibration standards for pharmacological dosing study were from 1 to 500 ng/ml. The results suggested that it was possible to obtain the plasma drug concentrations at all time points up to 12 hours after microdosing and the linear pharmacokinetic profiles were obtained for fexofenadine between microdose of 100 μg and therapeutic dose of 60 mg (Yamazaki *et.al.*, 2010). Similarly, a sample treatment procedure and LC-MS/MS method for quantitative determination of nicardipine in human plasma were developed for a microdose clinical trial with nicardipine (Yamane *et al.*, 2009). Bioanalytical methods were validated in the calibration ranges from 1 to 500 pg/ml and from 0.2 to 100 ng/ml to support microdosing and pharmacological dosing, respectively. Each method was successfully applied to measure drug concentrations in plasma using LC-MS/MS after administration of 100 μg microdose and 20 mg pharmacological dose to each of six healthy volunteers.

In order to obtain information on absolute oral bioavailability, a technique utilizing simultaneous intravenous microdosing of ^{14}C-labeled drug with oral dosing of non-labeled drug in dogs was exemplified using an investigational compound R-142086 (Miyaji *et al.*, 2009). Plasma concentrations of R-142086 were measured by LC-MS/MS and plasma concentrations of ^{14}C-R-142086 were measured by AMS following R-142086 oral dosing at 1 mg/kg and simultaneous ^{14}C-R-142086 intravenous dosing at 1.5 μg/kg (71.25 nCi/kg). Using this strategy, the oral bioavailability of R-142086 was calculated as 16.1% in dogs. In addition, the correlation between the plasma R-142086 concentration data obtained by AMS and LC-MS/MS was examined at an intravenous dose of 0.3 mg/kg (71.25 nCi/kg). The plasma concentration-time curves for ^{14}C-R-142086 determined by AMS and for R-142086 determined by LC-MS/MS in each dog are compared in Figure 4. Although plasma concentrations of R-142086 determined by LC-MS/MS were approximately 20% higher than those of ^{14}C-R-142086 as determined by AMS, there was excellent correlation (r=0.994) between both concentrations.

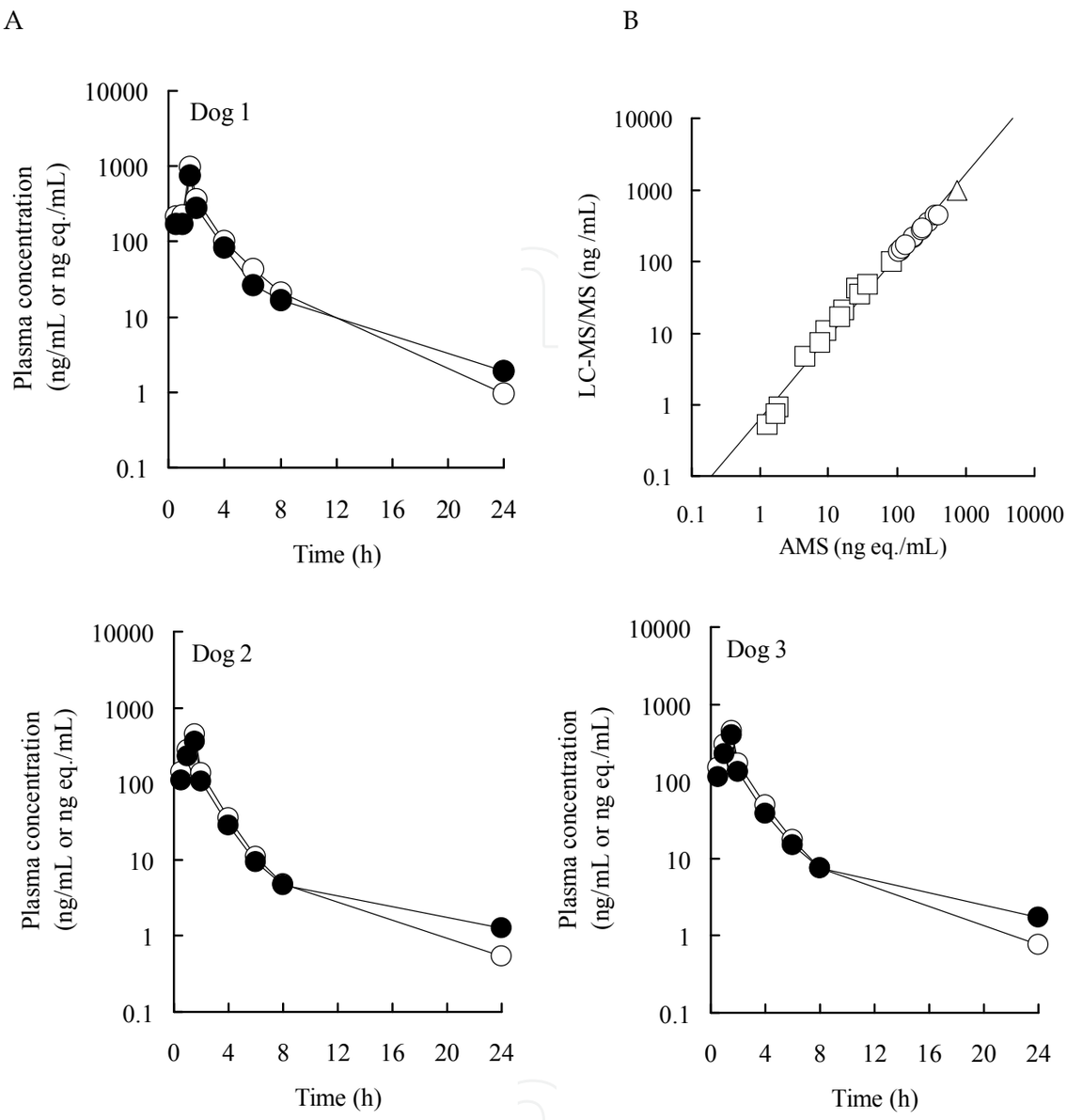


Fig. 4. Correlation between AMS and LC-MS/MS analyses. Panel A: Comparison of plasma concentrations of R-142086 determined by LC-MS/MS (○) versus those of ^{14}C-R-142086 determined by AMS (●) after intravenous administration of ^{14}C-R-142086 at a higher dose (0.3 mg/kg, 71.25 nCi/kg) in each of three dogs. Panel B: Relationship of concentration of R-142086 in all dogs determined by LC-MS/MS and those of ^{14}C-R-142086 determined by AMS after intravenous dosing of a higher dose (0.3 mg/kg, 71.25 nCi/kg). The coefficient of correlation (r) was 0.994. The regression line was y = 1.14 x -0.191.For AMS analysis, the plasma samples were diluted 5-fold (open square), 20-fold (open circle) or 50-fold (open triangle).
(Reprinted with permission from [Miyaji 2009],©2009, The Japanese Society for the Study of Xenobiotics)

6. Application of liquid chromatography-tandem mass spectrometry to support microdosing metabolism studies

LC-MS/MS technology has also been used to characterize and quantify metabolites in microdosing animal and clinical studies. Ni *et al.* (Ni *et al.*, 2008) reported the characterization of carbamazepine metabolites in both *in vitro* liver microsomes and *in vivo* rat at ultra-low concentrations or dose level. Concentrations of 100 nM or 3 nM carbamazepine were incubated in rat liver microsomes, and metabolites were characterized by LC-MS/MS. Incubation concentration at 3 nM was selected because of its close equivalency with plasma C_{max} of carbamazepine at the microdose of 1.67 μg/kg in rats. *In vitro* metabolism data showed the presence of oxidative and conjugated metabolites following incubations at 3 nM and 100 nM. Four metabolites of carbamazepine were detected and characterized in the plasma of rats dosed with 1.67 μg/kg of carbamazepine. The carbamazepine epoxide, among the four metabolites characterized, was the major human circulating metabolite of carbamazepine at the therapeutic doses. Through comparing with carbamazepine metabolism reported in the literature (Lertratanangkoon & Horning, 1982), study results suggested that the metabolic profile *in vivo* at a microdose is, in general, similar to that at therapeutic doses in rats for carbamazepine.

The metabolites of nicardipine were characterized using linear ion trap-fourier transform ion cyclotron resonance mass spectrometry for *in vitro* human liver microsomal incubation with 10 μM nicardipine, where the chemical structures and possible fragmentation patterns for nine metabolites were proposed. These nine metabolites were subsequently monitored and detected in human plasma in a microdosing clinical study (Yamane *et al.*, 2009).

Further evaluation took place on the sensitivity requirement for LC-MS/MS as an analytical tool to characterize metabolites in plasma and urine at microdose level in rats. In addition, the investigation of the proportionality of metabolite exposure from microdose of 1.67 μg/kg to a high dose of 5000 μg/kg was conducted for four model compounds of atorvastatin, ofloxacin, omeprazole and tamoxifen (Ni *et al.*, 2010). For all targeted metabolites based upon literature reports, only a few metabolites including the glucuronide metabolite of ofloxacin, the hydroxylation metabolite of omeprazole and hydration metabolite of tamoxifen were detected by LC-MS/MS in rat plasma following microdosing. The exposure of detected metabolites of omeprazole and tamoxifen appeared to increase in a non-proportional manner with increasing doses. For atorvastatin metabolites, the exposure of atorvastatin lactone increased non-proportionally with increasing doses while the exposure of ortho- and para-hydroxyatorvastatin did show proportional increase (Table 3). Following a single oral microdose or high dose to rats, the exposure of area under the curve of detected metabolites of atorvastatin, omeprazole or tamoxifen did not always display a proportional relationship from a microdose of 1.67 μg/kg to high dose of 5000 μg/kg. Therefore, it was concluded that the exposure of metabolites at the microdose level cannot simply be used to predict their exposure at higher doses.

7. Discussion

Microdosing could provide tremendous value to the drug development, particularly for the evaluation of pharmacokinetics and metabolism properties of compounds. In cases where human pharmacokinetic prediction becomes difficult due to conflicting animal

	Dose	C_{max} (ng/mL)		T_{max} (hour)		$AUC_{0\text{-}tlast}$ (ng*hours/ml)	
	µg/kg	Mean	SD	Mean	SD	Mean	SD
Atorvastatin	1.67	0.158	0.0508	0.556	0.193	0.208	0.0116
	25	0.508	0.193	0.444	0.193	0.426	0.0474
	350	2.68	1.32	0.333	0.00	2.54	0.806
	5000	34.8	17.7	0.555	0.385	36.5	21.0
ortho-Hydroxy atorvastatin	1.67	0.0985	0.124	0.777	0.507	0.0628	0.0485
	25	0.463	0.185	0.333	0.00	0.497	0.249
	350	5.12	2.76	0.665	0.576	6.63	2.89
	5000	50.9	35.5	0.777	0.507	67.4	47.6
para-Hydroxy atorvastatin	1.67	NC	NC	NC	NC	NC	NC
	25	0.0233	0.0133	0.665	0.576	0.0208	0.0111
	350	0.241	0.149	0.665	0.576	0.258	0.110
	5000	2.13	1.33	0.888	0.508	2.18	1.25
Lactone of atorvastatin	1.67	2.33	1.20	0.999	0.332	2.82	1.39
	25	4.08	0.427	0.444	0.193	5.33	0.648
	350	7.58	1.06	0.444	0.193	11.4	0.862
	5000	23.8	10.7	0.556	0.193	27.3	8.90

NC: not calculable

Table 3. The pharmacokinetic parameters of atorvastatin and its metabolites following a single oral dose to male Sprague-Dawley rats.

pharmacokinetic data, a microdose clinical study could help to determine if a drug has desirable pharmacokinetic properties that warrant further development. Highly sensitive and selective analytical tools such as LC-MS/MS and AMS have made it possible to characterize pharmacokinetics and metabolism of drug candidates at the microdose level. In the past several years, a lot of attention has been focusing on evaluating pharmacokinetic linearity of drug molecules from microdose to therapeutic doses in animals as well as in the clinic. It has been summarized that out of 26 drugs examined so far, 21 compounds, approximately 80%, have demonstrated linear pharmacokinetics between microdose to therapeutic doses (Lappin, 2010). For compounds which have failed to demonstrate pharmacokinetic linearity, there are a number of possible causes. For instance, drug candidates with saturable first-pass metabolism or saturable elimination at therapeutic doses would often result in under-prediction of exposure based upon microdose data. On the other hand, drug candidates with poor solubility would produce over-prediction of exposure based upon microdose data. Therefore, the understanding of physical and chemical properties of compounds and of enzyme kinetics *in vitro* could be very important prior to the commitment to a microdosing study. In practice, if there is a concern that

compound would display nonlinear pharmacokinetics from a microdose to therapeutic doses in the clinic, a pharmacokinetic study could be performed to exam pharmacokinetic linearity in a relevant animal species.

Microdosing could also be very valuable to obtain an earlier understanding of metabolism of drug candidates in the clinic. This has become more important with the release of the recent guidance document "safety testing of drug metabolites" by the FDA (Food and Drug Administration, 2008). The guidance document stated that metabolites found only in human plasma or metabolites present at disproportionately higher levels in humans than in any of the animal test species should be considered for safety assessment. In particular, human metabolites that are formed at greater than 10% of parent drug systemic exposure at steady state can raise a safety concern. As a result, it has become very important to obtain human drug metabolism information as early as possible in the drug development stage, and to compare with preclinical metabolism data. Although microdosing studies in the clinic would be ideal to understand the metabolism of drug candidates early on, caution must be exercised to extrapolate the learning from microdose to therapeutic doses. This could be particularly true for compounds where metabolism enzymes have low substrate capacities and can be saturated at low substrate concentrations. For example, the P450 isoform CYP2D6 is a low capacity enzyme and if a novel drug candidate is metabolized primarily through the CYP2D6 pathway, the metabolic pathway of this drug candidate at microdose may be different from that at therapeutic doses. The levels of a particular metabolite relative to parent drug, as the means to identify major metabolites, may be different from microdose to therapeutic doses. In this case, a thorough understanding of metabolic pathways with animals and *in vitro* would be very useful to assess reliability of drug metabolism prediction from microdose to therapeutic doses. In addition, a microdose clinical study would help to identify if human-specific metabolites are present so that a thorough evaluation of these human unique metabolites could take place in the relevant toxicological species.

8. Conclusion

The highly selective and sensitive technology of LC-MS/MS has become a powerful analytical tool that provides the opportunity to understand clinical pharmacokinetics of compounds using the microdosing approach. Furthermore, LC-MS/MS has demonstrated its usefulness for detecting and characterizing metabolites in plasma and urine at microdose level. Although the extrapolation of parent drug exposure from a microdose to a therapeutic dose appears to be promising, such extrapolation for metabolites may be compound and/or metabolite dependent. Extrapolation of metabolite exposure would particularly be difficult if there is involvement of enzyme inhibition, induction or saturation.

9. References

Adams, C. & Vu Brantner.V. (2010). Spending on New Drug Development. *Health Economics*, Vol.19, No.2, pp. 130-141.

Balani, S. K., Nagaraja, N. V., Qian, M. G., Costa, A. O., Daniels, J. S., Yang, H., Shimoga, P. R., Wu, J. T., Gan, L. S., Lee, F. W. & Miwa, G. T. (2006). Evaluation of Microdosing to Assess Pharmacokinetic Linearity in Rats Using Liquid Chromatography – Tandem Mass Spectrometry. *Drug Metabolism and Disposition*, Vol.34, No.3, pp. 384-388.

Bateman, K. P., Kellman, M., Muenster, H., Papp, R. & Taylor, L. (2009). Quantitative-Qualitative Data Acquisition Using a Benchtop Orbitrap Mass Spectrometer. *Journal of American Society on Mass Spectrometry*, Vol. 20, No.8, pp. 1441-1450.

Cech, N. B. & Enke, C. J. (2002). Practical Implications of Some Recent Studies in Electrospray Ionization Fundamentals. *Mass Spectrometry Reviews*, Vol. 20, No.6, pp. 362-387.

Chen, J., Korfermacher, W. A. & Hsieh, Y. (2005). Chiral Liquid Chromatography-Tandem Mass Spectrometric Methods for Stereoisomeric Pharmaceutical Determinations. *Journal of Chromatography B* Vol. 820, No.1, pp. 1-8.

Cole, R. B. (2000). Some Tenets Pertaining to Electrospray Ionization Mass Spectrometry. *Journal of Mass Spectrometry*, Vol. 35, pp. 763–772.

European Medicines Evaluation Agency (2004). European Agency for Evaluation of Medicines for Human Use. A position paper on the nonclinical safety studies to support clinical trials with a single microdose. CPMP/SWP2599/02/Rev 1. http://www.emea.eu.int/pdfs/human/swp/259902en.pdf.

Food and Drug Administration. (2006). Guidance for Industry, Investigators, and Reviewers. Exploratory IND Studies. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM078933.pdf.

Food and Drug Administration. (2008). Safety Testing of Drug Metabolites. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM079266.pdf.

Frank, R. & Hargreaves, R. (2003). Clinical biomarkers in drug discovery and development, *Nature Reviews Drug Discovery*, No.2, pp. 566-580.

Gao, S., Zhang, Z. & Karnes, H. T. (2005). Sensitivity Enhancement in Liquid Chromatography/Atmospheric Pressure Ionization Mass Spectrometry Using Derivitization and Mobile Phase Additives. *Journal of Chromatography B,* Vol. 825, No.2, pp. 89-110.

Garner, R.C. (2010). Practical experience of using human microdosing with AMS analysis to obtain early human drug metabolism and PK data, *Bioanalysis*, Vol.2, No.3, pp. 429-440.

Guillarme, D., Schappler, J., Rudaz, S. & Veuthey, J. (2010). Coupling Ultra-High-Pressure Liquid Chromatography with Mass Spectrometry. *Trends in Analytical Chemistry* Vol.29, No.1, pp. 15-27.

Ings, R. (2009). Microdosing: a valuable tool for accelerating drug development and the role of bioanalytical methods in meeting the challenge, *Bioanalysis,* Vol.1, No.7, pp. 1293-1305.

Kamel, A. & Prakash, C. (2006). High Performance Liquid Chromatography/Atmospheric Pressure Ionization /Tandem Mass Spectrometry (HPLC/API/MS/MS) in Drug Metabolism and Toxicology. *Current Drug Metabolism*, Vol. 7, No.8, pp. 837-852.

King, R. & Fernandez-Metzler, C. (2006). The Use of Qtrap Technology in Drug Metabolism. *Current Drug Metabolism*, Vol. 7, No.5, pp. 541-545.

Korfermacher, W. A. (2004). Using Mass Spectrometry for Drug Metabolism Studies. CRC Press, Boco Raton.

Kostiainen, R. & Kauppila, T. J. (2009). Effect of Effluent on the Ionization Process in Chromatography-Mass Spectrometry. *Journal of Chromatography A*, Vol. 1216, No.4, pp. 685-699.

Lappin, G. (2010). Microdosing: Current and the Future. *Bioanalysis* Vol.2, No.3, pp. 509-517.

Lappin, G. & Garner, R. C. (2005). The Use of Accelerator Mass Spectrometry to Obtain Early Human ADME/PK Data. *Expert Opinion on Drug Metabolism & Toxicology*, Vol. 1, No.1, pp. 23-31.

Lappin, G., Kuhnz, W., Jochemsenm R., Kneer, J., Chaudhary, A., Oosterhuis, B., Drijfhout, W. J., Rowland, M. & Garner, R. C. (2006). Use of Microdosing to Predict Pharmacokinetics at the Therapeutic Dose: Experience with Five Drugs. *Clinical Pharmacology & Therapeutics*, Vol.80, No.3, pp. 203-215.

Lappin, G., Wagner, C. C. & Merbel, N. (2009). New Ultrasensitive Detection Technologies for Use in Microdosing Studies. *Bioanalysis*. Vol.1, No.2, pp. 357-366.

Lee, H. (2005). Pharmaceutical Applications of Liquid Chromatography Coupled with Mass Spectrometry (LC/MS). *Journal of Liquid Chromatography & Related Technologies*, Vol.28, No.7-8, pp. 1161-1202.

Lertratanangkoon, K., & Horning, M. G. (1982). Metabolism of Carbamazepine. *Drug Metabolism and Disposition*, Vol. 10, No.1, pp. 1-10.

Marzo, A. & Dal Bo, L. (2007). Tandem Mass Spectrometry (LC-MS-MS): a Predominant Role in Bioassays for Pharmacokinetic Studies. *Arzneimittel Forschung*, Vol.57, No.2, pp. 122-128.

Ministry of Health, Labor and Welfare. Guidance (2008). Microdose clinical studies, Ministry of Health, Labor and Welfare, Pharmaceutical and Medical Safety Bureau, Tokyo, Japan.

Miyaji, Y., Ishizuka, T., Kawai, K., Hamabe, Y., Miyaoka, T., OH-Hara, T., Ikeda, T. & Kurihara, A. (2009). Use of an Intravenous Microdose of ^{14}C-Labeled Drug and Accelerator Mass Spectrometry to Measure Absolute Oral Bioavailability in Dogs; Cross-Comparison of Assay Methods by Accelerator Mass Spectrometry and Liquid Chromatography-Tandem Mass Spectrometry. *Drug Metabolism and Pharmacokinetics,* Vol. 24, No.2, pp. 130-138.

Ni, J., Ouyang, H., Aiello, M., Seto, C., Borbridge, L., Sakuma, T., Ellis, R., Welty, D. & Acheampong, A. (2008). Microdosing Assessment to Evaluate Pharmacokinetics and Drug Metabolism in Rats Using Liquid Chromatography-Tandem Mass Spectrometry. *Pharmaceutical Research*, Vol. 25, No.7, pp. 1572-1582.

Ni, J., Ouyang, H., Seto, C., Sakuma, T., Ellis, R., Rowe, J., Acheampong, A., Welty, D. & Szekely-Klepser, G. (2010). Sensitivity and Proportionality Assessment of

Metabolites from Microdose to High Dose in Rats Using LC-MS/MS. *Bioanalysis,* Vol.2, No.3, pp. 407-419.

Niessen, W. M. A. (2006). Liquid Chromatography-Mass Spectrometry, third ed., CRC Press, Boco Raton.

Sandhu, P., Vogel, J. S., Rose, M. J., Ubick, E. A., Brunner, J. E., Wallace, M. A., Adelsberger, J. K., Baker, M. P., Henderson, P. T., Pearson, P. G. & Baillie, T. A. (2004). Evaluation of Microdosing Strategies for Studies in Preclinical Drug Development: Demonstration of Linear Pharmacokinetics in Dogs of a Nucleoside Analog Over a 50-Fold Dose Range. *Drug Metabolism and Disposition*, Vol. 32, No.11, pp. 1254-1259.

Song, W., Pabbisetty, D., Groeber, E. A., Steenwyk, R. C. & Fast, D. M. (2009). Comparison of Fused-Core and Conventional Particle Size Columns by LC-MS/MS and UV: Application to Pharmacokinetic Study. *Journal of Pharmaceutical and Biomedical Analysis*, Vol. 50, No.3, pp. 491-500.

Sugiyama, Y. & Yamashita, S. (2011). Impact of microdosing clinical study – why necessary and how useful ? *Advanced Drug Delivery Reviews*, Vol.63, No.7, pp. 494-502.

Wells, D. A. (2003). High Throughput Bioanalytical Sample Preparation. Methods and Automation Strategies. Elsevier Science, Amsterdam, The Netherlands.

Wilding, I. R. & Bell, J. A. (2005). Improved Early Clinical Development Through Human Microdosing Studies. *Drug Discoveries & Therapeutics*, Vol. 10, No.13, pp. 890-894.

Williamson, L. N. & Bartlett, M. G. (2007). Quantitative Liquid Chromatography/Time-of-Flight Mass Spectrometry. *Biomedical Chromatography* Vol. 21, No.6, pp. 567-576.

Williamson, L. N., Zhang, G., Alvin, T. V. & Bartlett, M. G. (2008). Comparison of Time-of-Flight Mass Spectrometry to Triple Quadropole Tandem Mass Spectrometry for Quantitative Bioanalysis: Application to Antipsychotics. *Journal of Liquid Chromatography & Related Technologies*, Vol. 31, No.18, pp. 2737-2751.

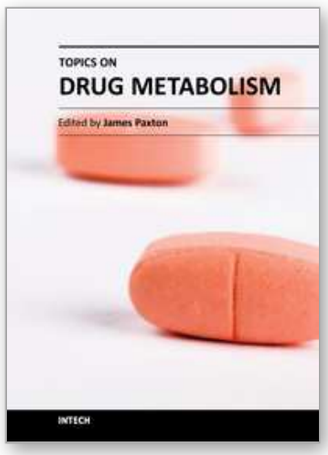
Xu, R. N., Fan, L., Rieser, M. & El-Shourbagy, T. (2007). Recent Advances in High Throughput Quantitative Bioanalysis by LC-MS/MS. *Journal of Pharmaceutical and Biomedical Analysis*, Vol. 44, No.2, pp. 342-355.

Yamane, N., Takami, T., Tozuka, Z., Sugiyama, Y., Yamazaki, A. & Kumagai, Y. (2009). Microdose Clinical Trial: Quantitative Determination of Nicardipine and Prediction of Metabolites in Human Plasma. *Drug Metabolism and Pharmacokinetics*, Vol. 24, No.4, pp. 389-403.

Yamane, N., Tozuka. Z., Sugiyama, Y., Tanimoto, T., Yamazaki, A. & Kumagai, Y. (2007). Microdose Clinical Trial: Quantitative Determination of Fexofenadine in Human Plasma Using Liquid Chromatography/Electrospray Ionization Tandem Mass Spectrometry. *Journal of Chromatography B*, Vol. 858, No.1-2, pp. 118-128.

A. Yamazaki, Y. Kumagai, N. Yamane, Z. Tozuka, Y. Sugiyama, T. Fujita, S. Yokota & M. Maeda. (2010) "Microdose study of a P-glycoprotein substrate, fexofenadine, using a non-radioisotope-labelled drug and LC/MS/MS", *Journal of Clinical Pharmacy and Therapeutics*, Vol. 35, pp. 169-175.

Zhang, N. R., Yu, S., Tiller, P., Yeh, S., Mahan, E. & Emary, W. B. (2009). Quantitation of Small Molecules Using High-Resolution Accurate Mass Spectrometers-a Different Approach for Analysis of Biological Samples. *Rapid Communications in Mass Spectrometry* Vol.23, No.7, pp. 1085-1094.



Topics on Drug Metabolism

Edited by Dr. James Paxton

ISBN 978-953-51-0099-7
Hard cover, 294 pages
Publisher InTech
Published online 22, February, 2012
Published in print edition February, 2012

In order to avoid late-stage drug failure due to factors such as undesirable metabolic instability, toxic metabolites, drug-drug interactions, and polymorphic metabolism, an enormous amount of effort has been expended by both the pharmaceutical industry and academia towards developing more powerful techniques and screening assays to identify the metabolic profiles and enzymes involved in drug metabolism. This book presents some in-depth reviews of selected topics in drug metabolism. Among the key topics covered are: the interplay between drug transport and metabolism in oral bioavailability; the influence of genetic and epigenetic factors on drug metabolism; impact of disease on transport and metabolism; and the use of novel microdosing techniques and novel LC/MS and genomic technologies to predict the metabolic parameters and profiles of potential new drug candidates.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Jinsong Ni and Josh Rowe (2012). Microdosing Assessment to Evaluate Pharmacokinetics and Drug Metabolism Using Liquid Chromatography-Tandem Mass Spectrometry Technology, Topics on Drug Metabolism, Dr. James Paxton (Ed.), ISBN: 978-953-51-0099-7, InTech, Available from: http://www.intechopen.com/books/topics-on-drug-metabolism/microdosing-assessment-to-evaluate-pharmacokinetics-and-drug-metabolism-using-lc-mc-ms-technology

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen