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Microdose Clinical Trial: Quantitative Determination of Nicardipine and Prediction of Metabolites in Human Plasma

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Summary: A sample treatment procedure and high-sensitive liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for quantitative determination of nicardipine in human plasma were developed for a microdose clinical trial with nicardipine, a non-radioisotope labeled drug. The calibration curve was linear in the range of 1-500 pg/mL using 1 mL of plasma. Analytical method validation for the clinical dose, for which the calibration curve was linear in the range of 0.2-100 ng/mL using 20 μL of plasma, was also conducted. Each method was successfully applied to making determinations in plasma using LC/MS/MS after administration of a microdose (100 μg) and clinical dose (20 mg) to each of six healthy volunteers. We tested new approaches in the search for metabolites in plasma after microdosing. In vitro metabolites of nicardipine were characterized using linear ion trap-fourier transform ion cyclotron resonance mass spectrometry (LIT-FTICRMS) and the nine metabolites predicted to be in plasma were analyzed using LC/MS/MS. There is a strong possibility that analysis of metabolites by LC/MS/MS may advance to utilization in microdose clinical trials with non-radioisotope labeled drugs.

Keywords: microdosing; nicardipine; LC/MS/MS; liver microsomes; in vitro metabolism; LIT-FTICRMS

Introduction

What is the primary reason for discarding a new drug in clinical development? The main reasons are of course tied to efficacy and toxicity. The prediction of human pharmacokinetics (PK) becomes a key decision point in drug development.1) Although huge compounds have been produced through combinatorial chemistry and high throughputs screening technologies, newly approved drugs are not only low in proportion to these achievements, but are actually decreasing overall. Costs for development of new drugs at pharmaceutical companies have steadily increased in step with exhaustive development strategies.2) The strategy of hit and miss with large numbers is not working out so we are reconsidering what should be considered fundamental to advancing drug development. One outcome has been greater necessity for development based on pharmacokinetics (PK) and pharmacodynamics (PD) in humans. PK data in humans prior to a traditional first-in-human study (Phase I study) is highly advantageous in selecting candidate drugs. Microdosing studies match this need. The European Agency for the Evaluation of Medicinal Products released a position paper in 2003 which defines the amounts for microdosing in human to be less than 1/100th of the therapeutic dose predicted from animal and in vitro models, while also not exceeding 100 μg.3) Subsequently, the Food and Drug Administration (FDA) also released its Guidance for Industry, Investigators, and Reviewers in Exploratory IND Studies in January 2006.4) The Japanese authority introduce this paper to Japan in 2008.5) The limited quantity doses mandate ultra-sensitive analysis of drug concentration in human plasma. Although accelerator mass spectrometry (AMS) has been used in the EU, negatives of that approach include the fact that it makes dosing of 14C-labeled drugs absolutely essential and the syntheses are costly and time consuming for pharmaceutical companies.6) It was in this light that we took notice...
of liquid chromatography-tandem mass spectrometry (LC/MS/MS), used most frequently in the sensitive analysis of drugs in human matrices after administration of a non-radioisotope labeled drug. We proved in a study that it is possible to perform analysis of fexofenadine in human plasma at all times after dosing 100 μg in non-radioisotope labeled drug form. The lower limit of quantification (LLOQ) was 10 pg/mL. We reported on actual examples of determination of drugs at a picogram per milliliter level in human plasma using LC/MS/MS.

The safety and toxicity of metabolites have come to be viewed as ever more important in the drug development process. In February of 2008, the FDA released its Guidance for Industry: Safety Testing of Drug Metabolites, in which it is stipulated that nonclinical studies should be conducted to evaluate safety in humans for the purpose of studying unique metabolites (formed only in human) or major metabolites (formed at greater than 10% of parent drug systemic exposure at steady state) since there are quantitative and/or qualitative differences between human and animal metabolites used in toxicity studies. The FDA publication also considers the fact that being able to confirm metabolites in human at an early stage of development is quite useful. Mass spectrometry has been the most promising technology for metabolites identification.

This study demonstrates our sample treatment procedure and analytical methods for microdose clinical trials using LC/MS/MS after administration of a non-radioisotope labeled drug, specifically nicardipine in this case. The chemical structures of nicardipine and the internal standard (IS), nifedipine, are shown in Figure 1. We studied practical applications – usefulness and effectiveness – of determining drug concentrations through LC/MS/MS in a microdose study. For comparison, a clinical dose study was also conducted. We thus tried to investigate metabolites in human plasma through a study conducted with microdosing as follows: (1) predict chemical structures of in vitro metabolites using linear ion trap-fourier transform ion cyclotron resonance mass spectrometry (LIT-FTICRMS), (2) analyze metabolites predicted to be in human plasma using LC/MS/MS, and (3) compare PK profiles in microdosing with those in clinical dosing.

Materials and Methods

Chemicals: Nicardipine hydrochloride, nifedipine, and all other reagents and chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Kyoto, Japan). D-Glucose-6-phosphate disodium salt (G6P) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). β-Nicotinamide adenine dinucleotide phosphate (NADP) and 250 U/vial glucose-6-phosphate dehydrogenase (G6PDH) were purchased from Oriental Yeast (Tokyo, Japan).

Human liver microsomes: Pooled mixed gender human liver microsomes (20 mg/mL microsomal protein of human liver, HLM) were purchased from XenoTech, LLC (Lenexa, KS, USA).

Preparation of stock and standard solutions: Nicardipine stock solution (100 μg/mL) and nifedipine stock solution (100 μg/mL) for IS were prepared by dissolving in acetonitrile. The nicardipine stock and IS stock solutions were serially diluted with acetonitrile as follows. For validation of an analytical method for microdosing, we prepared standard solutions for the calibration curve at concentrations of 0.05, 0.25, 0.5, 1, 2.5, 5, 10 and 25 ng/mL, standard solutions for quality control (QC) samples at concentrations of 0.05, 0.15, 2 and 20 ng/mL and an IS solution at a concentration of 50 ng/mL. These solutions were treated to be used for microdosing. For validation of an analytical method for clinical dosing, we prepared standard solutions at concentrations of 5, 12.5, 25, 62.5, 125, 250, 625 and 2500 ng/mL, standard solutions for QC samples at concentrations of 5, 10, 150, and 2000 ng/mL and an IS solution at a concentration of 50 ng/mL. These solutions were treated for clinical dosing. These stock and standard solutions were stored in a refrigerator maintained at 5°C.

Preparation of calibration standards, zero sample and QC samples: Calibration standards for microdosing at concentrations of 1, 5, 10, 20, 50, 100, 200 and 500 pg/mL were prepared by spiking 1 mL blank human plasma with 20 μL standard solution. Calibration standards for clinical dosing at concentrations of 0.2,
0.5, 1, 2.5, 5, 10, 25 and 100 ng/mL were prepared by spiking 480 μL blank human plasma with 20 μL standard solution. A 20 μL aliquot of the mixture was used. The zero sample for microdosing was prepared by spiking 1 mL blank human plasma with 20 μL acetonitrile. The zero sample for clinical dosing was prepared using 20 μL blank human plasma. QC samples for accuracy and precision were prepared in human plasma at concentrations of 1, 3, 40 and 400 pg/mL for microdosing and at 0.2, 0.4, 6 and 80 ng/mL for clinical dosing. QC samples for stability studies were prepared in human plasma at concentrations of 3 and 400 pg/mL for microdosing, and at 0.4 and 80 ng/mL for clinical dosing.

**Sample preparation for nicardipine and its metabolites in human plasma:** A 1 mL aliquot of modeled microdose plasma sample was transferred into a glass tube and 20 μL IS solution were added. After 1 mL acetonitrile was added, the mixture was mixed with a vortex mixer for 30 sec and centrifuged at 1820 × g for 5 min. The supernatant from each tube was transferred to another glass tube and evaporated to dryness under a stream of nitrogen gas at 40°C until approximately 1 mL remained. To these samples, 10 μL 28% ammonium hydroxide and 4 mL tert-butyl methyl ether were added. The mixtures were shaken for 5 min and centrifuged at 1820 × g for 5 min. The organic layer from each tube was transferred to another glass tube and evaporated to dryness under a stream of nitrogen gas at 40°C. The residues were dissolved in 100 μL mobile phase.

A 20 μL aliquot of modeled clinical dose plasma samples was transferred into a micro tube and 20 μL IS solution were added. After 160 μL acetonitrile were added, the mixture was mixed with a vortex mixer for 30 sec and centrifuged at 1820 × g for 5 min. 100 μL supernatant from each tube were transferred to another micro tube and 100 μL 5 mmol/L ammonium acetate were added.

**Analytical validation:** The analytical method for microdosing and clinical dosing was validated for selectivity, accuracy and precision in accordance with FDA guidelines for validation of bioanalytical methods. Selectivity was assessed by extracting from six different sources of plasma. Matrix effects were investigated using six independent sources of plasma. Calibration standards for microdosing and clinical dosing consisted of a zero sample and eight non-zero samples (1–500 pg/mL) and a second grouping of a zero sample and eight non-zero samples (0.2–100 ng/mL), respectively. Linearity was assessed by weighted (1/y²) least regression analysis. Intraday and inter-day precision and accuracy were determined by analyzing five sets of QC samples at four concentrations on three different days. Recovery was evaluated using the samples at three concentrations (3, 40 and 400 pg/mL for microdosing, and 0.4, 6 and 80 ng/mL for clinical dosing), and carried out in triplicate. The stability (freeze and thaw cycles, short-term stability, and long-term stability) of nicardipine in human plasma or processed samples was determined by analyzing three sets of QC samples at two concentrations. The stability of nicardipine stock solution (100 μg/mL), IS stock solution (100 μg/mL), standard solution (0.05 ng/mL) and IS solution (50 ng/mL) was determined by analyzing three sets of each solution after six hours storage at room temperature and after 67 days storage at 5°C.

**Clinical trial:** This trial was conducted after approval by the Institutional Review Board at Kitasato East hospital, Kanagawa, Japan. The design of the trial was a randomized, crossover study. Twelve healthy male volunteers who provided written informed consent were randomized to a “microdose-then-clinical dose” sequence or a “clinical dose-then-microdose” sequence. Subjects were given a single oral dose of nicardipine hydrochloride (Wako Pure Chemical Industries, Ltd., Kyoto, Japan) under fasting. The dose levels were a microdose (100 μg) and a clinical dose (20 mg). Four subjects were given a clinical dose (20 mg) after a microdose because two subjects were discontinued before the second period. The times for blood collection were 0.5, 1, 1.5, 2, 3, 4, 6 and 8 hours postdosing.

**In vitro metabolism reaction:** The incubation mixture contained a final concentration of 1 mg/mL microsomal protein in 0.1 M sodium phosphate buffer (pH 7.4), 5 mM MgCl₂, NADPH generating system (2 mM NADP, 10 mM G6P, 5 mM MgCl₂, 0.7 mU/mL G6PDH) and 10 μM substrate in a total volume of 200 μL. The enzyme reaction was initiated by the addition of solutions of NADPH generating system after pre-incubation for 5 min at 37°C. The reaction mixture was incubated for 5, 10, 15, 30 and 60 min at 37°C until the reaction was stopped by the addition of 6% ammonia solution (40 μL). To the mixture, 240 μL acetonitrile were added for deproteinization and then centrifuged at 1000 × g for 5 min at 4°C. The supernatant was used as a sample.

**LC/MS/MS analysis of nicardipine in human plasma:** The samples for microdosing and clinical dosing were analyzed according to the same analytical methods. The LC/MS/MS system consisted of an ACQUITY Ultra Performance LC (UPLC) system and Quattro Premier XE system equipped with an electrospray interface (Waters Corporation, Milford, MA, USA). The analytical column was an ACQUITY UPLC BEH C₁₈ (100 mm × 2.1 mm i.d., particle size 1.7 μm; Waters Corporation, Milford, MA, USA). The column was heated to 30°C. The mobile phase, consisting of acetonitrile/5 mM ammonium acetate (7:3, v/v), was pumped at a flow rate of 0.5 mL/min. The injection volume was 5 μL. Analysis was performed in the positive ion mode. The source and desolvation temperatures were set at 150°C and 400°C, respectively. Drying gas flow (nitrogen) and cone gas flow...
were set at 800 L/h and 50 L/h, respectively. The capillary voltage was set at 0.80 kV. Quantitation was performed by multiple reaction monitoring (MRM). The mass transition was from $m/z$ 480 to 315 for nicardipine (collision energy 24 eV, cone voltage 35 V, dwell time 0.1 sec) and from $m/z$ 347 to 315 for the IS (collision energy 12 eV, cone voltage 20 V, dwell time 0.1 sec). Analytical data were processed with MassLynx 3.5 software (Waters Corporation, Milford, MA, USA).

**LC/LIT-FTICRMS analysis of nicardipine metabolites in human liver microsomes:** The system consisted of an ACQUITY UPLC system (Waters Corporation, Milford, MA, USA) and LTQ FT, LIT-FTICR mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The analytical column was an Inertsil ODS-3 (100 mm $\times$ 2.1 mm i.d., particle size 3 $\mu$m; GL Sciences, Osaka, Japan). The column was heated to 40°C. The mobile phase, consisting of 5 mM ammonium acetate (solution A) and acetonitrile (solution B), was pumped at a flow rate of 0.2 mL/min. HPLC separation was done using a linear gradient program of 10–95% solution B for 13 min, held at 95% solution B for 5 min, 95–10% solution B for 0.1 min and held at 10% solution B for 6.9 min. The injection volume was 10 $\mu$L. Analysis was performed in the positive ion mode. The heated capillary temperature was set at 275°C. Spray voltage and capillary voltage were 4.0 kV and 15 V, respectively. Three scans were used: (1) full scan measurement in a scan range $m/z$ 150–1500 with a resolution of 25000 by FTICR, (2) data-dependent MS$^1$ measurement on the most intense ion from the MS full spectrum using LTQ and (3) data-dependent MS$^3$ measurement on the most intense ion from the MS$^2$ full spectrum using LTQ.$^{14}$ A precursor ion that had acquired its MS$^2$ spectrum was then placed on a dynamic exclusion list for a period of 12 sec. MS/MS collision energy was set at 35%. All data were processed using MetWorks 1.0.1 (Thermo Fisher Scientific, Waltham, MA, USA) which is able to search for metabolites and analyze their chemical structures.

**LC/MS/MS analysis of nicardipine metabolites in human plasma:** The samples for microdosing and clinical dosing were analyzed according to the same analytical methods. The LC/MS/MS system consisted of an ACQUITY UPLC system and Quattro Premier XE system equipped with an electrospray interface. The analytical column was an Inertsil ODS-3 (100 mm $\times$ 2.1 mm i.d., particle size 3 $\mu$m; GL Sciences, Osaka, Japan). The column was heated to 40°C. The mobile phase, consisting of 5 mM ammonium acetate (solution A) and acetonitrile (solution B), was pumped at a flow rate of 0.2 mL/min. HPLC separation was done using a linear gradient program of 10–95% solution B for 13 min, held at 95% solution B for 5 min, 95–10% solution B for 0.1 min and held at 10% solution B for 6.9 min. The injection volume was 10 $\mu$L. Analysis was performed in the positive ion mode. The source and desolvation temperatures were set at 150°C and 400°C, respectively. The drying gas flow (nitrogen) and cone gas flow were set at 800 L/h and 50 L/h, respectively. The capillary voltage was set at 0.80 kV. Quantitation was performed by MRM. The mass transition was from $m/z$ 388 to 313 for M1, $m/z$ 390 to 315 for M2, $m/z$ 464 to 313 for M3, $m/z$ 466 to 315 for M4, $m/z$ 478 to 357 for M5, $m/z$ 494 to 357 for M6, $m/z$ 496 to 359 for M7, $m/z$ 514 to 349 for M8, $m/z$ 514 to 349 for M9 (each collision energy 24 eV, each cone voltage 35 V, each dwell time 0.05 sec) and from $m/z$ 347 to 315 for the IS (collision energy 12 eV, cone voltage 20 V, dwell time 0.05 sec). The analytical data were processed with MassLynx 3.5 software.

**Results**

**Analytical validation:** There were no interfering peaks at the elution positions of nicardipine or IS. The other five blank plasma samples were also similar. Typical MRM chromatograms of blank plasma samples, zero sample and LLOQ for microdosing are shown in Figure 2. The absolute injected amount of sample for LLOQ was 1 fg nicardipine.

The calibration curve was linear over a concentration range of 1–500 pg/mL for microdosing and 0.2–100 ng/mL for clinical dosing using weighted (1/$y^2$) least squares linear regression. The main equations of the calibration curve for microdosing and clinical dosing were $y = 0.00788(\pm 0.000612)x + 0.00301(\pm 0.000553)$ and $y = 0.341(\pm 0.0120)x + 0.00611(\pm 0.00253)$, respectively. The equation for the calibration curve, $y = ax + b$, was obtained from the relationship between the ratio ($y$) of peak area of nicardipine to the IS peak area and nominal concentrations ($x$) of nicardipine. The correlation coefficient ($r$) for both calibration curves was $>0.993$. The precision and accuracy of the calibration curve for microdosing were less than 9.8% for CV and within ± 8.8% for RE. The precision and accuracy of the calibration curve for clinical dosing were less than 4.9% for CV and within ± 9.3% for RE.

Intra-day and inter-day precision and accuracy were determined by analyzing five sets of QC samples at four concentration levels on three different days. The intra-day precision and accuracy of QC samples for microdosing were less than 14.7% for CV and within ± 6.0% for RE. Inter-day precision and accuracy of QC samples for microdosing were less than 14.0% for CV and within ± 5.3% for RE. Intra-day precision and accuracy of QC samples for clinical dosing were less than 9.4% for CV and within ± 4.7% for RE. Inter-day precision and accuracy of QC samples for clinical dosing were less than 7.9% for CV and within ± 7.5% for RE. These results satisfied the criteria.

Stability data of QC samples for microdosing and clinical dosing are shown in Tables 1 and 2, respectively. In
Fig. 2. Typical MRM chromatograms of calibration curves for microdose: blank sample (A), zero sample (B) and standards at LLOQ (1 pg/mL) (C)

(IS: nifedipine)

freeze (−80°C) and thaw stability, short-term stability at room temperature and post-preparative stability (in the autosampler set at 5°C) studies, nicardipine was stable for 3 cycles, 4 hours and 48 hours, respectively. Nicardipine was stable in human plasma for 38 days storage at −20°C or −80°C. Nicardipine stock solution (100 μg/mL), IS stock solution (100 μg/mL), standard solutions (0.2, 20 and 10000 ng/mL) and IS solutions (0.5 and 10 ng/mL) were stable for 6 hours at room temperature and for 67 days at 5°C.

Clinical trial: The validated methods were applied to the analysis of nicardipine in human plasma after microdosing and clinical dosing. Quality control of the analysis was done by analyzing the calibration curve and
Table 1. Stability studies on QC samples for microdose (n = 3)

<table>
<thead>
<tr>
<th>Parameters for stability studies</th>
<th>Concentrations of nicardipine in human plasma (pg/mL)</th>
<th>3</th>
<th>400</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (pg/mL) CV (%) RE (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stability in matrix during freeze and thaw cycles</td>
<td>Cycle 3</td>
<td>3.18</td>
<td>6.0</td>
</tr>
<tr>
<td>Long-term stability in matrix for 38 days at −20°C</td>
<td>2.60</td>
<td>1.5</td>
<td>−13.3</td>
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<tr>
<td>Long-term stability in matrix for 38 days at −80°C</td>
<td>2.75</td>
<td>2.9</td>
<td>−8.3</td>
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<tr>
<td>Short-term stability in matrix for 4 hours at room temperature</td>
<td>3.06</td>
<td>7.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Post-preparative stability in processed sample in autosampler set at 5°C</td>
<td>For 24 hours</td>
<td>2.93</td>
<td>10.2</td>
</tr>
<tr>
<td>For 48 hours</td>
<td>3.12</td>
<td>1.3</td>
<td>4.0</td>
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</tbody>
</table>

Table 2. Stability studies of QC samples for clinical dose (n = 3)

<table>
<thead>
<tr>
<th>Parameters for stability studies</th>
<th>Concentrations of nicardipine in human plasma (ng/mL)</th>
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<th>80</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Mean (ng/mL) CV (%) RE (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stability in matrix during freeze and thaw cycles</td>
<td>Cycle 3</td>
<td>0.387</td>
<td>7.2</td>
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<tr>
<td>Long-term stability in matrix for 38 days at −20°C</td>
<td>0.380</td>
<td>2.6</td>
<td>−5.0</td>
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<td>Long-term stability in matrix for 38 days at −80°C</td>
<td>0.357</td>
<td>5.9</td>
<td>−10.8</td>
</tr>
<tr>
<td>Short-term stability in matrix for 4 hours at room temperature</td>
<td>0.395</td>
<td>6.8</td>
<td>−1.3</td>
</tr>
<tr>
<td>Post-preparative stability in processed sample in autosampler set at 5°C</td>
<td>For 24 hours</td>
<td>0.420</td>
<td>3.6</td>
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<tr>
<td>For 48 hours</td>
<td>0.418</td>
<td>0.5</td>
<td>4.5</td>
</tr>
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</table>

Fig. 3. Mean plasma concentration-time curves of nicardipine after receiving 100 μg solution of nicardipine (A) (n = 6) and 20 mg solution of nicardipine (B) (n = 10)

QC samples with each run. The concentrations were from 4.66 to 278 pg/mL after microdosing and 0.622 to 39.9 ng/mL, for which the expected range of calibration curve was within appropriate limits. The mean plasma concentration-time curves of nicardipine after its addition at 100 μg or 20 mg are shown in Figures 3A and 3B, respectively. Subjects administered a microdose after a clinical dose showed fluctuating concentrations due to the initially-administered clinical dose. Therefore, microdose data in six subjects administered a microdose before a clinical dose were adopted. MRM chromatograms at 8 hours after microdosing are shown in Figure 4. By LC/MS/MS methods, plasma concentrations of nicardipine after a microdose were sufficiently measurable to assess the plasma concentration-time curves of nicardipine.

**LC/LIT-FTICRMS analysis of nicardipine metabolites in human liver microsomes:** First, full scan accurate mass spectra of nicardipine and its metabolites were measured using FTICRMS. Secondly, the molecular formula of each metabolite was estimated using MetWorks 1.0.1 based on comparison between theoretical exact mass and experimental accurate mass. Thirdly, metabolized sites were predicted based on spectra for MS2 measurement or MS3 measurement and metabolite structures were identified.

Nine metabolites (M1-M9) were predicted by comparison with an extracted ion chromatogram immediately after adding substrate to microsomes and after incubation for 60 min (Fig. 5). The retention time for a given metabolite is less than for an unchanged drug because polarity of the metabolite is usually higher than that of an unchanged drug. However the retention time of M5 a-
lone eluted after that of nicardipine (11.36 min). In the case of reverse phase chromatography, some types of oxidation or cyclization in metabolite reactions occur from time to time. Table 3 shows retention time, theoretical mass and experimental mass of nicardipine and each metabolite. The experimental accurate mass leads to a molecular formula and kind of metabolite reaction. Subsequently, the fragment pattern of unchanged drug was observed from MS² and MS³ spectra and the chemical formula of metabolites was elucidated (Fig. 6). MS² product ions at m/z 315 and 359 suggested the loss of the 2,3-benzyl-N-methylaminoethyl ester moiety and 2,3-benzyl-N-methylaminoo group, respectively. MS² product ion at m/z 148 and 166 indicated the 2,3-benzyl-N-methylaminoethyl group and 2,3-benzyl-N-methylaminoo ester moiety, respectively. MS³ product ions at m/z 226, 254, 267, 269, 283 and 298 were cleaved from the MS² product ion at m/z 315. MS² and MS³ product ions of unchanged drug and M1-M9 are also summarized in Table 3.

M1: MS² product ions at m/z 313, 331 and 357 suggested the loss of the N-methylaminoethyl ester moiety, N-methylaminoethyl group and N-methylamino group, respectively. M1 possibly formed by debenzylation of the side chain and chemical structure change from a dihydropyridine to a pyridine through oxidation.

M2: MS² product ions at m/z 315 suggested the loss of the N-methylaminoethyl ester moiety. MS³ product ions at m/z 226, 254, 267, 269, 283 and 298, which were cleaved from the MS² product ion at m/z 315, were the same as the fragment pattern for MS² product ion at m/z 315 for nicardipine. It was considered that the structure was the same. MS³ product ion at m/z 359 suggested the loss of the N-methylamino group. It was thought that M2 was formed by debenzylation of the side chain.

M3: MS² product ions at m/z 313, 331 and 357 suggested the loss of the N-benzylaminoethyl ester moiety, N-benzylaminoethyl group and N-benzylamino group, respectively. Possibly, M3 was formed by N-demethylation of the side chain and the chemical structure change from a dihydropyridine to a pyridine through oxidation.

M4: MS² product ions at m/z 315 and 359 suggested the loss of the N-benzylaminoethyl ester moiety and N-benzylaminoethyl ester moiety and N-benzylaminoethyl ester moiety, respectively. MS² product ion at m/z 152 indicated the N-benzylaminoethyl ester moiety. It was inferred that M4 was formed by N-demethylation of the side chain.

M5: MS² product ions at m/z 313 and 357 suggested the loss of the N-benzyl-N-methylaminoethyl moiety and N-benzyl-N-methylamino group, respectively. MS² product ion at m/z 148 indicated the N-benzyl-N-methylaminoethyl group. M5 was thought to form by the chemical structure change from a dihydropyridine to a pyridine through oxidation.

M6: MS² product ions at m/z 313 and 357 suggested the loss of the N-benzyl-N-methylaminoethyl ester moiety and N-benzyl-N-methylamino group, respectively. It was considered that the MS² product ion at m/z 164 was produced from the N-benzyl-N-methylaminoethyl group by adding oxygen to nitrogen or a benzene ring in the N-benzyl group. Possibly M6 was formed by chemical structure change from a dihydropyridine to a pyridine through oxidation and monoxygenation on the N-benzyl-N-methylaminoethyl group to N-oxide or phenol.

M7: MS² product ions at m/z 315 and 359 suggested the loss of the N-benzyl-N-methylaminoethyl ester moiety and N-benzyl-N-methylamino group, respectively. It was considered that the MS² product ion at m/z 164 was produced from the N-benzyl-N-methylaminoethyl group by addition of oxygen to nitrogen or a benzene ring in the N-benzyl group. It was considered that the MS² product ion at m/z 182 was produced from the N-benzyl-N-methylaminoethyl ester moiety by the addition of oxygen to nitrogen in the N-aminogroup or benzene ring in the N-benzyl group. It was inferred that M7 was formed by monoxygenation on the N-benzyl-N-methylaminoethyl group to N-oxide or phenol.
Fig. 5. Extracted ion chromatogram from NADPH-supplemental human liver microsomal incubation of nicardipine (10 μM)
Incubation times: 0 min (A) and 60 min (B).
Table 3. List of predicted nicardipine metabolites in human liver microsomes

<table>
<thead>
<tr>
<th>Unchanged drug and metabolites</th>
<th>Molecular formula</th>
<th>Retention time (min)</th>
<th>Experimental accurate mass</th>
<th>Theoretical exact mass [M + H]^+</th>
<th>Mass error (mmas)</th>
<th>MS2 product ion (m/z)</th>
<th>MS3 product ion (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicardipine</td>
<td>C_{26}H_{29}N_{3}O_{6}</td>
<td>11.36</td>
<td>480.21335</td>
<td>480.21291</td>
<td>0.44</td>
<td>148, 166, 315, 359</td>
<td>226, 254, 267, 269, 283, 298</td>
</tr>
<tr>
<td>M1</td>
<td>C_{19}H_{21}N_{3}O_{6}</td>
<td>6.82</td>
<td>388.15042</td>
<td>388.15031</td>
<td>0.11</td>
<td>313, 331, 357</td>
<td>--</td>
</tr>
<tr>
<td>M2</td>
<td>C_{19}H_{23}N_{3}O_{6}</td>
<td>6.87</td>
<td>390.16595</td>
<td>390.16596</td>
<td>-0.01</td>
<td>315, 331, 357</td>
<td>226, 254, 267, 269, 283, 298</td>
</tr>
<tr>
<td>M3</td>
<td>C_{25}H_{25}N_{3}O_{6}</td>
<td>10.14</td>
<td>464.18173</td>
<td>464.18161</td>
<td>0.12</td>
<td>313, 331, 357</td>
<td>--</td>
</tr>
<tr>
<td>M4</td>
<td>C_{25}H_{27}N_{3}O_{6}</td>
<td>9.73</td>
<td>466.19754</td>
<td>466.19726</td>
<td>0.28</td>
<td>152, 313, 357</td>
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<tr>
<td>M5</td>
<td>C_{26}H_{27}N_{3}O_{6}</td>
<td>11.68</td>
<td>478.19748</td>
<td>478.19726</td>
<td>0.22</td>
<td>148, 313, 357</td>
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<tr>
<td>M6</td>
<td>C_{26}H_{27}N_{3}O_{7}</td>
<td>7.08</td>
<td>494.19217</td>
<td>494.19218</td>
<td>-0.01</td>
<td>164, 313, 357</td>
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<tr>
<td>M7</td>
<td>C_{26}H_{29}N_{3}O_{8}</td>
<td>7.19</td>
<td>514.21838</td>
<td>514.21839</td>
<td>0.06</td>
<td>164, 182, 315, 359</td>
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<td>M8</td>
<td>C_{26}H_{31}N_{3}O_{8}</td>
<td>8.01</td>
<td>514.21844</td>
<td>514.21839</td>
<td>0.05</td>
<td>166, 349, 393</td>
<td>217, 247, 290, 331</td>
</tr>
<tr>
<td>M9</td>
<td>C_{26}H_{31}N_{3}O_{8}</td>
<td>8.44</td>
<td>514.21839</td>
<td>514.21839</td>
<td>0.05</td>
<td>166, 349, 393</td>
<td>217, 247, 290, 331</td>
</tr>
</tbody>
</table>

—: Not listed because there are not necessary in the elucidation of metabolites

M8: MS² product ions at m/z 349 and 393 suggested the loss of the N-benzyl-N-methylaminoethyl ester moiety and N-benzyl-N-methylamino group after dioxidation of the dihydropyridine structure, respectively. MS³ product ions at m/z 217, 247 and 290 of MS² product ion at m/z 349 were produced by cleavage after dioxidation of the dihydropyridine structure. The MS¹ product ion at m/z 331 was produced by dehydration from the MS² product ion at m/z 349. The MS² product ion at m/z 166 pointed to the N-benzyl-N-methylaminoethyl ester moiety. It was inferred that M8 was formed by dioxhydroxylation on dihydropyridine.

M9: Although the MS² spectra of M9 were the same as those of M8, there were differences for each intensity. Therefore, M9 was considered to be a diastereomer of M8.

As a result, the chemical structures and possible fragmentation positions of nine metabolites in human microsome were predicted (Fig. 7). The peak areas for nine metabolites in human microsome at each reaction time are shown in Figure 8. Metabolite reactions to M2, M4, M5 and M8 were rapid. In M1, M3, M6, M7 and M9, the metabolic reaction was slow.

**LC/MS/MS analysis of nicardipine metabolites in human plasma:** The nine predicted metabolites and IS in human plasma of four subjects after microdosing and clinical dosing were analyzed using LC/MS/MS. The mass transition of nine metabolites was from the precursor ion to the product ion (fragment peaks of the highest relative intensity were selected), obtained from LIT-FTICRMS data. LC conditions were the same as in LIT-FTICRMS analysis. MS conditions were the same as in quantitation analysis for nicardipine.

The extracted ion chromatograms for metabolites except for M6 and M7 in human plasma after microdosing are shown in Figure 9. The retention time for the IS was 8.9 min. We observed seven metabolites (M1, M2, M3, M4, M5, M8 and M9) in plasma after clinical dosing. Although the sensitivity of the metabolites in plasma after microdosing was lower than that after clinical dosing, six of the same metabolites (M1, M2, M4, M5, M8 and M9) in plasma after microdosing were observed. M3 in plasma after microdosing was not detected due to low sensitivity since the sensitivity of M3 was the lowest for all metabolites even in plasma after clinical dosing. M6 and M7 were thus not present because they could not be observed in plasma after clinical dosing or microdosing. Figures 10A-F shows the peak area ratios of metabolites (M1, M2, M4, M5, M8 and M9) to peak area of IS after microdose and clinical dose. The Tmax of M1 and M5 for microdosing and clinical dosing were the same and those of M4, M8 and M9 were similar. Tmax of M3 after clinical dosing was 0.5 hours (results not shown).

**Discussion**

The lower limit of quantification in plasma at a 100 µg dose was calculated based on Cmax, 0.032 µg/mL at 1.0 hour, at a 20 mg dose level.¹⁵ We calculated the 1/200th value of Cmax with a 20 mg dose as Cmax when administering the drug as a microdose (100 µg dose). The value less than 1/24 the estimated value, i.e. 1 pg/mL, was set as the LLOQ. To achieve this high-sensitivity, we
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Fig. 6. Full scan mass spectrum
MS2 and MS3 spectra of nicardipine

selected an ACQUITY UPLC system, Quattro Premier XE mass spectrometer and an ACQUITY UPLC BEH C18 analytical column. High resolution on a chromatogram is proportional to particle size in an analytical column. Particle size 1.7 μm gave us outstanding column efficiency when the system was used. The analytical column, which has tolerance to high pressure up to 15000 psi (103500 kPa), allowed a high flow rate of 0.5 mL/min. The mobile phase, consisting of acetonitrile/5 mM ammonium acetate (7:3, v/v), provided good peak shapes for nicardipine and IS. The retention times were 0.6 min for nicardipine and 0.35 min for IS.

We needed plasma samples of at least 1 mL to conduct analysis at an order of picogram per milliliter, which is reasonable considering the volume of blood samples collected from human. Solid phase extraction was tried first and an interference peak at the elution position of nicardipine was observed. Although wash and elute solvent were changed, the value could not be determined quantitatively at a 1 pg/mL level. Next, sample preparation was changed to liquid-liquid extraction with t-buty methyl ether. Plasma sample (1 mL) was previously deproteinized with acetonitrile because the protein combined rate of nicardipine was from 98% to 99.5% and removal of protein in plasma is necessary before liquid-liquid extraction. When nicardipine and IS were extracted from the supernatant, pH was alkaline because their pKa were 8.6 and < 1, respectively. As a result, there was no interference peak at all at eluting positions of nicardipine and IS. At the clinical dose, LLOQ (0.2 ng/mL) could be sufficiently determined using 20 μL plasma. Thus, we selected the easiest sample preparation: protein precipitation with acetonitrile. No interference peak was observed at the eluting positions of nicardipine and IS.

To increase assay sensitivity using LC/MS/MS, the removal of endogenous substances in plasma at the point of sample treatment, usage of the most suitable mobile phase for improvement of ionization efficiency, selection of the optimal linear velocity adapted to a high-resolution analytical column in combination with a UPLC system, and an increase in selectivity by operating in a MRM.
Fig. 7. Predicted chemical structures and fragmentation positions of nicardipine metabolites in microsomes.
mode were required.

Nifedipine, a chemically-related substance of nicardipine, was used as IS for the same reason as in the previous analysis.\textsuperscript{7)} Although a stable isotope-labeled form is generally used, it is not feasible for analysis at the microdosing study stage.

We selected nicardipine hydrochloride, a potent, orally active vasodilator (hypertension, angina, cerebrovascular diseases),\textsuperscript{21)} as the drug for administration because it is a substrate of CYP3A4.\textsuperscript{22)} In this study, by LC/MS/MS plasma concentrations of nicardipine after a microdose could be sufficiently measured to assess the plasma concentration-time curves of nicardipine. Details of the results in this study including pharmacokinetic analysis and fluctuating concentrations in plasma for subjects administered a microdose after a clinical dose will be reported in another paper.

Metabolites at the development stage of new drugs have been traced exhaustively using radioisotope labeled drugs in nonclinical studies, thus providing clear information on animals. Metabolites in human are initially confirmed in a Phase I study. Nicardipine metabolites in the urine of animals and human have been investigated using \textsuperscript{14}C-labeled nicardipine.\textsuperscript{23–25)} We hope to discover metabolites when a non-radioisotope labeled drug is administered to humans in a microdosing study. This should be an effective means for narrowing down to successful drug candidates. We took on the challenge of investigating metabolites in human plasma after microdosing.

Unfortunately, metabolites in human plasma in a microdosing study could not be detected directly using LIT-FTICRMS because low quantities of metabolites were undetectable. MS\textsuperscript{3} product ion data require ion intensity of more than 10\textsuperscript{4} in order to determine structure.\textsuperscript{14)} Thus, we decided to investigate \textit{in vitro} metabolites in human liver microsomes using LIT-FTICRMS and subsequently analyze the metabolites in human plasma using LC/MS/MS after microdosing. A linear ion trap mass spectrometer may be used for qualification analysis and possible MS\textsuperscript{5} measurement because it can trap specific ions. The data dependent exclusion measurement which produces MS\textsuperscript{2} fragmentation data for many MS\textsuperscript{2} product ions are useful for chemical structural analysis of metabolites because data dependent MS\textsuperscript{3} measurementcleaves only one MS\textsuperscript{2} product ion of the highest intensity regardless of whether the ion derives from metabolic sites or not.\textsuperscript{14)} The FTICR mass spectrometer has high resolution (the highest resolution of LTQ FT: 500000) and high mass accuracy. It is capable of determining the molecular formulas of compounds. These combination systems are quite useful for drug metabolism research.\textsuperscript{26)} Tandem mass spectrometry operating in the MRM mode provides higher selectivity and sensitivity because specific precursor ions and product ions for analytes are selected in the first and third quadrupoles, respectively. In this study it was possible to determine 10 analytes (M1-M9 and IS) simultaneously. We discovered seven new \textit{in vitro} metabolites (M1, M3, M4 and M6-M9) in human liver microsomes and six new \textit{in vivo} metabolites (M1-M4, M8 and M9) in human plasma. The existence of M2 and M5 in human liver microsomes and M5 in human plasma had been previously reported.\textsuperscript{27,28)} Regarding PK profiles for each metabolite, it makes no sense to compare the values of peak area ratios themselves in microdosing versus clinical dosing because there are differences in sensitivity for metabolites and IS in each sample after microdosing and clinical dosing. Wagner \textit{et al.} reported that nicardipine undergoes linear first-pass metabolism to M5 and other metabolic pathways are responsible for the saturable first-pass metabolism observed for nicardipine.\textsuperscript{29)} Our results for nicardipine, M1, M2, M4 and M5 after microdosing and clinical dosing showed the same profiles. Although M3 after microdosing could not be detect-
Fig. 9. MRM chromatograms for seven metabolites of nicardipine in human plasma at 0.5 hours after microdosing.
Fig. 10. Semi-log plots of peak area ratios versus time after microdosing (■) and clinical dosing (□)
M1 (A), M2 (B), M4 (C), M5 (D), M8 (E), and M9 (F). (n = 4).

ed, we predicted similar profiles. Figures for the peak area ratio-time curves of M1, M4, M5, M8 and M9 were generally similar across microdosing and clinical dosing. It was considered that those of M2 were not important because the metabolite was slow. We think the paired metabolites of dihydropyridine and pyridine such as M1 and M2, M3 and M4, and M6 and M7 are produced from the first-pass metabolism pair of unchanged nicardipine and M5. The metabolic pathway from nicardipine to M5 in humans has been reported.\(^{28}\) Metabolite reactions from M2 to M1 and from M4 to M3 also occur by oxidation of the dihydropyridine ring. The retention time of metabolite M3 formed by oxidation of the dihydropyridine structure was slower than that of M4. The behavior of M2, M4, M5 and M8 was similar to in vitro metabolism. It has been reported that oxidation is mediated by CYP2C8, 2D6 and 3A4, and debenzylation is mainly catalyzed by CYP2C8 and 3A4 in human liver microsomes.\(^{27}\) The metabolic pathways for 8 urinary metabolites in dog and rat, metabolic pathways for 6 urinary metabolites in humans, and the metabolic fate of nicardipine with rats, dogs and monkeys in in vivo and in vitro studies have also been reported.\(^{24,30,31}\) Analysis of metabolites using LC/MS/MS may thus come to be applicable to microdose clinical trials with non-radioisotope labeled drugs.

Accordingly, we predicted five metabolic pathways as follows: (1) metabolite from nicardipine to M5 by first-pass oxidation, (2) metabolite from nicardipine to M8 or M9 by dioxidation on dihydropyridine, (3) metabolite from nicardipine to M2 and from M5 to M1 by debenzylation, (4) metabolite from nicardipine to M4, and from M5 to M3 by demethylation, (5) metabolite from nicardipine to M7 and from M5 to M6 by oxidation on monoxygenation on the N-benzyl-N-methylaminoethyl group to N-oxide or phenol.

Even for metabolites discovered in nonclinical studies, if the precursor and product ions are known, PK profiles of the metabolites in plasma after a microdosing study can be analyzed. It is possible to analyze quantitatively if there are standard substances. However, when the polarity of metabolites is significantly higher than that of
the parent drug, the sample treatment procedure will have to be altered. It is thus quite advantageous to obtain information on human metabolites at the microdosing stage.

References