Pharm Res (2011) 28:1963–1972
DOI 10.1007/s11095-011-0423-8

RESEARCH PAPER

Clinical Relevance of Liquid Chromatography Tandem Mass Spectrometry as an Analytical Method in Microdose Clinical Studies

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Received: 13 December 2010 / Accepted: 4 March 2011 / Published online: 7 April 2011
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ABSTRACT

Purpose To investigate the potency of LC-MS/MS by means of sensitivity and the applicability for cassette dosing in microdose clinical trials.

Methods Thirty one top-selling 31 drugs were spiked to human plasma, extracted, and analyzed by LC–MS/MS.

Results The lower limits of quantification for each drug varied from 0.08 to 50 pg/mL, and were lower than one eighth of the assumed maximum plasma concentration at microdose in all drugs except for losartan, indicating the high performance in acquisition of full pharmacokinetic profiles at microdose. We also succeeded in simultaneous analysis of multiple compounds, assuming a situation of cassette dosing in which multiple drug candidates would be administrated simultaneously.

Conclusions Together with the features of LC–MS/MS, such as immediate verification, the utilization of non-radiolabeled drugs and no special facilities, we suppose that LC–MS/MS analysis would be widely applicable in conducting microdose clinical studies.

KEY WORDS cassette dosing · exploratory clinical trials · LC–MS/MS · lower limit of quantification · microdose study

Electronic Supplementary Material The online version of this article (doi:10.1007/s11095-011-0423-8) contains supplementary material, which is available to authorized users.

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ABBREVIATIONS

AMS	accelerator mass spectrometry
$Cmax_{Clin}$	Cmax in clinical PK study
$Cmax_{MD}$	Cmax in microdose study
CV	coefficient of variation
$Dose_{Clin}$	dose in clinical PK study
$Dose_{MD}$	dose in microdose study
EMEA	European Agency for the Evaluation of Medicinal Products
EUMAPP	European Union Microdose AMS Partnership Programme
FDA	Food and Drug Administration
HILIC	hydrophilic interaction chromatography
IS	internal standard
LC–MS/MS	liquid chromatography-tandem mass spectrometry
LLE	liquid-liquid extraction
LLOQ	lower limit of quantification
MHLW	Ministry of Health Labour and Welfare
MRM	multiple reaction monitoring
ODS	octadecylsilyl
PET	positron emission tomography
PK	pharmacokinetic
QC	quality control
RE	relative error
SPE	solid-phase extraction

INTRODUCTION

Guidance on microdosing has been released in the EU (1), the USA (2), and Japan (3), where three analytical methods are documented for quantification of extremely low drug concentrations after microdosing: accelerator mass spec-

trometry (AMS), liquid chromatography–tandem mass spectrometry (LC–MS/MS), and positron emission tomography (PET). Each analytical method has its own features. Pharmacokinetic data of the parent drugs are primarily obtained by LC–MS/MS, while pharmacokinetic data of both the parent drug and metabolites are obtained by AMS with parallel use of chromatography. On the other hand, organ distribution and receptor binding data can be acquired by PET. Irrespective of the analytical method used, the purpose of the microdose study remains the same: to select good drug candidates prior to the Phase I study.

Investment required for one successful drug launch has risen from US$ 1.1 billion (1995–2000) to US$ 1.7 billion (2000–2002) (4). Nevertheless, the success rate from the preclinical stage to the launch has declined from 14% (1995–2000) to 8% (2000–2002). The reasons for the attrition during nonclinical and clinical studies were tied mostly to the safety, efficacy, toxicology, and pharmacokinetics/bioavailability (5,6). Integration of microdosing prior to the conventional clinical studies should enhance optimized drug selection by excluding the compounds having undesired properties in pharmacokinetics and receptor binding.

Previously, we have reported the results of two microdose studies, where the plasma concentrations of fexofenadine and nicardipine were successfully determined by LC–MS/MS (7,8). The metabolites of nicardipine were also analyzed by estimating their chemical structures using linear ion trap–Fourier transform ion cyclotron resonance mass spectrometry. The results indicated that LC–MS/MS would be a useful analytical method for the microdose studies, on the premise that the dose is 100 μg of fexofenadine and nicardipine.

However, to date, AMS has been most frequently used to obtain the pharmacokinetic data in the microdose studies (9,10). The use of LC–MS/MS is rather rare in the microdosing studies presumably because of a biased view that LC–MS/MS would not be sensitive enough. While LC–MS/MS has been used most frequently as a sensitive assay method in the conventional clinical studies, there has been no systematic investigation on how low the lower limit of quantification could be brought down technically in human samples. The reasonable explanation for this is that the quantification sensitivity required in these cases was merely for the pharmacological dose levels. In order to examine whether or not LC–MS/MS is practically applicable in microdose studies in terms of sensitivity, we selected 31 drugs out of 47 top-selling drugs worldwide with a variety of chemical structures and therapeutic purposes (Uto Brain, News Release, 2007), and we determined the lower limit of quantification (LLOQ) for these drugs spiked in human plasma and examined the applicability of LC–MS/MS to the microdose studies. Based on the findings in the present study, we report here that LC–MS/MS is widely applicable to the microdose studies as the quantification method.

MATERIALS AND METHODS

Selection Criteria

Out of the 47 top-selling drugs worldwide, 31 drugs were selected according to the following four criteria: (i) exclusion of high-molecular weight drugs (epoetin alpha, darbepoetin alpha, etanercept, pegfilgrastim, rituximab, infliximab, trastuzumab, bevacizumab, adalimumab, insulin glargine, and enoxaparin injection) because the guidance for microdosing studies for high-molecular weight drugs have not yet been established; (ii) exclusion of combination drugs (salmeterol/fluticasone) because the guidance for microdosing studies has not been defined for such drugs; (iii) exclusion of drugs which cannot be quantified without derivatization, such as diethyldithiocarbamation or methylation (oxaliplatin and alendronate (11)), because its optimal ionization is difficult by electrospray ionization (Meng M. *et al*., the 54th American Society for Mass Spectrometry Conference, 2006); and (iv) exclusion of the fourth and higher in class drugs by the order of sales. Therefore, the fourth proton-pump inhibitor rabeprazole and the fourth angiotensin II receptor antagonist irbesartan were excluded. We investigated the parent drugs, although it is known that clopidogrel, oseltamivir, and losartan are rapidly converted into their active metabolites.

Apparatus

The LC system used was ACQUITY UPLC system (Waters Corporation, Milford, MA, USA). The mass spectrometer was API5000 system (Applied Biosystems/MDS Analytical Technologies, Foster City, CA, USA) equipped with a turbo electrospray ionization source.

Reagents and Materials

All compounds except for oseltamivir phosphate were purchased from LKT Laboratories, Inc. (Milford, MA, USA), AvaChem Scientific LLC. (San Antonio, TX, USA), MP Biomedicals Inc. (Irvine, CA, USA), Toronto Research Chemicals Inc. (North York, ON, Canada), or Sigma-Aldrich Co. (St. Louis, MO, USA). Oseltamivir phosphate was synthesized before use (12,13). All other reagents used were purchased from Wako Pure Chemical Industries, Ltd. (Kyoto, Japan). Solid-phase extraction (SPE) cartridges, InertSep Pharma (60 mg/3 mL) and InertSep MC-1

(60 mg/3 mL), were purchased from GL Science Inc. (Osaka, Japan). Oasis hydrophilic-lipophilic-balanced (HLB) cartridge (30 mg/1 mL) used for SPE and the columns used for analysis, ACQUITY UPLC BEH C18 column, ACQUITY UPLC BEH Shield RP18 column, and Atlantis HILIC Silica column, were purchased from Waters Co. (Milford, MA, USA). Cadenza CD-C18 column of Imtakt Co. (Kyoto, Japan), Phenomenex Aqua C18 column and Phenomenex Luna C18(2) column from Phenomenex Inc. (Torrance, CA, USA), and TSK-gel ODS-100 V column from TOSOH Co. (Kyoto, Japan) were also used (Table S2).

Preparation of Stock Solutions and Standard Solutions

All stock solutions used (200 μg/mL) were serially diluted with acetonitrile, methanol, or water to prepare standard solutions for the calibration curves and quality control (QC) samples.

LC–MS/MS Analysis

Octadecylsilyl (ODS) column was used for primary investigation. The mobile phase, consisting of acetonitrile or methanol/0.1% formic acid or 10 mM ammonium formate, was basically isocratic (Table S2). The turbo ion spray interface was operated in either the positive ion mode or negative ion mode (Table S1). Quantification was performed from multiple reaction monitoring (MRM). Analytical data were processed using the Analyst software version 1.4.2 (Applied Biosystems/MDS Analytical Technologies, Foster City, CA, USA).

Sample Treatment

The SPE cartridges having sulfonate/cation-exchange groups (InertSep MC-1) or mixed phase with methacrylate and stylen divinylbenzene-N-vinyl pyrolidone copolymer (InertSep Pharma) were selected first (Tables I and II). We performed SPE as follows: after adding 0.1 mL of formic acid and 1.5 mL of water, plasma samples were loaded onto SPE cartridges, washed with 3 mL of water and 3 mL of wash solvent, and then eluted with 2 mL of organic solvent. The loading volume of the plasma samples was 1 mL except for cetiridine and levofloxacin, whose sample volumes were 0.1 mL. After the samples for cetiridine and levofloxacin were diluted with 0.5 mL of water, the mixed solution was loaded and washed with 1 mL of water and 1 mL of wash solvent, and then eluted with 1 mL of organic solvent. For SPE with hydrophilic-lipophilic-balanced cartridges (Oasis HLB), samples were diluted with 0.5 mL of water, and the mixed solution was loaded, washed and then eluted. After the eluted solution had been evaporated to dryness under nitrogen gas, the residue was reconstituted in 0.1 mL of dissolved solution.

In cases of low recovery or any other problems, liquid-liquid extraction (LLE) was adopted (Table III). Plasma samples (1 mL) were deproteinized with 1 mL of acetonitrile, and the supernatant was evaporated under nitrogen gas until a volume of approximately 1 mL remained. Deproteinization was necessary before extraction because a cleanup using LLE alone was insufficient when employing a plasma volume of 1 mL. Then the samples were extracted using 4 mL of organic solvent under acidic or alkali conditions. The organic layer was evaporated to dryness under nitrogen gas, and the residues were dissolved in 0.1 mL of reconstitution solution.

When internal standard (IS) was used for the quantification, each IS solution was added to the plasma samples before the sample preparation.

Validation Parameters

In order to determine the LLOQ, validation parameters were determined as follows. Selectivity was assessed using pooled blank plasma from six subjects. The calibration standards consisted of a zero sample and six to eight non-zero samples, or standards which were prepared on Day 1. The equation for the calibration curve was prepared using the least squares method with $1/y^2$ weighting. The linearity of the calibration curves was assessed by the correlation coefficient and the relative error (RE) at each concentration level. Intra-day precision and accuracy were determined by analyzing five sets of QC samples at three concentration levels on Day 1. Recovery was evaluated in triplicate at each concentration.

RESULTS

Mass Spectrometric Conditions

In analyzing organic compounds by LC–MS/MS, it is necessary to ionize the compounds, and the sensitivity of quantification is attributed to the extent of ionization of the functional group that the chemical structure holds (Fig. S1a–e). The extent of the ionization depends on the volatility of the organic solvent and the pH of the solution. Therefore, acetonitrile was selected as the mobile phase because its ionization efficiency is high. To adjust the pH, formic acid and ammonium formate were used because they are volatile. The precursor ion, or product ion, was automatically selected at the highest peak intensity in the mass spectrum or the tandem mass spectrum under infusion of both acetonitrile/0.1% formic acid and acetonitrile/10 mM ammonium formate, and the collision energy was

Table I Sample Treatment Conditions for Compounds Extracted by SPE (Solid Phase Extraction) Using InertSep MC-1

Drugs	pKa for compound	IS	Second wash solvent	Elution solvent	Dissolved solution	Recovery (%)	CV (%)
Bupropion	7.9	Quetiapine				94.4	6.6
(S)-Citalopram	9.6	Tamsulosin				92.1	2.5
Clopidogrel	4.55	Imatinib				82.5	2.6
Imatinib	pKa_1 8.07, pKa_2 3.73 pKa_3 2.56, pKa_4 1.52	Quetiapine				86.0	1.5
Olanzapine	7.37, 4.69	Sertraline	Methanol	Acetonitrile containing 4% ammonia/methanol (9:1, *v/v*)	Mobile phase	86.5	1.9
Pioglitazone	5.8, 6.4	Quetiapine				85.0	6.8
Quetiapine	3.3, 6.8	Imatinib				81.2	7.7
Risperidone	pKa_1 8.24, pKa_2 3.11	Quetiapine				97.0	0.7
Rosiglitazone	6.1, 6.8	Quetiapine				74.4	1.8
Sertraline	8.9	Quetiapine				75.6	8.2
Tamsulosin	8.73, 10.23	Quetiapine				88.0	2.1

optimized simultaneously. The solution that gave the highest intensity for the product ion was selected as the temporal mobile phase, and then the optimum conditions such as nebulizer gas, turbo gas, curtain gas, collision gas, ion spray voltage, and turbo gas temperature were automatically searched (Table S1).

Analytical Column and Mobile Phase

The retention time and the peak of each drug were determined using an ODS column (ACQUITY UPLC BEH C18 column) with the temporal mobile phase. The resolution of the interference peaks was also confirmed using human plasma sample spiked with the drug. When the ODS column or the temporal mobile phase were not appropriate, analytical conditions were rearranged. All the drugs were quantified using the isocratic mode, and the retention times were within 4.0 min (Table S2). In the case of amlodipine, atorvastatin, and oseltamivir, which were analyzed with a mobile phase containing formic acid, their column retention tended to be weaker in the ODS column than other compounds because of the increased ionization under acidic condition. Therefore, in order to increase the retention time of these compounds, we selected the ACQUITY UPLC BEH Shield RP18 column, which has a carbamate group in the stationary phase. The Phenomenex Aqua C18 column with polar endcapping was used in order to obtain good resolution between the angiotensin II receptor antagonists (sartans) and interference peaks. Furthermore, the use of methanol instead of acetonitrile was effective in increasing the retention of drugs such as docetaxel with an ODS column. Cetiridine was difficult to retain on an ODS column because this hydrophilic amphoteric compound does not interact much with this hydrophobic column. Therefore, a hydrophilic interaction chromatography (HILIC) silica analytical column was selected. With utilization of HILIC, the retention of compounds was strengthened in highly organic mobile

Table II Sample Treatment Conditions for Compounds Extracted by SPE (Solid Phase Extraction) Using InertSep Pharma or Oasis HLB

Drugs	IS	Type of cartridge	Second wash solvent	Elution solvent	Dissolved solution	Recovery (%)	CV (%)
Atorvastatin	Clopidogrel		10% Methanol			82.9	8.7
Topiramate	Celecoxib				Mobile phase	92.3	2.2
Venlafaxine	–					80.9	3.5
Zolpidem	–	InertSep Pharma		Methanol		100.8	0.1
Esomeprazole	Zolpidem		30% Methanol		Acetonitrile/2% ammonium hydroxide (7:3, *v/v*)	53.5	2.4
Lansoprazole	Zolpidem					41.3	6.2
Pantoprazole	Zolpidem					68.9	0.7
Cetirizine	–	Oasis HLB	30% Methanol	Methanol	Methanol	65.9	0.6
Levofloxacin	–			Ethyl acetate/methanol (1:9, *v/v*)	Mobile phase	42.0	1.4

Table III Sample Treatment Conditions for Compounds Extracted by LLQ (Liquid-Liquid Extraction)

Drugs	pKa for compound	IS	Added reagents before extraction	Organic solvent	Dissolved solution	Recovery (%)	CV (%)
Candesartan	2.1, 4.6	Valsartan				63.1	0.8
Losartan	4.3	Candesartan				67.5	0.6
Valsartan	3.9, 4.73	Candesartan	Formic acid		Mobile phase	65.4	2.3
Celecoxib	11.1	Topiramate		t-Butyl methyl ether		57.3	3.6
Rosuvastatin	4.6	–				90.4	1.1
Simvastatin	4.3	–	10 mM Ammonium acetate-acetic acid buffer (pH 4.5)		10 mM Ammonium acetate-acetic acid buffer (pH 4.5) (5:5, *v/v*)	61.2	1.8
Amlodipine	8.6	Oseltamivir		t-Butyl methyl ether	Mobile phase	82.7	6.6
Oseltamivir	7.75	Amlodipine				56.6	1.8
Montelukast	6.5 ± 0.8	–	Ammonium solution	Ethyl acetate		83.8	3.2
Docetaxel	2.5-4.5	Amlodipine		t-Butyl methyl ether	Methanol/acetonitrile/water (45:45:10, *v/v*)	82.0	3.0
Donepezil	8.90	–		Hexane contained 3% 2-propanol	Mobile phase	80.7	2.6

phase because of the existence of a water layer in the stationary phase. The retention was stronger in mobile phases consisting of methanol rather than acetonitrile.

Sample Preparation Procedure

The established sample preparation procedures and recovery from spiked human plasma are summarized in Tables I, II and III. For basic and weak basic compounds, typical procedure for InertSep MC-1 could be applied, because the nitrogen atom of each compound was retained by the sulfonate group of the stationary phase. InertSep Pharma or Oasis HLB was used if the retention of the compound to the columns was stronger than InertSep MC-1. We investigated the optimal wash solvents of SPE cartridges by changing the ratios of methanol and water. Plasma sample was loaded onto the SPE cartridge after addition of formic acid, in order to load the ionic form. In the case of the benzimidazole derivatives, such as esomeprazole, pantoprazole, and lansoprazole, which degrade under acidic condition, formic acid was not added to the plasma sample, and, instead, ammonia solution was added to alkalify these samples to prevent degradation. The volume of plasma samples used for cetiridine and levofloxacin was 0.1 mL, because the LLOQ did not decrease proportionately to the sample volume. When the recovery was low, or the cleanup was insufficient, LLE was selected. If the pKa of the compound was known, the pH of the sample was adjusted to "pKa – 2" for acidic compounds and "pKa + 2" for basic compounds, in order to diminish their ionized forms which could not be extracted into the organic solvent. For most compounds, we selected t-butyl methyl ether as the extraction solvent, which has a polarity index of 2.5 (water = 9), relative density of 0.7, and boiling point of 55°C, which meant that it was easy to handle. For donepezil extraction, we adopted n-hexane containing 2-propanol as previously reported because 2-propanol prevented the formation of emulsion during shaking (14). Ethyl acetate (polarity index = 4.4) was used for montelukast, because the recovery was higher than t-butyl methyl ether. For simvastatin, acetic acid buffer (pH = 4.5) was used for sample treatment and preparation of mobile phase because this pH value allows the minimum conversion of simvastatin to simvastatin acid (15).

IS was either selected within the drugs investigated or was not used. Assuming that the synthesis of the stable radioisotope-labeled forms of the candidate drugs would not be carried out at the time of microdose studies, we used the drugs of the same analytical conditions as the IS.

Simultaneous Analysis and Sample Preparation

The samples spiked with lansoprazole and pantoprazole were analyzed simultaneously because the sample treatment, analytical methods, and range of the calibration curves were completely the same. Both calibration curves were linear at range from 5 to 5000 pg/mL, and the LLOQ was defined as 5 pg/mL.

Validation Parameters

The selectivity was assessed using pooled blank plasma from six subjects. Peaks from endogenous substances did not interfere with the analyte or the IS. The LLOQ was defined as "the lowest concentration of the calibration curve where the RE was within ±20% and the coefficient of

variation (CV) did not exceed 20%." The calibration curves showed good linearity over a range of 400-times or more. The correlation coefficient was ≥0.993, and the RE was ±20% at LLOQ and ±15% above the LLOQ, respectively, which met the acceptance criteria. When there was an interference peak at the eluting position of the analyte, the response at the LLOQ was investigated at least five times with blank samples. The intra-day precision and accuracy were ≤13.7% and ±12.9% respectively, and met acceptance criteria. The precision of recovery was ≤8.7% throughout the investigation, which met the acceptance criteria of 15%. Therefore, we concluded that the quantification was not influenced by the recovery, even when it was <50%. When bioanalysis is performed by electrospray ionization mass spectrometry, internal method is usually adopted to minimize variation on sample treatment or ionization efficiency. On the other hand, though we applied absolute calibration method to our analysis, the recovery as well as the CV (%) showed small deviation from a precise value in some cases. This may indicate that endogenous substances were successfully removed from the plasma samples at the stage of sample treatment, and analytical conditions were well optimized in our study. The samples for calibration curves and QC were prepared from the same stock solutions in the present study because we simply mentioned at the investigational stage how much extent of drugs can be used for the microdosing clinical study with LC–MS/MS in terms of the quantification limit. However, in the actual bioanalytical analysis, it is desirable to prepare stock solutions for the calibration curves and QC samples separately as long as the stability and accuracy of drug solutions have not been verified. The obtained LLOQ values are shown in Table IV. The MRM chromatograms for clopidogrel showed the lowest LLOQ value among 31 drugs investigated (Fig. 1).

Applicability to Microdosing Clinical Study

The predicted Cmax in microdose study ($Cmax_{MD}$) was calculated proportionately from the Cmax after the clinical PK studies ($Cmax_{Clin}$), on the assumption of linearity (Table IV). $Cmax_{Clin}$ were collected from package inserts, or the appendix of the Goodman & Gilman's The Pharmacological Basis of Therapeutics (16), or other literature data (17,18). The ratios of $Cmax_{MD}$ to the LLOQ value are shown in Fig. 2. We set the acceptance criterion of this ratio in microdose studies to ≥ 8 (2^3), referring to the Guideline for Bioequivalence Studies of Generic Products (19), which requires quantification of human samples over more than three times of elimination half-time in order to calculate basic PK parameters, such as the area under the curve (AUC), elimination half-time (t1/2), clearance (CL), and volume of distribution (Vd). All the compounds met this criterion except for losartan. To examine the ability for a sufficient calculation, we set a higher criterion of the ratios at ≥ 32 (2^5) and found that 24 out of 31 compounds (77%) met this criterion. Each upper concentration of the calibration curve was within the range of estimated $Cmax_{MD}$.

DISCUSSION

In the microdose studies conducted by a consortium using AMS as the ultrasensitive analytical method, it has been reported that the detection limits were as low as 300 fg/mL (ZK253, an investigational drug) to approximately 10 pg/mL (warfarin, diazepam, midazolam, and erythromycin) using 0.5 mL of plasma (20). In other microdose studies using AMS conducted by the European Union Microdose AMS Partnership Programme (EUMAPP), the $Cmax_{MD}$ values ($Dose_{MD}$ = 100 μg each) for paracetamol, phenobarbital, fexofenadine, propafenone, sumatriptan, and clarithromycin were 1.1, 2.6, 0.306, 0.015, 0.1, and 0.188 ng/mL, respectively (21). For the purpose of obtaining the full pharmacokinetic profile of the compound given, the analytical method should be sensitive enough to measure the drug concentrations down to a level of about one eighth of the Cmax value, and AMS is definitely a method sensitive enough for the microdose study as demonstrated by these previous studies.

Considering the Cmax values actually observed in the previous microdose studies and a potential high sensitivity of LC–MS/MS in our previous study, however, we thought that LC–MS/MS would be relevant for microdose study. We quantified 31 drugs in human plasma at low concentrations with LC–MS/MS and resulted in established LLOQ for each drug which varied from 0.08 to 50 pg/mL. The sample cleanup method employed was nothing particularly distinctive, and was simply the extraction by solid phase extraction column or the deproteinization by acetonitrile followed by the liquid-liquid extraction. Using the LC–MS/MS methods established in the present study, full pharmacokinetic profiles of nearly all the drugs were theoretically capable to be measured.

Losartan was the only drug of the 31 drugs investigated where the ratio of $Cmax_{MD}$ to the LLOQ value did not meet the acceptance criteria. The Cmax value for losartan is low because the compound is rapidly transformed to the pharmacologically active metabolite, the carboxylic acid-form metabolite (EXP-3174) (18). In addition, the LLOQ levels of the same drug group (i.e., angiotensin II receptor antagonists) ranged from 10 to 50 pg/mL, whose range was higher than those levels of other drugs investigated. We believe that this was caused by endogenous substances such

Table IV Dose and Cmax in Clinical Dose and Microdose, and the Investigated LLOQ (Lower Limit of Quantification) of Each Drug

Drugs	$Dose_{Clin}$[a] (mg)	$Cmax_{Clin}$[b] (ng/mL)	$Dose_{MD}$[c] (μg)	$Cmax_{MD}$[d] (pg/mL)	LLOQ (pg/mL)
Amlodipine	5	2.81	50	28	2
Atorvastatin	5	2.64	50	26	2
Bupropion	100	141	100	141	2
Candesartan	4[e]	55.1	40	551	10
Celecoxib	100	553	100	553	4
Cetirizine	10	214.5	100	2145	5
(S)-Citalopram	20	21	100	105	0.8
Clopidogrel	75	2.29×10^3	100	3053	0.08
Docetaxel	100[f]	2×10^3	100	1966	5
Donepezil	5	9.97	50	98	0.5
Esomeprazole	20	725×10^3	100	3627	4
Imatinib	400	1.64×10^3	100	438	0.8
Lansoprazole	15	442.7	100	3533	5
Levofloxacin	500	8.04×10^3	100	1608	30
Losartan	25	84.5	100	340	50
Montelukast	10	526	100	5260	2
Oseltamivir	75	360	100	480	2
Olanzapine	5	10.5	50	105	1
Pantoprazole	40	2.5×10^3	100	6250	5
Pioglitazone	30	1.4×10^3	100	4667	0.2
Quetiapine	25	65.29	100	261	0.4
Risperidone	1	7.01	10	70	0.2
Rosiglitazone	2	156	20	1560	0.2
Rosuvastatin	10	7.87	100	79	4
Sertraline	50	15.1	100	30	2
Simvastatin	5	5.1	50	51	3
Tamsulosin	0.1	3.2	1	32	0.4
Topiramate	50	0.84×10^3	100	1680	4
Valsartan	20	0.86×10^3	100	4300	20
Venlafaxine	150	150	100	100	4
Zolpidem	5	76.2	50	762	0.8

[a] $Dose_{Clin}$: clinical dose; [b] $Cmax_{Clin}$: clinical maximum plasma concentration after admistration of $Dose_{Clin}$; [c] $Dose_{MD}$: dose at microdose study (100 μg, or one hundredth of the pharmacological dose); [d] $Cmax_{MD}$ (pg/mL) = $Cmax_{Clin} \times Dose_{MD}/Dose_{Clin}$; [e] As candesartan cilexetil. [f] Original dose 60 mg/m^2 (body surface was calculated as approx. 1.7 m^2 /body)

as phospholipids that are extracted at the same time by liquid-liquid extraction under acidic condition, emerging as interference peaks on chromatograms and also acting as suppressants of ionization.

In the present study, we excluded combination drugs of salmeterol/fluticasone in our investigation based on our selection criteria of tested drugs. We never intentionally exclude this case because of low plasma concentrations of these drugs after their topical administration. Cmax values of salmeterol and fluticasone in the clinical situation were reported to be 103.7 pg/mL and 87.0 pg/mL after administration of 50 μg of salmeterol and 250 μg of fluticasone propionate as inhaled powder (package insert of the Adoair® Diskus®/Aerosol). Assuming that we need to measure 1/8 of Cmax value of salmeterol and fluticasone administered at 1/100 of their pharmacological doses, the required LLOQ would be 0.13 and 0.11 pg/mL, respectively. As the LLOQs of all the test compounds except clopidogrel were above these concentrations, we think it would be difficult to measure the plasma concentration of salmeterol and fluticasone at microdose study, though we have never checked the LLOQs of these compounds. We excluded polar drugs from the investigation because these drugs as such show little or no ionization in electrospray ionization. Chemical derivatization is a routine procedure to give an ionizable property to the polar compounds, and the reported LLOQ levels of alendronate and oxaliplatin, for instance, were 50 pg/mL and 1 ng/mL, respectively,

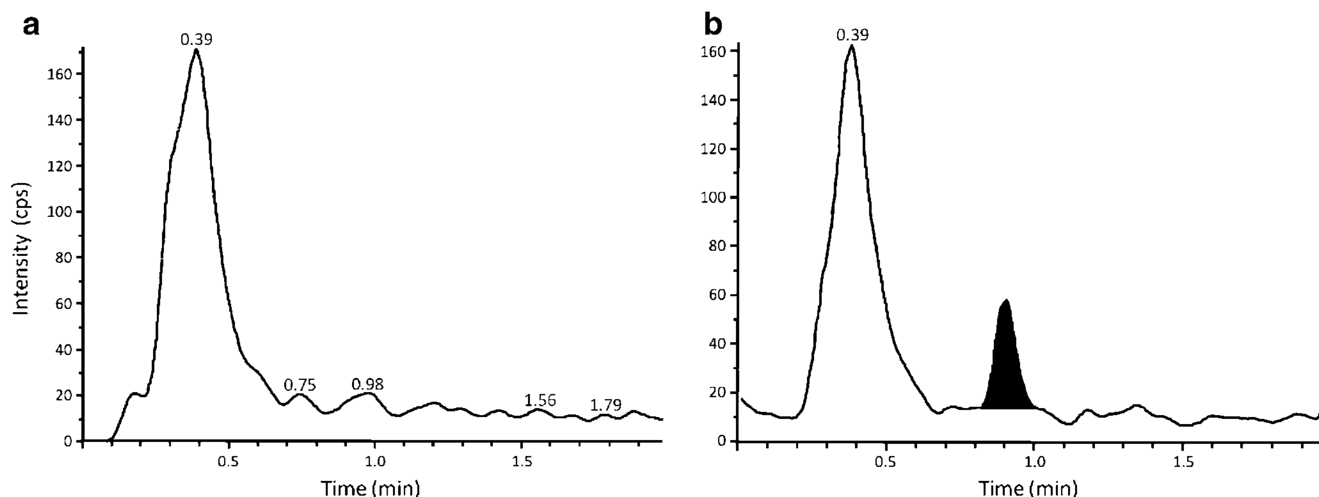

Fig. 1 Example of MRM chromatograms at the LLOQ (lower limit of quantification): (**a**) blank; (**b**) clopidogrel at 0.08 pg/mL.

after derivatization, using 0.1 mL of human serum and plasma, respectively. By increasing the amount of sample to a volume of 1 mL, therefore, full pharmacokinetic profiles of these two drugs could be assessed by LC–MS/MS after microdosing, as the $Cmax_{MD}$ values of alendronate and oxaliplatin are calculated to be 92 and 663 pg/mL, respectively. In case the compounds are difficult to ionize in mass spectrometry, appropriate derivatization would make LC–MS/MS applicable to the microdose study, although we did not systematically investigate in the present study.

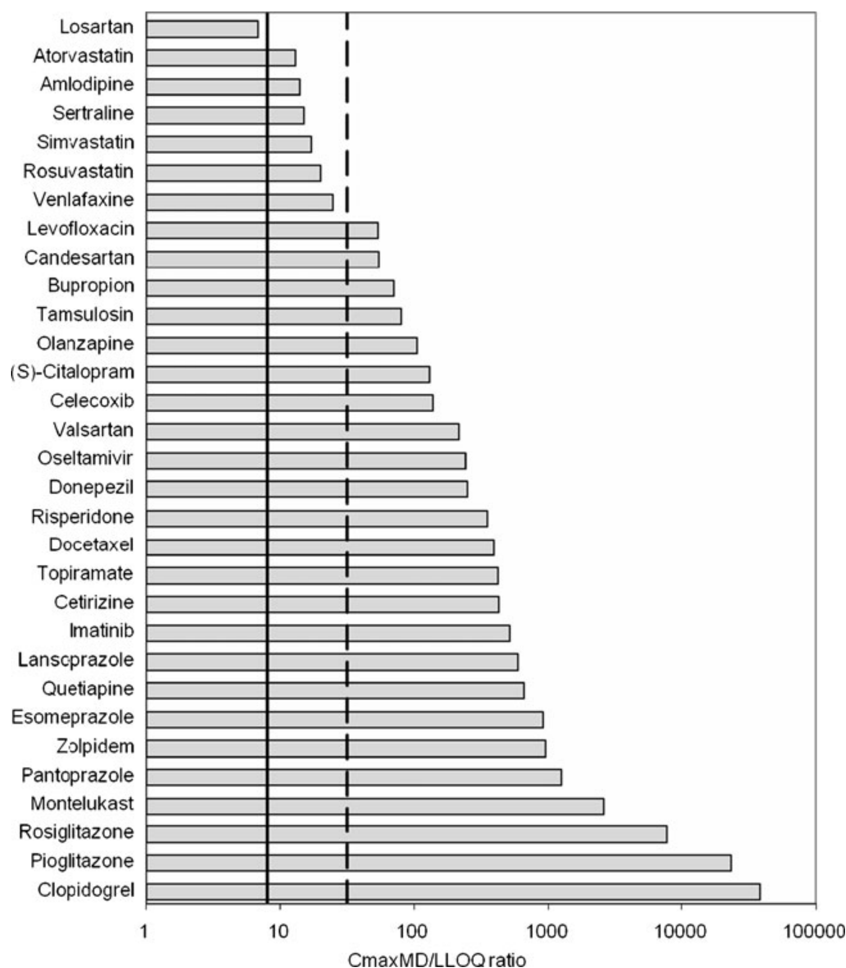

Fig. 2 The ratios of $Cmax_{MD}$ to LLOQ (lower limit of quantification) for each drug. Solid line: ratio at 8 (2^3), dotted line: ratio at 32 (2^5).

Both AMS and LC–MS/MS have pros and cons when used as the assay method in the microdose studies. Synthesis of the radioisotope-labeled drugs is essential for AMS, and it is necessary to assess the radiation exposure in humans based on the animal data prior to the clinical study. AMS provides the mass balance data and, in combination with chromatography and fraction collection, allows the comprehensive analysis of both the known and unknown metabolites together with that of the parent compound. Complete separation of the parent compound from the metabolites and/or of one metabolite from others is needed in chromatography to obtain purely the pharmacokinetic data of the parent compound or of each metabolite. AMS needs a relatively long time of data acquisition, however, since the carbon-containing substances in the sample should be converted to a graphite form-carbon before analysis. Considering a predicted increase in the number of samples to be measured by AMS in the future, a limited number of AMS instruments might be a problem. The data acquisition by LC–MS/MS is quite rapid compared to AMS, on the other hand, since there is no need of complete separation of the analytes in chromatography due to a high assay specificity of the tandem mass spectrometry based on the specific detection of the precursor and product ions of each analyte. However, the assay method by LC–MS/MS should be validated before use in the clinical studies (22). The assay validation is a time-consuming process, while LC–MS/MS can rapidly analyze the parent drug with the known metabolites or even with other drugs simultaneously. Increased number of samples as a risk could be ignored due to the dissemination of LC–MS/MS instruments to many laboratories.

LC–MS/MS is sensitive enough in analyzing most of the compounds after microdosing. Whether to use AMS or LC–MS/MS in a microdose study should be decided according to the study purpose but not according to the sensitivity, as we described in this paper. If one wants to detect unknown metabolites or wants to obtain mass balance data, AMS is the best assay method to be chosen. If one wants to obtain the pharmacokinetic profiles of the parent drug and/or the known metabolites, such as the bioavailability and other pharmacokinetic parameters, or the contribution of hepatic and renal clearances to the total clearance, LC–MS/MS can carry out the given task with a greater performance than AMS.

Cassette microdosing is considered to be useful in finding the optimal drug candidate from multiple compounds with similar structures. We successfully conducted the simultaneous analysis of the compounds with similar chemical structures, lansoprazole and pantoprazole. Multiple compounds in cassette dosing would have similar physicochemical properties, allowing not only simultaneous sample preparation but also simultaneous analysis. We found that the same protocol for the preparation and quantification of samples for quantification could be applied to all these compounds with varying physicochemical properties: bupropion, imatinib, pioglitazone, risperidone and tamsulosin. In these cases, the compounds would have to undergo different sample preparation and/or analysis, which would take more time. Although it would depend on the physicochemical similarity of the compounds in cassette dosing, LC–MS/MS could be used in most cases of cassette dosing microdose study as long as a slight decreased sensitivity is acceptable. Simultaneous analysis of multiple compounds by AMS after cassette dosing is time- and cost-consuming, because the ^{14}C-containing fractions constituting each of the peaks of the radiolabeled parent compounds and their metabolites must be perfectly separated and collected by chromatography before their conversion to graphite-form samples. For the cassette microdosing study, the assay performance of LC–MS/MS is considered clearly higher than that of AMS. In such studies, the maximum amount of dose is 100 μg in total. Assuming that not more than five candidate drugs would be administrated as cassette dose, the $Dose_{MD}$ for each drug would be not more than 20 μg, and the $Cmax_{MD}/LLOQ$ ratio of more than 40 would be needed, which is cleared by the majority of drugs investigated (Fig. 2).

CONCLUSIONS

The present study using 31 marketed drugs with different physicochemical properties indicated that quantification with LC–MS/MS would be sensitive enough to microdose clinical studies, and could be used for simultaneous acquisition of the pharmacokinetic data of multiple drugs after cassette dosing. Considering the advantage in that the radioisotope-labeled compound is unnecessary, and the rapid analysis of the parent compound together with known metabolites, LC–MS/MS would be more widely applied in the future in order to obtain the pharmacokinetic profiles of the parent drugs in microdose studies.

ACKNOWLEDGMENTS

Naoe Yamane is an employee of JCL Bioassay Corporation, Hyogo, Japan. We thank Drs. M. Shibasaki, M. Kanai, and K. Yamatsugu of the Graduate School of Pharmaceutical Sciences at the University of Tokyo, Japan, for donating the gift of oseltamivir phosphate. We also thank to Dr. T. Tanimoto of the School of Pharmaceutical Sciences, Mukogawa Women's University, Japan, for sharing the reagents with us, and T. Hakui and M. Nishimura of GL Science Inc. for technical support of SPE methods. This work

was performed as part of "Innovative Strategies for Drug Development Using Microdosing Clinical Studies," financed by the New Energy and Industrial Technology Development Organization (NEDO). A part of this work has been presented at the 23rd JSSX (The Japanese Society for the Study of Xenobiotics) Annual Meeting "Study on practical application for liquid chromatography/tandem mass spectrometry for determination of drug concentration in human plasma in microdose study."

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