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Research Paper

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

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Purpose. To evaluate the sensitivity requirement for LC-MS/MS as an analytical tool to support human microdosing study with sub-pharmacological dose, investigate proportionality of pharmacokinetics from the microdose to therapeutic human equivalent doses in rats and characterize circulating metabolites in rats administered with the microdose.
Materials and Methods. Five drugs of antipyrine, metoprolol, carbamazepine, digoxin and atenolol were administered orally to male Sprague–Dawley rats at 0.167, 1.67, 16.7, 167 and 1,670 μg/kg doses. Plasma samples were extracted using either solid phase extraction or liquid–liquid extraction, and analyzed using LC-MS/MS.
Results. Using 100 μl of plasma sample, the lower limit 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 using an API 5000™. Proportional pharmacokinetics were observed from 0.167 μg/kg to 1,670 μg/kg for antipyrine and carbamazepine and from 1.67 to 1,670 μg/kg for atenolol and digoxin, while metoprolol exhibited a non-proportional pharmacokinetics relationship. Several metabolites of carbamazepine were characterized in plasma from rats dosed at 1.67 μg/kg using LC-MS/MS.
Conclusions. This study has shown the promise of sensitive LC-MS/MS method to support microdose pharmacokinetics and drug metabolism studies in human.

KEY WORDS: LC-MS/MS; metabolism; microdosing; pharmacokinetics; rats.

INTRODUCTION

In order to obtain human pharmacokinetics and drug metabolism (PKDM) information as early as possible, a new experimental approach has been introduced prior to conducting phase I clinical trials, referred to as human microdose pharmacokinetics (PK), to accelerate and optimize drug development (1,2). Microdosing studies involve the administration of sub-pharmacological doses to human subjects to evaluate the pharmacokinetics and metabolism of a drug candidate. A microdose is defined as less than 1/100th of the dose of a test substance calculated (based on animal data) to yield a pharmacologic effect of the test substance with a maximum dose of ≤100μg (1). The data from human PK microdosing studies could provide several benefits in drug development (3,4): (a) assist in selecting drug candidates for clinical trials, (b) determine the first dose for the subsequent phase I study, (c) establish the likely pharmacological dose and (d) estimate the required amount of active pharmaceutical ingredient for further clinical trials. Drug candidates, therefore, can be evaluated in exploratory IND studies to accelerate the selection of promising candidates for further drug development. In addition, the results from microdosing studies could be used to establish whether the metabolic pathways observed in human *in vivo* are also seen in animal species *in vivo*. This would help to further justify the selection of best species for long-term toxicological evaluation in the absence of human mass balance study with radiolabeled material (5,6).

Microdosing studies may not predict the pharmacokinetic behavior of the drug at clinical doses since it is not known whether or not dose proportionality is maintained between a microdose and a clinical dose. Non-proportionality of pharmacokinetics could be induced when absorption is dose-dependent or when binding, metabolism or elimination becomes saturated. Therefore, it is important to demonstrate proportional pharmacokinetics between the microdose and the predicted therapeutically equivalent dose in an appropriate preclinical species. Combined with *in vitro* and *in vivo* metabolism comparisons between animals and human, the human microdosing data could then be utilized to predict human pharmacokinetics at the therapeutic dose. Such a strategy has been successfully used to examine marketed drugs and new chemical entities (7,8). In addition, it is important to compare *in vivo* metabolism between animal

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species and human to evaluate similarities and differences in the *in vivo* metabolite profiles. If unique metabolites are found in human, it may be necessary to test these unique metabolites *in vitro* and/or in relevant toxicological species *in vivo* (9,10). Therefore, it is desirable to obtain *in vivo* human metabolism information early in the drug development process.

Microdosing studies for PKDM investigations rely on an analytical technique with adequate sensitivity. Currently, accelerator mass spectrometry (AMS) is the technology of choice due to its excellent sensitivity with limits of quantitation at the femtogram or attogram per ml level (7). However, AMS has its limitations. Drug concentration measurement using AMS requires the synthesis of ^{14}C-radiolabeled drug, which can be costly and time-consuming (10) and necessitate extra precautions 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 concentration, the parent drug in plasma extracts must first be separated by high performance liquid chromatography (HPLC) with fraction-collection followed by subsequent analysis by AMS (8). 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 relatively low throughput and high operating cost when performing sample analysis by AMS (6).

With the ever increasing sensitivity of liquid chromatography-tandem mass spectrometry (LC-MS/MS) instruments, there is growing interest in the ability of LC-MS/MS to support human microdosing studies. In a feasibility study, Balani *et al.* demonstrated that pharmacokinetics of the known drugs fluconazole and tolbutamide and an investigational compound could be readily characterized in rats at a microdose of 1 μg/kg using LC-MS/MS (11). Another recent study used LC-MS/MS to report the pharmacokinetics of an experimental agent following a microdose in cynomolgus monkeys (12). Good proportionality was observed in plasma pharmacokinetics for oral doses from 0.5 μg/kg (microdose) to 10 mg/kg (therapeutic dose) of the test agent. In both studies, it was concluded that LC-MS/MS assays would have adequate sensitivity in supporting human microdosing studies of these compounds.

It was our interest to further evaluate the sensitivity requirement of LC-MS/MS for microdosing PK studies in rats, to investigate the proportionality of pharmacokinetics from a microdose to the equivalent of a therapeutic human dose in rats, and to evaluate the use of a hybrid triple quadrupole linear ion trap instrument for the characterization of metabolites to support human microdosing studies. The pharmacokinetics of chemical entities could be affected by a variety of parameters such as permeability, solubility, molecular size, metabolic stability and active transport processes. The Biopharmaceutical Classifications System (BCS) classifies compounds according to their permeability and solubility as class I-IV (13). In this study, we selected five drugs from three different classes of the BCS with diverse chemical structures (Fig. 1 and Table I). The five compounds were antipyrine and metoprolol (Class I), carbamazepine and digoxin (Class II) and atenolol (Class III). Since BCS class IV compounds have low permeability and low solubility, they pose great challenges to the drug development process and therefore BCS class IV compounds were not selected for this study. Moreover, the selected compounds also cover a broad range of molecular weight (188–780), cLogP (−0.11–1.98), and solubility (0.015–>1000 mg/ml). In terms of biopharmaceutical properties, antipyrine has a low hepatic extraction ratio and metoprolol has a high hepatic extraction ratio (14). The selected compounds also cover a range of oral bioavailability (carbamezapine > atenolol> metoprolol) (15,16). Digoxin is a well-known P-glycoprotein inhibitor and exhibits variable bioavailability in human individuals (17). These diverse compounds were selected to evaluate both the utility of LC-MS/MS for the analytical support of microdosing studies, as well as the proportionality of pharmacokinetics with respect to dose.

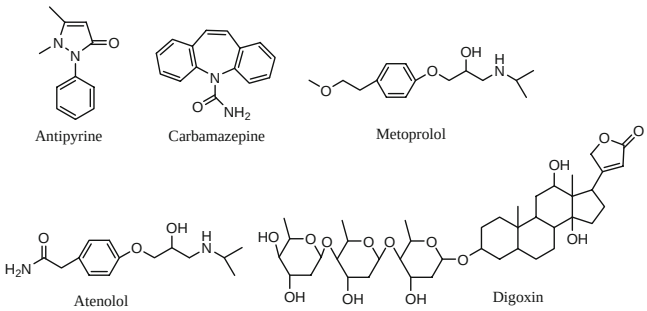

Fig. 1. The chemical structures of antipyrine, carbamazepine, metoprolol, atenolol, and digoxin.

MATERIALS AND METHODS

Drugs and Reagents

Antipyrine (Lot no. G), digoxin (Lot no. O0B096), metoprolol (Lot no. F), carbamazepine (Lot no. K0E209) and atenolol (Lot no. H1C320) were obtained from US Pharmacopeia (Rockville, MD, USA). The internal standards of anitpyrine-d_3, carbamazepine-d_{10} and atenolol-d_7 were obtained from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). Oleandrin and propanolol, internal standards of digoxin and metoprolol, were purchased from ChromaDex (Santa Ann, CA) and Sigma-Aldrich (St. Louis, MO), respectively. Chemicals for HPLC analyses were at least of HPLC grade. All other reagents and solvents were procured from commercial sources in the highest grade. Rat liver microsomes (Lot no. 0510153) were obtained from XenoTech LLC (Lenexa, KS). NADPH regenerating system (Solution A, Lot no. 64735; Solution B, Lot no. 64734) and UDPGA (Solution A, Lot no. 64196; Solution B, Lot no. 63034) were purchased from BD Biosciences (Bedford, MA). EDTA-treated rat plasma was obtained from Bioreclamation Inc. (East Meadow, NY).

Animals

Sixty-six jugular vein-cannulated male Sprague–Dawley rats (three rats per dose per test article) were used in this study. This study complied with all requirements of the US Department of Agriculture (USDA), and all regulations

Table I The Physicochemical Properties of Antipyrine, Carbamazepine, Metoprolol, Atenolol, and Digoxin

Compound	BCS Class	MW	ClogP	Aqueous Solubility (mg/ml)	Note
Antipyrine	I	188	0.38	>1,000	Low hepatic extraction
Carbamazepine	II	236	1.98	0.11	High oral bioavailability
Metoprolol	I	267	1.35	>1,000	High hepatic extraction
Atenolol	III	266	−0.11	26.5	Medium bioavailability
Digoxin	II	780	1.62	0.015	Variable oral absorption

issued by the USDA implementing the Animal Welfare Act, 9 CFR, Parts 1, 2, and 3. The animal procedures also complied with the applicable Allergan standard operating procedures (SOPs). Each animal was identified with an individually numbered ear tag. At the time of dose administration, the animals weighed 260–360 g. Animals were individually housed in suspended, stainless steel wire-mesh cages and were used the second day upon arrival at Allergan. The animals were fasted overnight until four hours post dose and then were fed with certified Rodent Diet *ad libitum*. Upon completion of the in-life portion of the study, animals were sacrificed by overdose of isoflurane anesthesia.

Study Design for Metabolism and Pharmacokinetic Studies

In Vivo Pharmacokinetic Studies

Antipyrine and metoprolol formulations were prepared in pure de-ionized water. Carbamazepine, digoxin, and atenolol formulations were prepared in 40% propylene glycol/10% ethanol/50% water. The test articles were administered to Sprague–Dawley rats (three rats per dose per test article) via oral gavage. Atenolol, metoprolol, and digoxin were administered at 1.67, 16.7, 167, or 1,670 μg/kg. Antipyrine and carbamazepine were administered at 0.167, 1.67, 16.7, 167, or 1,670 μg/kg. Blood samples were collected at the following post-dose time points: antipyrine (15, 30 min, 1, 2, 3, 4, 6, and 8 h), carbamazepine (10, 20, 30 min, 1, 2, 4, 6, and 8 h), metoprolol (5, 10, 20, 40 min, 1, 2, 3, and 4 h), atenolol (1, 2, 3, 4, 6, 8, 10 and 12 h), and digoxin (1, 2, 4, 6, 8, 10, 12 and 24 h). A volume of blood of 0.25 ml was drawn from each animal at each sampling time. Plasma was then separated by centrifugation at 2,500 rpm at 4°C for 15 min. Maximum observed plasma concentration (C_{max}), the time at which C_{max} occurs (T_{max}), the area under the concentration-time curve through the last quantifiable sampling time ($AUC_{0\text{-tlast}}$), and apparent oral half-life ($t_{1/2}$) were calculated for each compound at each dosage using Watson software (version 6.3, Thermo Electron, Waltham, MA).

In Vitro Rat Liver Microsome Incubation

Rat liver microsomes at 1 mg/ml of total microsomal protein in 100 mM potassium phosphate buffer (pH 7.4) were incubated with carbamazepine at concentrations of 3 and 100 μM at 37°C in the presence of NADPH or UDPGA regenerating system, respectively. Sample of 0.2 ml at 0, 60 and 120 min were removed from the incubations and precipitated with 0.8 ml of acetonitrile for the UDPGA samples. For the NADPH samples, the incubations were stopped after 20 min and precipitated with an equal volume of acetonitrile. The mixtures were then centrifuged at 1,500×*g* for 10 min and analyzed by LC-MS/MS.

Analytical Techniques

Standards and Quality Controls in Plasma

Separate stock solutions were prepared for each analyte at a concentration of 500 μg/ml in methanol and stored at −20°C. Working standard solutions of each analyte were prepared by diluting stock solutions in 50:50 methanol/water to concentrations of 0.01 ng/ml to 10 μg/ml and stored at 4°C. Calibration standards were prepared in duplicate at concentrations of 1 pg/ml to 500 ng/ml by adding 10 μl of working solution to 0.1 ml plasma. Individual internal standard (IS) stock solutions were prepared at a concentration of 500 μg/ml in methanol and stored at −20°C. The internal standard spiking solutions were prepared at a concentration of 25 ng/ml in 50:50 methanol/water and stored at 4°C.

Plasma Sample Preparation

Both solid-phase and liquid–liquid extractions were utilized for plasma sample preparation to support the pharmacokinetic studies. All methods consisted of 0.1 ml rat plasma spiked with 0.01 ml of appropriate internal standard. The extraction method for antipyrine utilized solid-phase extraction (SPE), which was modified from the procedure described by Coolen *et al.* (18). Prior to extraction, 0.2 ml of water was added to each sample. The solid phase extraction (SPE) was conducted using a Tomtec Quadra 96 system (Hamdon, CT) with Isolute C18 SPE extraction cartridges (Bellafonte, PA). The cartridge was conditioned with 0.3 ml methanol followed by 0.9 ml water. After loading the diluted sample, the cartridge was washed with 0.3 ml of 25 mM ammonium acetate. After drying for 0.5 min, the analytes were eluted with 0.3 ml methanol. Sample eluent was dried with nitrogen using a Zymark Turbovap 96 (Hopkinton, MA) at 40°C. Prior to injection on LC-MS/MS, the samples were reconstituted with 0.1 ml of 90:10 acetonitrile/water with 0.1% formic acid. The extraction method for carbamazepine utilized liquid-liquid extraction (LLE) with ethyl acetate. The extraction method was modified from the procedure described by Zhu *et al.* (19). Following sample dilution with 0.1 ml water, 2 ml of ethyl acetate was added to each sample and then shaken by vortex for 1 min. Samples were centrifuged at ~3,000 rpm for 5 min before the organic layer

>was transferred to another sample tube. Following evaporation with nitrogen using a Zymark Turbovap (Hopkinton, MA) at a temperature setting at 40°C, the samples were reconstituted with 0.1 ml 40:60 acetonitrile/water with 0.1% formic acid. Similar liquid–liquid extractions were carried out for atenolol and digoxin using chloroform and isopropanol, and for metoprolol using methyl-*tert*-butyl ether. Following sample dilution with 0.025 ml 1 M sodium hydroxide (pH 11), 1 ml 80:20 chloroform/isopropanol, for atenolol, or 1 ml 95:5 chloroform/isopropanol, for digoxin, was added to each sample and then shaken by vortex for 1 to 3 min. For metoprolol, the sample was diluted with 0.1 ml sodium carbonate: ammonia (pH 11) and extracted with 2 ml methyl-*tert*-butyl ether. Following centrifugation, the organic layer was transferred and evaporated to dryness. The samples were reconstituted with appropriate solvent for injection on the LC-MS/MS system.

LC-ESI-MS Conditions

Both an API 4000™ and an API 5000™ triple quadrupole mass spectrometer systems (Applied Biosystems/SCIEX, Concord, ON, Canada) were used for the analysis of plasma samples from the pharmacokinetic studies. The API 4000™ mass spectrometer was interfaced with an HPLC system consisting of SCL-10A vp system controller, LC-10AD vp pumps (Shimadzu, Columbia, MD) and a CTC HTS PAL autosampler (Leap Technologies, Carrboro, NC). The API 5000™ mass spectrometer was interfaced with Shimadzu LC-20AD pumps and SIL-20AC autosampler (Shimadzu, Columbia, MD). Reversed-phase gradient HPLC methods were used for sample analysis. The mobile phases consisted of water and acetonitrile with 0.1% formic acid. A Zorbax Eclipse XDB-C18 column (50×4.6 mm, 1.8 μm; Agilent, Santa Clara, CA) was used for the separation of cabamazepine, metoprolol, atenolol and digoxin while a Waters Atlantic HILIC Silica column (100×2 mm, 3 μm; Waters, Milford, MA) was used for the separation of antipyrine. Flow rates ranged from 0.4 to 0.8 ml/min and gradient for HPLC separation varied slightly depending upon the analyte. The LC-MS/MS conditions were optimized for each compound to establish quantification methods using the multiple reaction monitoring (MRM) mode. The detailed experimental conditions for these five analytes using API 5000™ mass spectrometer were summarized in Table II.

A 4000 QTRAP® (Applied Biosystems/MDS Sciex, Concord, ON, Canada) hybrid triple quadrupole-linear ion trap mass spectrometer, was used to analyze the *in vitro* and *in vivo* samples for metabolite characterization. The mass spectrometer was interfaced to either a Shimadzu Prominence HPLC system (Columbia, MD) or an Agilent 1200 HPLC system (Palo Alto, CA). The mobile phases used for elution were 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). A mobile phase gradient was used with a flow rate of 0.25 ml/min. A Phenomenex Luna C18(2) (100×2 mm, 3 μm) column (Torrance, CA) or an Agilent XDB (150×2 mm, 5 μm) column (Palo Alto, CA) was used for sample analysis. Multiple reaction monitoring (MRM) experiments were used as a survey scan to generate MS/MS data with independent data acquisition (IDA) to detect and characterize the metabolites in a single injection.

Table II LC-MS/MS Experimental Conditions for Antipyrine, Carbamazepine, Metoprolol, Atenolol, and Digoxin Using API 5000™

	Antipyrine	Carbamazapine	Metoprolol	Atenolol	Digoxin
Flow Rate (ml/min)	0.5	0.8	0.4	0.4	0.4
HPLC Column	Waters Atlantic HILIC Silica, 2×100 mm, 3u	Zorbax Eclipse XDB-C18, 4.6×50 mm, 1.8u	Zorbax Eclipse XDB-C18, 4.6×50 mm, 1.8u	Zorbax Eclipse XDB-C18, 4.6×50 mm, 1.8u	Zorbax Eclipse XDB-C18, 4.6×50 mm, 1.8u
Mobile Phase A	0.1% formic acid in 90:10 water/acetonitrile	0.1% formic acid in water	0.1% formic acid in water	0.1% formic acid in water	5 mM ammonium formate and 0.1% formic acid in water
Mobile Phase B	0.1% formic acid in 90:10 acetonitrile: water	0.1% formic acid in acetonitrile	0.1% formic acid in acetonitrile	0.1% formic acid in acetonitrile	Acetonitrile
Gradient	hold at 99% B for 1.1 min; step to 60% B and hold for 0.4 min	Isocratic at 40% B	hold at 10% B for 0.5 min; slow gradient to 80% B over 2.5 min, hold for 1.0 min	hold at 7% B for 5.0 min; step gradient to 90% B, hold for 1.0 min	hold at 30% B for 0.1 min; slow gradient to 90% B over 3 min, hold for 1.0 min
Retention Time (min)	1.1	1.8	3.2	4.3	2.5
Ionization Mode	(+) ESI	(+) ESI	(+) ESI	(+) ESI	(+) ESI
MRM Transition	189.1/106.1	237.1/194.1	268.2/191.1	267.2/145.1	798.5/651.4
Collision Energy (eV)	35	26	25	39	20
QC (n=2); ng/ml	0.2, 100	0.2, 100	0.2	0.2, 100	0.2, 100
Acceptance Criteria	20%	15%	15%	15%	20%

Table III Calibration Curve Parameters for Antipyrine, Carbamazepine, Metoprolol, Atenolol, and Digoxin Using API 5000™

Analyte	Concentration Range (pg/ml)	Regression Model	Curve Fit (*r* Value)	Slope	Intercept
Antipyrine	10–5,000	No weighting	0.9999	13.5	0.259
Carbamezepine	1–1,000	Linear (1/*x* weighting)	1.0000	0.0345	0.0066
Metoprolol	5–20,000	Linear (1/*x* weighting)	0.9990	0.00125	0.000271
Atenolol	10–20,000	Linear (1/*x* weighting)	1.0000	0.000835	0.00139
Digoxin	50–20,000	Linear (1/*x* weighting)	0.9907	0.182	0.000342

Data Analysis

Bioanalytical Analysis

Analyst software (version 1.4, Applied Biosystems, Foster City, CA) was used to integrate the peak areas of analyte and internal standard. Watson LIMS software (version 6.3, Thermo Electron, Waltham, MA) was used for construction of calibration curves relating peak area ratios of analyte/internal standard to plasma concentration of analyte and for the final determination of sample concentrations.

Pharmacokinetic Analysis

Non-compartmental PK parameters including maximum observed plasma concentration (C_{max}), the time at which C_{max} occurs (T_{max}), the area under the concentration-time curve through the last quantifiable sampling time ($AUC_{0\text{-tlast}}$), and apparent oral half life ($t_{1/2}$) were calculated for each compound at each dosage using Watson (version 6.3, Thermo Electron, Waltham, MA).

RESULTS

Bioanalytical Assay

Selective and sensitive bioanalytical assays were developed for antipyrine, carbamazepine, metoprolol, atenolol and digoxin using both API 4000 ™ and API 5000 ™ LC-MS/MS systems. The lower limit of quantitation (LLOQ) in rat plasma achieved using the API 5000 ™ mass spectrometer, in general, was two to ten times more sensitive than the API 4000 ™ mass spectrometer for the five analytes. Calibration curve parameters including concentration range, regression model, curve fit, slope and intercept for antipyrine, carbamazepine, metoprotol, atenolol and digoxin using API 5000 ™ are tabulated in Table III. More than three-quarter of standard curve concentrations and two-third of quality control concentrations for all five analytes are within acceptance criteria. Linear standard curves were achieved for all five compounds with r value above 0.99. Representative LC-MS/MS chromatograms for digoxin of 5 pg/ml and metoprolol of 5 pg/ml using the API 5000 ™ mass spectrometer were displayed in Fig. 2.

In vivo PK Studies

Since the human microdose is capped at 100 μg per human (1), the corresponding microdosage would be 1.67 μg/kg assuming human bodyweight of 60 kg. In the current study, the lowest dose administered to rats for metoprolol, atenolol and digoxin was 1.67 μg/kg, the equivalent maximum human microdose without allometric correction. For antipyrine and carbamazepine, a ten fold lower dose of 0.167 μg/kg was also administered. In addition, up to 1,000 fold higher doses compared to 1.67 μg/kg were selected to evaluate pharmacokinetic proportionality in rats. Following oral administration of metoprolol, atenolol, and digoxin at doses of 1.67, 16.7, 167, or 1,670 μg/kg and antipyrine and carbamazepine at doses of 0.167, 1.67, 16.7, 167, or 1,670 μg/kg, the mean plasma concentration versus time profiles in male Sprague–Dawley rats are shown in Fig. 3. The resulting pharmacokinetic parameters are summarized by mean values in Table IV. Antipyrine was rapidly detected in plasma, with T_{max} at 0.333–0.833 h and $t_{1/2}$ at 1.21–1.60 h post dose. Antipyrine concentrations were measurable in rat plasma for up to 8 h following the lowest dose of 0.167 μg/kg. Carbamazepine was rapidly detected in plasma, with T_{max} at 0.222–0.5 h and $t_{1/2}$ at 1.16–2.13 h post dose. Carbamazepine concentrations were measurable in rat plasma after 8 h following the lowest dose of 0.167 μg/kg. Digoxin was detected in plasma, with T_{max} at 1.00–1.67 h and $t_{1/2}$ at 1.67–2.58 h post dose. Digoxin concentrations were measurable in rat plasma after 12 h following 16.7 μg/kg oral dose and 8 h following 1.67 μg/kg oral dose. The $AUC_{0\text{-last}}$ and C_{max} values for antipyrine,

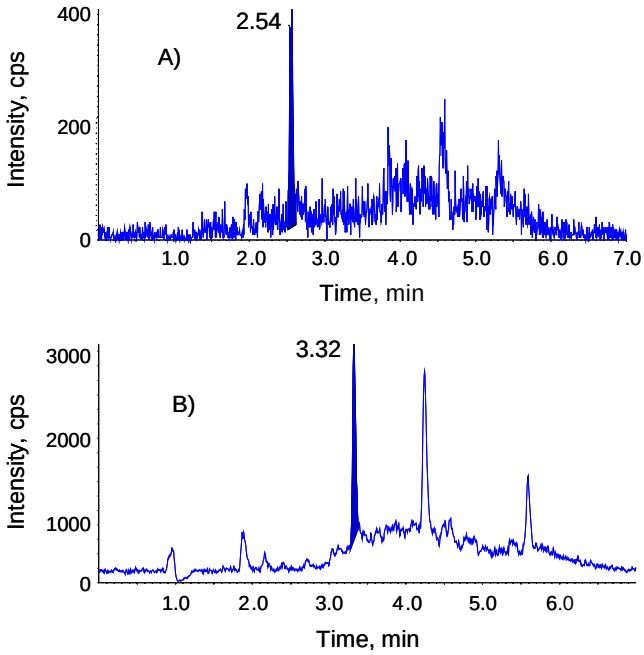

Fig. 2. Representative LC-MS/MS chromatograms for **A** digoxin of 5 pg/ml at retention time of 2.54 min and **B** metoprolol of 5 pg/ml at retention time of 3.32 min using the API 5000 ™ mass spectrometer.

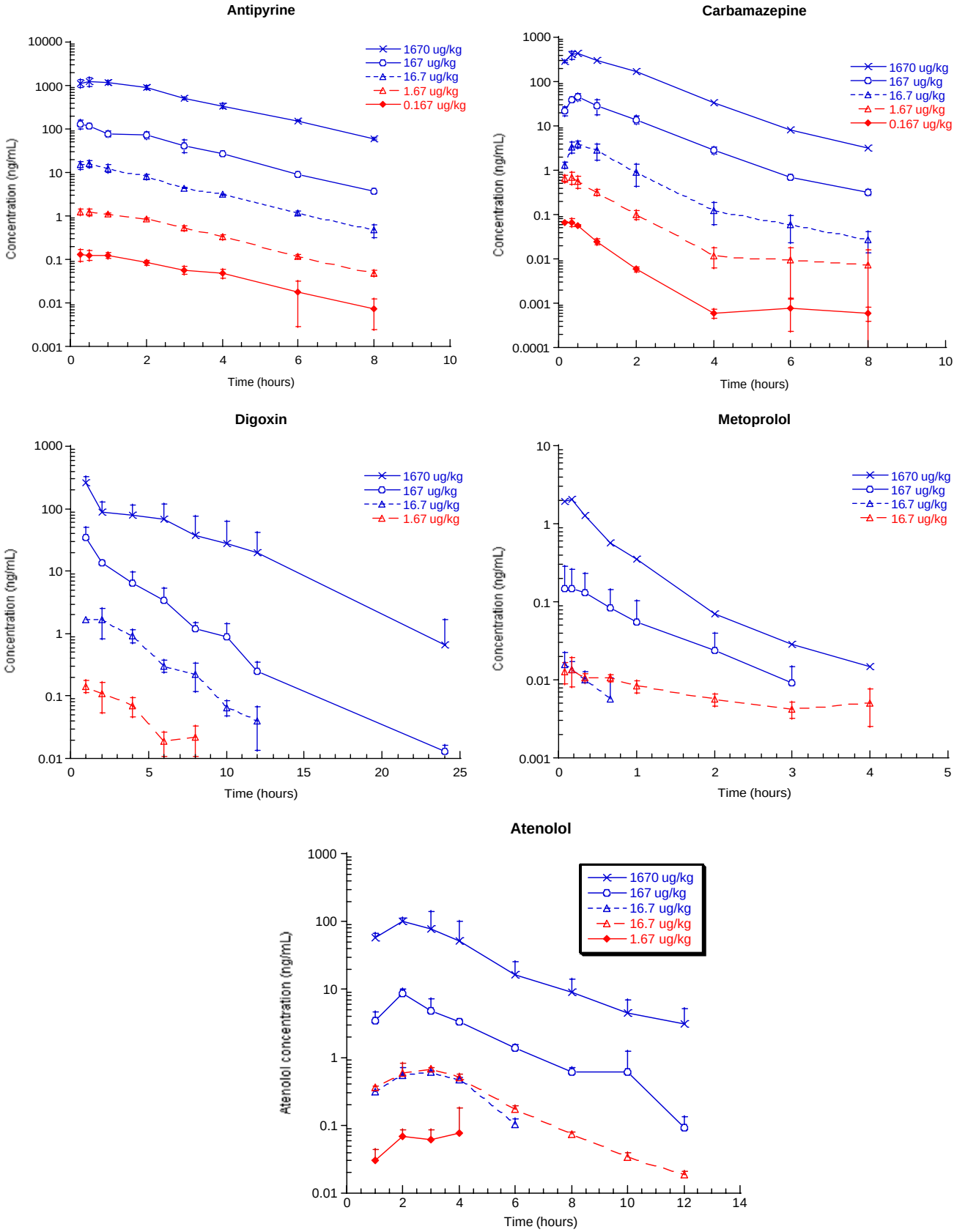

Fig. 3. The pharmacokinetic profiles of antipyrine, carbamazepine, metoprolol, atenolol, and digoxin following a single oral dose to male Sprague–Dawley rats (*blue color* indicates that the data were obtained from API 4000 ™ mass spectrometer; Red color indicates that the data were obtained from API 5000 ™ mass spectrometer.).

Table IV The Pharmacokinetic Parameters of Antipyrine, Carbamazepine, Metoprolol, Atenolol, and Digoxin Following a Single Oral Dose to Male Sprague–Dawley Rats

Compounds	Dose (μg/kg)	AUC (ng·h/ml)	C_{max} (ng/ml)	T_{max} (h)	$t_{1/2}$ (h)
Antipyrine	1,670	3,790±130	1,330±160	0.833±0.289	1.60±0.11
	167	271±78	134±29	0.333±0.144	1.21±0.32
	16.7	37.2±3.53	16.8±2.9	0.333±0.144	1.47±0.26
	1.67	3.63±0.26	1.28±0.19	0.417±0.144	1.45±0.06
	0.167	0.417±0.091	0.136±0.036	0.750±0.433	1.57±0.72
Carbamazepine	1,670	809	446	0.500	1.16
	167	73.3±12.3	45.2±8.2	0.500±0.000	1.27±0.04
	16.7	5.83±1.92	3.91±0.70	0.444±0.096	1.98±0.39
	1.67	0.796±0.197	0.699±0.190	0.222±0.096	1.62±0.71
	0.167	0.0661±0.0059	0.0722±0.0082	0.222±0.096	2.13±0.52
Atenolol	1,670	351±167	119±27	2.33±0.58	2.49±0.85
	167	24.5±4.4	8.69±1.42	2.00±0.00	1.48±0.21
	16.7	2.12±0.14	0.629±0.054	1.47±0.26	2.67±0.58
	1.67	0.476±0.466	0.130±0.114	3.67±2.08	3.16±0.49
Digoxin	1,670	830±527	258±68	1.00±0.00	2.22±0.49
	167	63.2±16.4	34.1±15.9	1.00±0.00	2.58±0.38
	16.7	6.06±2.09	1.75±0.86	1.67±0.58	1.67±0.41
	1.67	0.443±0.112	0.163±0.008	1.33±0.58	2.39±0.45
Metoprolol	1,670	1.18	2.14	0.125	0.880
	167	0.155±0.121	0.158±0.131	0.139±0.048	0.923±0.209
	16.7	0.00795	0.0170	0.125	0.584
	1.67	NC[a]	NC[a]	NC[a]	NC[a]

[a] NC indicates that the PK parameters are not calculated due to insufficient data.

carbamazepine and digoxin were proportional for all tested doses. Atenolol was detected in plasma, with T_{max} at 2.00–3.67 h and $t_{1/2}$ at 0.916–3.16 h post dose. Atenolol concentrations were measurable in rat plasma after 10 h following 1.67 μg/kg oral dose. The $AUC_{0\text{-last}}$ and C_{max} values for atenolol were proportional for the three highest dosing levels. Metoprolol was rapidly detected in plasma, with T_{max} at 0.111–0.139 h and $t_{1/2}$ at 0.670–0.923 h post dose. Metoprolol concentrations were measurable in rat plasma after 4 h following 16.7 μg/kg oral dose, but not measurable following 1.67 μg/kg oral dose.

In vitro and *in vivo* Metabolism Study

Metabolites of carbamazepine were characterized in rat liver microsomal incubations in the presence of NADPH or UDPGA regenerating system using the predictive approach of using multiple reaction monitoring-independent data acquisition (MRM-IDA). A summary of the metabolites detected and characterized in both in the *in vivo* and *in vitro* experiments are given in Table V. In the presence of NADPH, four different hydroxyl metabolites and one di-hydroxyl metabolite were detected in the 100 nM carbamazepine incubation in rat liver microsomes. The same metabolites, except for two of the hydroxyl metabolites, were also detected in the 3 nM incubation of carbamazepine. The chromatograms in Fig. 4 illustrate the detection of the hydroxyl metabolites under both incubation conditions. Table VI summarizes the MS/MS spectra that were used to characterize these metabolites. In the presence of UDPGA, four glucuronide conjugates were detected in the 3 nM and 100 nM incubations of carbamazepine. A summary of the MS/MS spectra that were used to characterize these metabolites is given in Table VII.

Four metabolites of carbamazepine were detected and characterized in plasma of rats dosed with 1.67 μg/kg of carbamazepine using the MRM-IDA technique (Table VIII). They are single hydroxylation of carbamazepine, oxidation of carbamazepine followed by *O*-glucuronidation, the addition of 14 mass units to carbamazepine followed by *O*-glucuronidation and the addition of 14 mass units and oxidation to carbamazepine followed by *N*-glucuronidation. Except the metabolite with the

Table V Summary of the Carbamazepine Metabolites Identified and Characterized in Rat Liver Microsomes and *In Vivo* Rat Plasma

Metabolite	*In Vitro* with NADPH	*In Vitro* with UDPGA	*In Vivo Plasma*
Oxidation	4		1
Di-Oxidation	1		
Glucuronidation		1	
O–2H + Glucuronidation		1	1
Oxidation + Glucuronidation		1	1
2O–2H + *N*-Glucuronidation			1
Di-Oxidation + Glucuronidation		1	

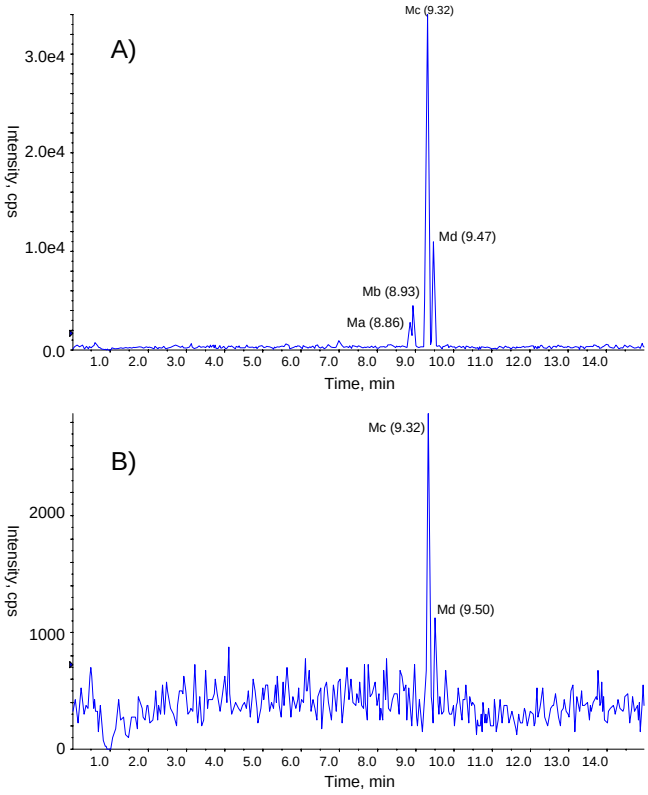

Fig. 4. Detection of hydroxyl metabolites of carbamazepine in rat liver microsomes in the presence of NADPH using MRM (transition m/z 253.1>210.1) for detection. **A** 4 hydroxyl metabolites detected in the 100 nM incubation, **B** 2 hydroxyl metabolites detected in the 3 nM incubation.

addition of 14 mass units and oxidation to carbamazepine followed by N-glucuronidation, the other metabolites observed *in vivo* could also be observed in *in vitro* microsomal incubation with either NADPH or UDPGA. The single hydroxylation of carbamazepine was characterized as carbamazepine epoxide, the major human circulating metabolite of carbamazepine at the clinical dose, and was confirmed with synthetic standard (Fig. 5).

DISCUSSION

LC-MS/MS could potentially provide an alternative to AMS because of its ease of use, highly automated sample preparation process, direct on-line linkage between HPLC and MS and relatively low expense. Although LC-MS/MS is not as sensitive as AMS, it was the purpose of this study to investigate if LC-MS/MS has adequate sensitivity to support microdosing studies. The microdose for rats was approximated based on a mg/kg body weight basis from the maximum clinical microdose, i.e., 100 μg/per person (average 60 kg human body weight). Thus, an oral microdose of 1.67 μg/kg was selected for studies in rats.

For antipyrine and carbamazepine, assays were developed with lower limits of quantitation (LLOQ) of 10 and 1 pg/ml, respectively, using an API 5000 ™ LC-MS/MS system. Because of low hepatic clearance and high oral bioavailability of these two compounds, the plasma concentrations in rats could be quantified up to the last collection time point of 8 h post-dose for the lowest dose of 0.167 μg/kg. Thus, LC-MS/MS assays were adequate to support microdose studies in rats for these compounds. For digoxin, an LLOQ of 5 pg/ml was obtained using the API 5000™ LC-MS/MS system. The assay sensitivity was sufficient to obtain the pharmacokinetic profile at the microdose level of 1.67 μg/kg for digoxin, which is known to have low oral absorption. For atenolol, an LLOQ of only 20 pg/ml was obtained. Combined with moderate oral bioavailability for atenolol, it is not surprising to observe only a partial pharmacokinetic profile at the microdose of 1.67 μg/kg. For metoprolol, an LLOQ of 5 pg/ml was achieved. However, due to its high hepatic clearance, which results in low drug exposure, no pharmacokinetic profile was obtained at the microdose of 1.67 μg/kg. Thus, the sensitivity was not sufficient to support the microdose study in rats for metoprolol. In addition, our experience with these five compounds found that the API 5000 ™ was up to tenfold more sensitive than the API 4000 ™, allowing the quantitation of drug in samples for lower doses and later time points.

An important aspect of microdosing is to extrapolate the pharmacokinetic parameters from a microdose level to those at the therapeutically equivalent dose level. All extrapolations are based on the assumption of a proportional

Table VI. Summary of the Carbamazepine Metabolites Characterized in Rat Liver Microsomes in the Presence of NADPH at Both 3 and 100 nM Incubation Concentrations

Metabolite (RT)	Substrate, 100 nM	Substrate, 3 nM	M/z of Fragment Ions (Ion Assignment, Relative Intensity to Base Peak)[a]
Oxidation (8.86 min)	✓		253.0 ($[M+H]^+$, 89), 210.1 ($[M+H–CONH]^+$, 100), 194.8 (33)
Oxidation (8.93 min)	✓		253.0 ($[M+H]^+$, 50), 210.1 ($[M+H–CONH]^+$, 100), 208.1 (70), 182.0 (25), 180.1 (($[M+H–CONH–COH_2]^+$, 25)
Oxidation (9.32 min)	✓	✓	252.8 ($[M+H]^+$, 27), 210.1 ($[M+H–CONH]^+$, 20), 180.3 (($[M+H–CONH–COH_2]^+$, 100), 107.2 (47)
Oxidation (9.47 min)	✓	✓	253.1 ($[M+H]^+$, 23), 235.0 ($[M+H–H_2O]^+$, 8), 210.2 ($[M+H–CONH]^+$, 100), 208.2 (25), 190.2 (8), 180.1 ($[M+H–CONH–COH_2]^+$, 20)
Di-oxidation (8.57 min)	✓	✓	269.0 ($[M+H]^+$, 52), 252.2 (7), 226.0 (100), 225.0 (31), 224.0 (72), 208.2 (14), 198.2 (21), 180.2 (45)

[a] Intensities are for the enhanced product ion spectra at the 100 nM incubation.

Table VII Summary of Carbamazepine Metabolites Characterized in Rat Liver Microsomes in the Presence of UDPGA at Both 3 and 100 nM Incubation Concentrations

Metabolite (Retention Time)	Substrate 100 nM	Substrate 3 nM	M/z of Fragment Ions (Ion Assignment, Relative Intensity to Base Peak)[a]
Glucuronidation (8.80 min)	✓	✓	413.1 ($[M+H]^+$, 2), 237.2 ($[M+H–176]^+$, 24), 194.0 ($[M+H–176–CONH]^+$ (28), 192.0 (100) 191.1(22)
O – 2H + Glucuronidation (6.67 min)	✓	✓	427.3 ($[M+H]^+$, 100), 409.4 ($[M+H–H_2O]^+$, 14), 356.2 (16), 251.2 ($[M+H–176]^+$, 16)
O + Glucuronidation (13.59 min)	✓	✓	429.1 ($[M+H]^+$, 0), 313.1 (100), 253.3 ($[M+H–176]^+$, 39), 91.2 (100)
Di-oxidation + Glucuronidation (16.59 min)	✓	✓	445.2 ($[M+H]^+$, 13), 341.4 (3), 269.4 ($[M+H–176]^+$, 100), 190.9 (3)

[a] Intensities are for the enhanced product ion spectra at the 100 nM incubation.

relationship of pharmacokinetic parameters across various dosing levels. Without proportionality, the value of microdosing studies would be questionable. However, the current literature references yield little data to verify the proportionality assumption, be it in humans or in animal species (7,8,11,20). In this study, we set out to test the proportional relationship of pharmacokinetic parameters in rats using a limited selection of compounds. Although it is understandable that proportional PK relationship in rats or other animal species not necessarily translates into proportional PK relationship in humans, nevertheless, proportional PK relationship in rats or other animal species does lead to higher confidence in extrapolating microdose PK parameters to normal dose in human.

The pharmacokinetic parameters are summarized in Table IV. Antipyrine, carbamazepine, atenolol, and digoxin demonstrated a relatively good proportional relationship of AUC and C_{max} following oral administration of 0.167 to 1670 μg/kg to rats, while metoprolol exhibited a non-proportional relationship in AUC exposure but proportional relationship in C_{max}. Metoprolol is a compound with low metabolic stability and high liver extraction ratio, which generally leads to low AUC exposure. The lack of sensitivity by LC-MS/MS led the observation of only partial pharmacokinetic profile up to 40 min post-dose at 16.7 μg/kg for metoprolol. The incomplete PK profile at 16.7 μg/kg dose may contribute to the non-proportional AUC increases compared to higher doses of 167 and 1,670 μg/kg, where PK profile was obtained up to 3–4 h post-dose. Therefore, the overall success rate for extrapolating exposure (AUC and C_{max}) in rats was found to be four out of the five compounds. In this study, drug was not administered via an intravenous route to obtain the absolute oral bioavailability at the microdose level. In the 'CREAM' (Consortium for Resourcing and Evaluating AMS Microdosing) trial, five compounds (midazolam, diazepam, ZK253, warfarin, and erythromycin) were administered via both oral and intravenous routes at microdose level in humans (7). Midazolam, diazepam and ZK253 demonstrated comparable oral bioavailability in humans at microdose level to those at therapeutic doses, while warfarin did not show a correlation.

It is important to obtain *in vivo* human metabolism data early in the drug development process, particularly in the case where a metabolic pathway is identified in *in vitro* human hepatocytes or liver microsome incubation, but not in *in vivo* animals. Microdosing can potentially be used to establish whether or not the unique metabolic pathway identified in human *in vitro* occurs in human *in vivo*. In addition, it is equally important to compare metabolism between the microdose and the projected therapeutic dose in the relevant animal species. Potential metabolism differences in terms of the type and the amount of metabolites generated between the microdose and the projected therapeutic dose may be due to enzyme inhibition and/or enzyme saturation at higher doses.

Carbamazepine was selected as an example to investigate metabolism differences from the microdose to the therapeutic dose in rats. Carbamazepine is extensively metabolized and metabolic pathways are well understood in both rats and humans (21,22). The major metabolic pathways of carbamazepine are through oxidation to carbamazepine-10,11-epoxide, hydroxylation of six-membered aromatic ring, *N*-glucuronidation at the carbamoyl side chain and *O*-glucuronidation of hydroxylated metabolite. C_{max} for carbamazepine at the microdose of 1.67 μg/kg in rats was calculated to be approximately 3 nM. *In vitro* rat liver microsome incubation of 3 nM carbamazepine in the presence of NADPH or UDPGA showed the presence of oxidative or conjugated metabolites, which are similar to those found at the higher substrate concentration of 100 nM

Table VIII Summary of the Carbamazepine Metabolites Characterized in Rat Plasma

Metabolite (Retention Time)	M/z of Fragment Ions (Ion Assignment, Relative Intensity to Base Peak)
Oxidation (8.32 min)	253.3 ($[M+H]^+$, 5), 210.0 ($[M+H–CONH]^+$, 17), 151.9 (10), 180.1 ($[M+H–COH_2]^+$, 100), 91.0 (12), 79.2 (14)
O – 2H + Glucuronidation (13.88 min)	427.1 ($[M+H]^+$, 9), 250.9 ($[M+H–176]^+$, 100), 184.1 (58), 99 (52), 86.2 (52)
Oxidation + Glucuronidation (13.94 min)	429.3 ($[M+H]^+$, 2) 329 (12) 273 (22), 271 (18), 255 (82), 253 ($[M+H–176]^+$, 59), 99.0 (100)
2O – 2H + *N*-Glucuronidation (16.21 min)	443.3 ($[M+H]^+$, 94), 425.3 ($[M+H–H_2O]^+$, 26), 225.4 (73), 224.2 ($[M+H–176–CONH]^+$, 67), 220.3 (82), 219.2 (100)

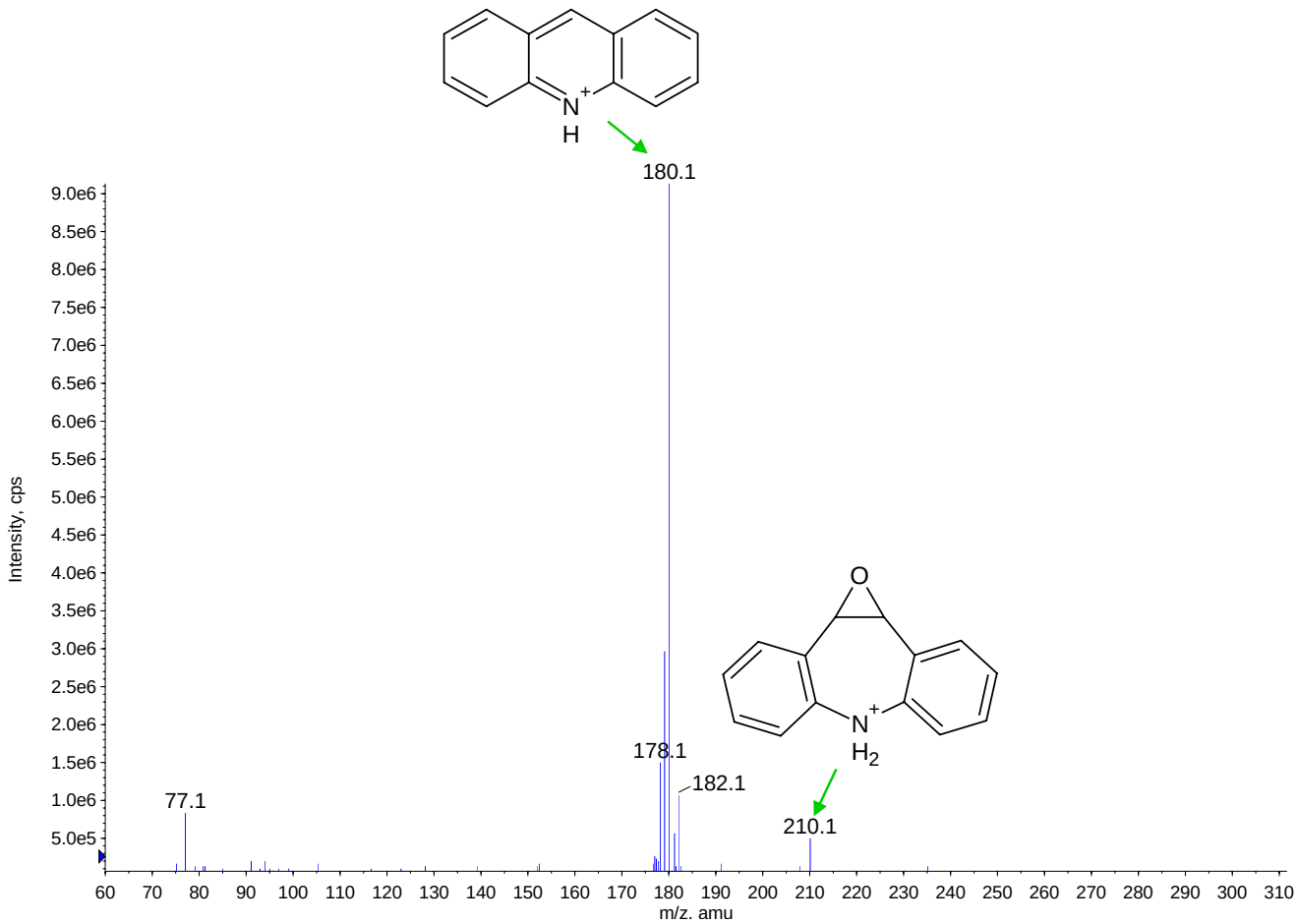

Fig. 5. Interpretation of the MS/MS spectrum of the carbamazepine epoxide metabolite identified in plasma *in vivo*.

and in the literature (22). Rat plasma samples were subsequently profiled for metabolites at the microdose of 1.67 μg/kg. Four metabolites were identified as single oxidation, oxidation of carbamazepine followed by *O*-glucuronidation, the addition of 14 mass units to carbamazepine followed by *O*-glucuronidation and the addition of 14 mass units and oxidation to carbamazepine followed by *N*-glucuronidation, which are similar to the major metabolites at therapeutic doses reported in the literature (21,22). This suggests that the metabolic profile *in vivo* at a microdose is, in general, similar to that at therapeutic doses in rats for carbamazepine. However, further comprehensive studies need to be carried out to investigate the similarities and differences of the *in vivo* metabolic profiles from the microdose to the therapeutic doses for a variety of compounds.

LC-MS/MS has enough sensitivity to be used to profile metabolites *in vivo* at a microdose using the predictive approach of MRM-IDA. The observation of *N*-glucuronidation of oxidative metabolite of carbamazepine only *in vivo* without prior knowledge from *in vitro* studies supports the utility of 4000 QTRAP® for profiling of unknown or unique metabolites in microdosing samples. However, the technology has its limitation and one could potentially miss unexpected, MS-unionizable or MS-insensitive metabolites. In addition, the direct quantitative assessment of metabolites will be difficult using LC-MS/MS due to the lack of synthetic standards. On the other hand, accelerator mass spectrometer together with ^{14}C-labeled compound could ensure the detection and quantitation of all major metabolites in metabolite profile, which is an advantage over LC-MS/MS.

CONCLUSION

The LC-MS/MS assays developed provided sufficient sensitivity to study microdose pharmacokinetics of antipyrine, carbamazepine, atenolol and digoxin in rats. Proportionality of drug exposure between the microdose and greater than 1,000 fold higher doses in rats were demonstrated for antipyrine, carbamazepine, atenolol and digoxin. LC-MS/MS also demonstrated its usefulness for detecting and confirming circulating metabolites in microdosing studies in rats. The 4000 QTRAP® mass spectrometer with the function of multiple reaction monitoring and independent data acquisition was used for metabolite profile and characterization *in vitro* and *in vivo*. Through triggering MS/MS and MS^3 analysis in the trap from MRM transitions via single injection, metabolites were rapidly identified and characterized even at low abundance.

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