ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
24 (2000) 237–249

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Determination of pibutidine metabolites in human plasma by LC-MS/MS

Koji Kato *, Shigeji Jingu, Naoyoshi Ogawa, Shohei Higuchi

Drug Metabolism Laboratory, Pharmaceutical Research Laboratories, Taisho Pharmaceutical Co., Ltd., Omiya, Saitama 330-8530, Japan

Received 15 March 2000; received in revised form 14 June 2000; accepted 22 June 2000

Abstract

A novel metabolite-screening procedure for pibutidine, an H_2-receptor antagonist, which uses high-performance liquid chromatography/tandem mass spectrometry (LC-MS/MS), demonstrated the presence of pibutidine and its four metabolites in plasma from volunteers who received a single dose of pibutidine hydrochloride. In order to quantitatively examine the metabolism of pibutidine, an assay based on LC-MS/MS was subsequently developed for the simultaneous determination of its metabolites in human plasma. Target analytes consisted of M-5, M-7 and M-8, which were prominently detected by the screening procedure, and M-9, which has pharmacological activity as an H_2-receptor antagonist. Metabolites and their deuterated internal standards were extracted from human plasma using an Oasis HLB extraction cartridge, and chromatographed on a Monitor C18M column. No isotope effects on chromatographic retention time were observed for any deuterated compounds, which were ionized using an electrospray ionization (ESI)- interface and detected by MS/MS in the selected reaction-monitoring (SRM) mode simultaneously with the corresponding metabolites. The assay was validated over the concentration range of 0.1 to 25.6 ng ml^{-1} and used to determine the plasma levels of metabolites in volunteers following oral administration of a 20-mg dose of pibutidine hydrochloride. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Pibutidine; Metabolism; Human plasma; LC-MS/MS; Electrospray ionization; Isotope effect

1. Introduction

Pibutidine hydrochloride, 3-amino-4-[[(Z)-4-[[4-(piperidinomethyl)-2-pyridyl]oxy]-2-butenyl]amino]-3-cyclobutene-1,2-dione monohydrochloride or IT-066 (Fig. 1), is a novel histamine H_2-receptor antagonist with potent and long lasting anti-secretory and anti-ulcer effects [1,2], in addition to gastric mucosal protective effect [3]. Pre-clinical metabolic studies in rats and dogs using [piperidino-2, 6-^{14}C] pibutidine hydrochloride and TLC showed that this drug was extensively metabolized [4,5]. The major metabolites were isolated by TLC and identified by electron ionization (EI) and chemical ionization (CI) mass spectrometry, and ^{1}H-NMR. The postulated metabolites (M-1–M-9, M-11–M-13) were subse-

* Corresponding author. Fax: +81-48-6527254.
E-mail address: s15379@ccm.taisho.co.jp (K. Kato).

0731-7085/00/$ - see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0731-7085(00)00398-8

quently synthesized and compared with chromatographic and spectral data to confirm their identification. Based on this knowledge of animal metabolism, in a previous study, the metabolism of pibutidine in humans was investigated using urine obtained from volunteers following oral administration of the unlabelled drug [6]. A novel screening method for pibutidine metabolites, using two-stage collision-induced dissociation (CID) in a triple quadrupole mass spectrometer, demonstrated that the unchanged drug and eight metabolites were present in urine. Metabolism of pibutidine in humans, which was proposed based on the results of analysis of urine, was similar in pattern to that in rats, but the extent of *O*-dealkylation producing M-1 in humans was undoubtedly lower than that in rats.

Generally, identification and quantification of metabolites in plasma are required for investigation of their potential contribution to the activity and/or safety profile of a drug being examined. However, the plasma metabolites of pibutidine in humans have not yet been reported in detail. In the present study, qualitative and quantitative analyses of pibutidine metabolites in plasma at much lower levels than those in urine were carried out. As internal standards for quantitative analysis, the corresponding piperidinyl-D_{10} analogues of each metabolite were used. Utilization of isotopically labeled analogues for quantitative analysis by LC-ESI-MS can yield great efficiency by co-elution of an internal standard with an analyte in order to eliminate matrix effect [7,8] and to obtain calibration curve linearity [7]. However, molecules uniformly deuterium-labeled at the piperidine ring (D_{10}-compounds) are eluted before the corresponding unlabeled compounds in reversed-phase high-performance liquid chromatography (RP-HPLC), because deuterated compounds are less lipophilic than their protium isomers [9]. The isotope effect on HPLC behavior is influenced by HPLC conditions [10,11]. In order to minimize differences in retention time between labeled and unlabeled compounds, HPLC conditions were examined in the present study.

This paper describes studies of the usefulness of LC-MS/MS for examination of the metabolism of pibutidine in humans, including the following: screening for pibutidine metabolites in plasma; development and validation study of an assay system for the simultaneous determination of the significant metabolites of pibutidine in plasma; and application of the method to analysis of plasma samples obtained from a clinical study trial.

2. Experimental

2.1. Materials

Pibutidine hydrochloride was synthesized at Sumitomo Seika Chemical (Osaka, Japan). Au-

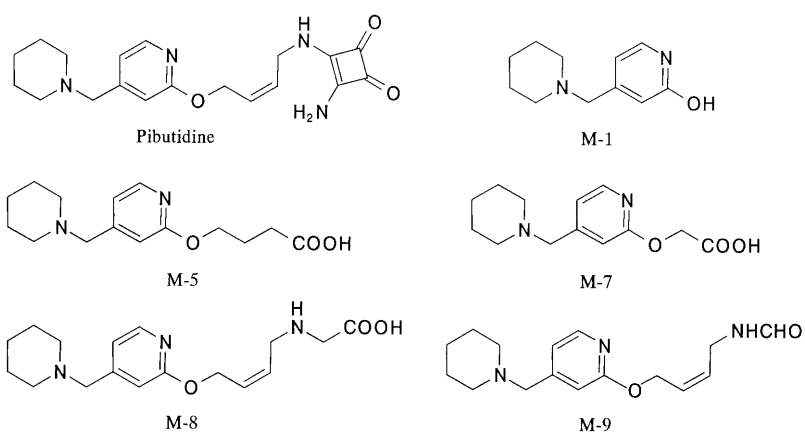

Fig. 1. Chemical structures of pibutidine and its metabolites.

thentic standards of pibutidine metabolites (M-1 – M-9, M-11 – M-13) and of deuterated analogues (D_{10}-pibutidine, D_{10}-M-8 and D_{10}-M-9) were synthesized at Taisho Pharmaceutical Research Laboratories (Saitama, Japan). The standards of M-5, M-7, M-8 and M-9 were hydrogen chloride (HCl) salts. All the D_{10}-analogues were uniformly deuterium-labeled at the piperidine ring. A stock solution of each of the standards was prepared at 100 μg ml^{-1} in methanol, kept refrigerated (approximately 4°C) when not in use, and was stable for several months.

HPLC-grade methanol, acetonitrile and chloroform, analytical grade ammonia solution and acetic acid, and reagent-grade ammonium formate and formic acid were obtained from Wako Pure Chemical Industries (Osaka, Japan). HPLC-grade distilled water and reagent-grade ammonium acetate were obtained from Kanto Chemical (Tokyo, Japan). Amberlite XAD-2 resin was purchased from Organo (Tokyo, Japan) and prepared for use by washing successively with methanol, distilled water and 2% ammonia solution. Oasis HLB extraction cartridge was purchased from Waters Corporation (Milford, MA). Nitrogen was generated by a Balston 75-760 nitrogen-generation system (Whatman, Haverhill, MA). The purity of the nitrogen was 99.8% or higher, depending on the flow rate of the gas. Argon was of ultra-high purity grade (99.999%, Taiyo Toyo Sanso, Osaka, Japan).

2.2. Instrumentation

Chromatographic separations were performed with an HP 1050 LC system (Hewlett-Packard, Palo Alto, CA). Mass spectrometric analyses were performed with a TSQ 700 triple quadrupole mass spectrometer equipped with a Finnigan ESI interface (Finnigan MAT, San Jose, CA). Data were manipulated on Finnigan ICIS ver.8.2.1 and LC_{QUAN} ver.1.2 software.

2.3. Screening for plasma metabolites

A 0.5-ml aliquot of plasma sample was basified with 80 μl of 25% ammonia solution and submitted to liquid–liquid extraction with 5 ml of chloroform. The organic phase was evaporated with a Yamato RD-41 centrifugal evaporator (Tokyo, Japan), and the residue was reconstituted in 25 μl of ammonium acetate (20 mM, pH 7.0)-methanol (90:10, v/v). The aqueous phase was loaded onto an XAD-2 resin column (bed volume: 10 ml) and allowed to pass through by gravity flow. The column was then washed with 10 ml of 2% ammonia solution. Analytes trapped on the column were eluted with 20 ml of methanol. The eluate was dried by the evaporator, and the residue was reconstituted in 25 μl of ammonium acetate (20 mM, pH 7.0)–methanol (90:10, v/v) and combined with the above extract. The combined extracts were filtered through a 0.22-μm Ultrafree membrane (Millipore, Bedfold, MA), and 20 μl of the filtrate was injected into the LC-MS/MS system.

The HPLC column was a Capcell PAK C_{18} UG120 S5 column (250×2.0 mm, Shiseido, Tokyo, Japan) maintained at a temperature of 50°C. Solvents A, B and C were 20 mM ammonium acetate adjusted to pH 7.0 with 25% ammonia solution, methanol and acetonitrile, respectively. All the HPLC solvents were degassed using helium. Mobile phases were delivered at 0.16 ml min^{-1} using 10% B and 0% C between 0 min and 10 min, followed by a linear gradient to 17% B and 35% C at 38 min, and then isocratic to 40 min with 17% B and 35% C. Electrospray ionization was performed with a spray voltage of $+4.0$ kV in the positive ion mode. The heated capillary temperature was maintained at 210°C. Nitrogen served both as sheath gas with an operating pressure of 70 psi and as auxiliary gas with a flow-rate of 10 units. Two-stage CID scanning was performed with an octapole-offset voltage of -30 V and a second quadrupole (Q_2)- offset voltage of -25 V. Precursor and product ion scannings were performed with Q_2-offset voltages of -20 and -25 V, respectively. Argon was used as the collision gas at 1.9 mTorr in Q_2. These scanning modes were operated with dwell times of approximately 4 ms per ion.

2.4. Isolation of D_{10}-M-5 and D_{10}-M-7

D_{10}-M-5 and D_{10}-M-7 were isolated from urine (total volume: ca. 960 ml) collected over a 24-h period after a single oral administration of 200-mg D_{10}-pibutidine to each of two dogs. Urine subdivided into 50-ml portions was transferred into a separatory funnel together with 4 ml of 25% ammonia solution, and submitted to extraction with 250 ml of chloroform. The resultant organic phase was discarded, and then the aqueous phase was loaded onto an Oasis cartridge (6 g) preconditioned sequentially with 100 ml each of methanol and distilled water. The cartridge was then washed with 100 ml of distilled water. Both D_{10}-M-5 and D_{10}-M-7 trapped on the cartridge were eluted with 150 ml of methanol. The eluate was concentrated by a Yamato RE52 rotary evaporator and then lyophilized using a Christ Gamma 2–20 (Martin Christ, Osterode, Germany). The residues obtained from all of the subdivided urine samples were combined and dissolved in 6 ml of ammonium acetate (20 mM, pH 7.0). The resulting solution containing the D_{10}-metabolites of interest was divided into 450 μl portions, each of which was separated by RP-HPLC on a Wakosil–II 5C18 AR column (250 × 6.0 mm, Wako Pure Chemical Industries, Osaka, Japan) at ambient temperature. Solvents A, B and C were ammonium acetate (20 mM, pH 7.0), methanol and acetonitrile, respectively. Mobile phases were delivered at 1.0 ml min^{-1} using 10% B and 0% C between 0 min and 10 min, followed by a linear gradient to 20% B and 40% C at 60 min. The fractions corresponding to D_{10}-M-5 and D_{10}-M-7 were obtained with a Gilson model 201 fraction collector (Villiers-le-Bel, France) and evaporated by the centrifugal evaporator, separately. The residues of D_{10}-M-5 and D_{10}-M-7 were redissolved in 2.4 and 1.6 ml of 0.5% acetic acid, respectively. The individual solutions were subdivided into 400 μl portions, and each was further separated on the Wakosil–II column described above, using the following conditions. Solvents A and B were 0.5% acetic acid and methanol, respectively. Mobile phases were delivered at 1.0 ml min^{-1} using 0% B between 0 min and 10 min, followed by a linear gradient to 30% B at 60 min. Each fraction of D_{10}-M-5 and D_{10}-M-7 was obtained and evaporated to dryness. The individual D_{10}-metabolite residues were reconstituted in methanol, and further diluted with methanol until the signal intensities of their respective D_{10}-metabolites were equal to those of the corresponding unlabeled standards in stock solutions (100 μg ml^{-1}) on LC-MS chromatograms obtained by monitoring the protonated molecules. Finally, 50 ml for D_{10}-M-5 and 15 ml for D_{10}-M-7 were prepared as 100 μg ml^{-1} stock solutions.

2.5. Simultaneous determination of metabolites in plasma

2.5.1. Preparation of standard solutions

For the simultaneous determination of M-5, M-7, M-8 and M-9 (Fig. 1), an internal standard solution was prepared by mixing four D_{10}-compounds (D_{10}-M-5, D_{10}-M-7, D_{10}-M-8 and D_{10}-M-9) at concentrations of 0.2 μg ml^{-1} each in distilled water–methanol (9:1, v/v). A working solution of the metabolite standards (512 ng ml^{-1} as HCl salts) was prepared by mixing 51.2-μl portions of each of four stock solutions of M-5, M-7, M-8 and M-9 (100 μg ml^{-1}) in a 10-ml volumetric flask, diluting with distilled water and making up to volume. Moreover, this solution was diluted in series with distilled water to four concentrations of 128, 32, 8 and 2 ng ml^{-1}.

2.5.2. Assay procedure

To prepare calibration samples, 25 μl of the appropriate working solution were added to 500 μl of human blank plasma, followed by the addition of 250 μl of carbonate buffer (0.1 M, pH 10) and 20 μl of the internal standard solution. The concentrations of the calibration samples corresponded to 0.1, 0.4, 1.6, 6.4 and 25.6 ng ml^{-1} as HCl salts in plasma. The quality control (QC) samples were prepared in the same fashion as the calibration samples at concentrations corresponding to 0.1, 1.6 and 25.6 ng ml^{-1}. A blank sample (no standard) was prepared by spiking 25 μl of distilled water (to substitute for the working solution), 250 μl of carbonate buffer and 20 μl of the internal standard solution into 500 μl of human

blank plasma. The study samples were prepared by adding 250 μl of carbonate buffer and 20 μl of the internal standard solution to 500 μl of the clinical samples. Each sample was loaded onto an Oasis cartridge (30 mg) preconditioned with 1 ml each of methanol, distilled water and carbonate buffer. The cartridge was washed with 1 ml each of carbonate buffer and distilled water. Analytes of interest were then eluted from the cartridge with 1 ml of methanol. The eluate was dried by the centrifugal evaporator, and the residue was reconstituted in 100 μl of 5 mM ammonium formate adjusted to pH 3.1 with formic acid. This solution was filtered through a 0.22-μm membrane, and 10 μl was injected into the LC-MS/MS system.

2.5.3. LC-MS/MS conditions

The HPLC column was a Monitor C18M (50 × 2.0 mm, Column Engineering, Ontario, CA) maintained at a temperature of 40°C. Solvents A and B were ammonium formate (5 mM, pH 3.1) and acetonitrile, respectively. Mobile phases were delivered at 0.20 ml min^{-1} using a linear gradient from 0% B to 30% B between 0 min and 7 min, and then an isocratic condition to 10 min with 30% B. Equilibration of the column with solvent A was then performed for 10 min. An automated post-column switching valve (Valco, Houston, TX) was employed to divert the eluent from the analytical column to waste for the first 5.5 min following injection and then into the MS/MS system for the rest of the run. ESI was performed in the positive ion mode with a spray voltage at + 5.0 kV. The heated capillary temperature was maintained at 230°C. Nitrogen served both as sheath gas with an operating pressure of 85 psi and as auxiliary gas with a flow-rate of 10 units. SRM mode was used for the simultaneous detection of M-7, D_{10}-M-7, M-8 and D_{10}-M-8 until 8 min, and then of M-5, D_{10}-M-5, M-9 and D_{10}-M-9 until 10 min, with dwell times of 500ms. The first quadrupole (Q_1) was set to transmit the protonated molecules at m/z 279 (M-5), 251 (M-7), 320 (M-8), 290 (M-9), 289 (D_{10}-M-5), 261 (D_{10}-M-7), 330 (D_{10}-M-8) and 300 (D_{10}-M-9). These ions were fragmented by CID with argon (approximately 1.7 mTorr) at 20 eV for M-5 and M-9, at 25 eV for M-7 and at 15 eV for M-8 in Q_2. The product ions attributed to the [(piperidinomethylpyridyloxy moiety) + 2H]$^+$ were monitored via the third quadrupole (Q_3) at m/z 193 and 203 for the metabolites and their internal standards, respectively.

2.5.4. Assay validation

The assay for simultaneous determination of the four metabolites was validated over the concentration range 0.1–25.6 ng ml^{-1} as HCl salts. Calibration lines for each batch analyzed were determined using five calibration standards plus blank matrix (blanks were not used in the calculation). The concentrations as free base of the standards at the respective points on the calibration graphs (x) were calculated as follows: nominal concentration [ng base ml^{-1}] = desired concentration as HCl salt [ng ml^{-1}] × weighing factor × molecular weight as base/molecular weight as HCl salt. The lines of best fit for calibration standards were calculated by $1/y^2$-weighted least-squares regression analysis based on the peak area ratios of analytes to respective internal standards (y). The precision and accuracy of the assay were determined by replicate analyses of QC samples. The precision was evaluated by the relative standard deviations (RSD) occurring within (intra-) and between (inter-) run analysis. The accuracy was expressed as the percentage of the theoretically determined concentration. In the intra-run analysis, six replicates of QC samples were analyzed at each concentration level. The inter-run precision was determined by analyzing QC samples in duplicate during six analytical runs ($n = 12$). The lower limit of quantification (LLOQ) of the assay was defined as the lowest concentration on the calibration curve, with acceptable precision ($< 20\%$) and accuracy ($100 \pm 20\%$). Assay specificity for endogenous interfering peaks was assessed by running individual drug-free plasma samples from six volunteers. The ion suppression by matrix material was evaluated at three concentrations in triplicate, based on the difference in signal response between the standard solutions and the processed blank plasma residues spiked with the standards after sample preparation. Extraction recovery was determined in triplicate at three

concentrations by comparing the signal intensities of samples spiked with the standards prior to extraction with those of blank plasma extracts spiked with the standards. The stability of metabolites in the injection sample at approximately 4°C was determined by reanalyzing the same injection samples at three days after preparation with a calibration curve newly determined on the day of analysis. The freeze-thaw stability and long-term storage stability of metabolites in plasma were also assessed. The freeze-thaw stability was determined by repeatedly (three cycles) freezing and thawing QC samples at three levels and analyzing the samples in quadruplicate. The long-term storage stability was evaluated by analyzing QC samples after 5 months of storage at below −20°C. The LC-MS/MS system was routinely checked for carry-over by injecting a blank sample after the injection of a sample with highest concentrations of metabolites.

2.6. Clinical samples

Plasma samples were collected from healthy male volunteers who received a single oral dose of 20 or 40 mg pibutidine hydrochloride, and were stored frozen at below −20°C until analysis.

3. Results and discussion

3.1. Pibutidine metabolites in plasma

Two-stage CID scanning [6] was performed as follows. First, non-mass selected ions derived by ESI were fragmented by in-source CID promoted by octapole-offset potential. Second, the diagnostic fragment ions attributed to the [(piperidinomethylpyridyloxy moiety) + 2H]$^+$ (m/z 193, 207 and 209) were isolated in Q_1 and fragmented by CID in Q_2 to yield metabolite-specific ions, which were monitored via Q_3. The structurally characteristic ions at m/z 193, 207 and 209 depended on the oxidized form of the piperidine ring, implying the unchanged form, N-α-oxide, and hydroxylated form/N-oxide, respectively. Fig. 2A shows typical two-stage CID mass chromatograms obtained from blank human plasma spiked with 10 ng ml^{-1} of the authentic standards of pibutidine, M-1 to M-9 and M-11 to M-13. M-10 could not be identified structurally before the present study, although it had been found in canine urine [5]. The traces presented clear baselines and exhibited no endogenous substances, with the exception of some peaks on the m/z 207-related chromatogram. This method was therefore used for the analysis of pibutidine metabolites in clinical plasma samples. Reconstructed chromatograms of plasma obtained from subjects receiving a single oral dose of pibutidine hydrochloride (40 mg) are shown in Fig. 2B. The chromatogram related to m/z 193 included five major peaks; these peaks were identified as dose-related compounds (M-7, M-1, M-5, M-8 and parent drug, in the order of HPLC elution) by matching their precursor ion spectra of m/z 193, product ion spectra of the protonated molecules and HPLC retention times with those of authentic standards. Most peaks with m/z 207 were assigned to endogenous substances present in drug-free plasma.

Accordingly, M-5, M-7 and M-8, which were prominently detected by the screening, were considered target compounds for the quantitative analysis described below. On comparison of the peak intensity ratios to respective standards among the metabolites detected, the plasma level of M-1 was estimated one order lower than those of the others. Although M-9 could not be detected on screening, it was considered a target because it has pharmacological activity equivalent to the parent drug as an H_2-receptor antagonist.

3.2. D_{10}-metabolites as internal standards

When external standard calibrations were used in ESI-MS/MS quantitative analyses for pibutidine and its metabolites in human urine, the linear dynamic ranges appeared to be relatively narrow [6]. On the other hand, calibration with isotopically labeled internal standard for pibutidine provided linearity over a wide concentration range [7]. Accordingly, in the present assay as well the respective isotope-labeled analogues of metabolites were used as internal standards, of which two D_{10}-compounds (D_{10}-M-5, D_{10}-M-7)

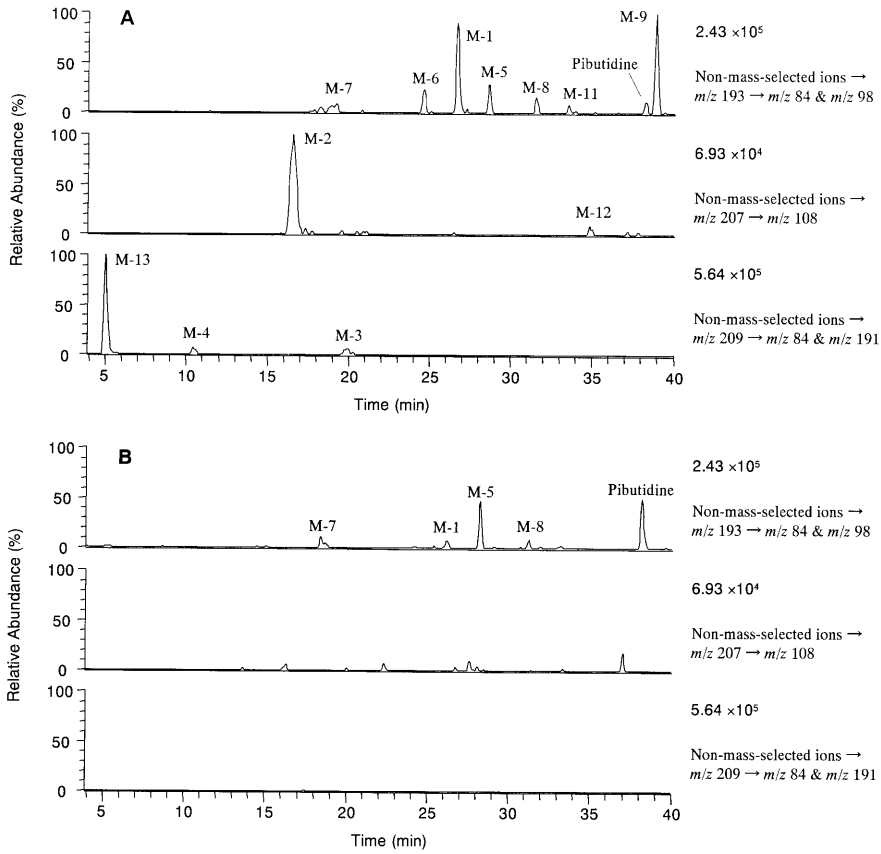

Fig. 2. Two-stage CID mass chromatograms of (A) blank human plasma spiked with 10 ng ml^{-1} of the standards, and (B) plasma obtained at 2 h after oral administration of pibutidine hydrochloride (40 mg) to healthy subjects.

that were difficult to synthesize were isolated from urine of dogs following administration of D_{10}-pibutidine. The purities of these compounds were checked mass-spectrometrically. The positive electrospray mass spectra of D_{10}-M-5 and D_{10}-M-7 are shown in Fig. 3. The spectra yielded predominantly the corresponding protonated molecules, and provided no evidence that the unlabeled compounds coexisted with their D_{10}-analogues.

3.3. Pretreatment procedures

In order to detect pibutidine metabolites present at trace levels in plasma, a pretreatment procedure with enrichment of analytes was required. Unfortunately, pibutidine undergoes extensive biotransformation in humans [6], and the resulting metabolites have a wide variety of physical properties. For comprehensive recovery in the metabolite screening, the hydrophobic metabolites were first extracted by chloroform, and subsequently the hydrophilic metabolites were recovered from the aqueous phase with an Amberlite XAD-2 column. This tedious procedure was not suitable for quantitative analysis requiring handling of a large number of samples. In order to overcome this difficulty, an Oasis HLB extraction cartridge packed with a macroporous copolymer made from a balanced ratio of two monomers, hydrophilic *N*-vinylpyrrolidone and lipophilic divinylbenzene [12], was employed. The extraction cartridge was able to extract almost all pibutidine metabolites from plasma with satisfactory recoveries by a general procedure for solid-phase ex-

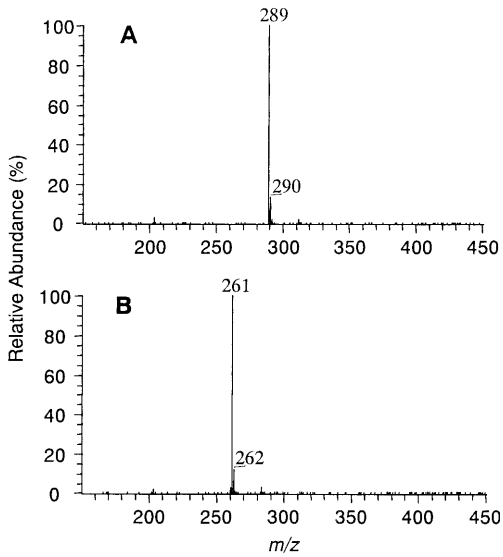

Fig. 3. Positive electrospray mass spectra of (A) D_{10}-M-5 and (B) D_{10}-M-7 isolated from canine urine.

traction. This relatively simple method without tedious multiple sample preparation allowed higher sample throughput for quantitative analysis.

3.4. Chromatographic separation

When a neutral solvent system (ammonium acetate (20 mM, pH 7.0)-methanol-acetonitrile) and a Capcell PAK C_{18} column were used for RP-HPLC separation of pibutidine metabolites and their internal standards, the isotope effect was observed as the difference in retention time between the D_0- and D_{10}-compounds (Fig. 4). For quantitative analysis by LC-ESI-MS, co-eluting an isotopically labeled internal standard with an analyte is useful for enhancing the reliability of the assay, by eliminating matrix effect and obtaining the calibration curve linearity [7]. Therefore, in order to reduce the isotope effect, an acidic solvent system (ammonium formate (5 mM, pH 3.1)-acetonitrile) and a Monitor C18M column with a length of 50 mm were selected. With use of mobile phase of decreased ionic strength (5 mM) as well, this column could always elute analytes of interest as sharp peaks. M-5, M-7, M-8 and M-9 were eluted at 8.5, 6.7, 6.9 and 8.5 min, respectively, and were well separated from pibutidine (8.9 min), which could be present in clinical samples at a much higher plasma level than those of metabolites.

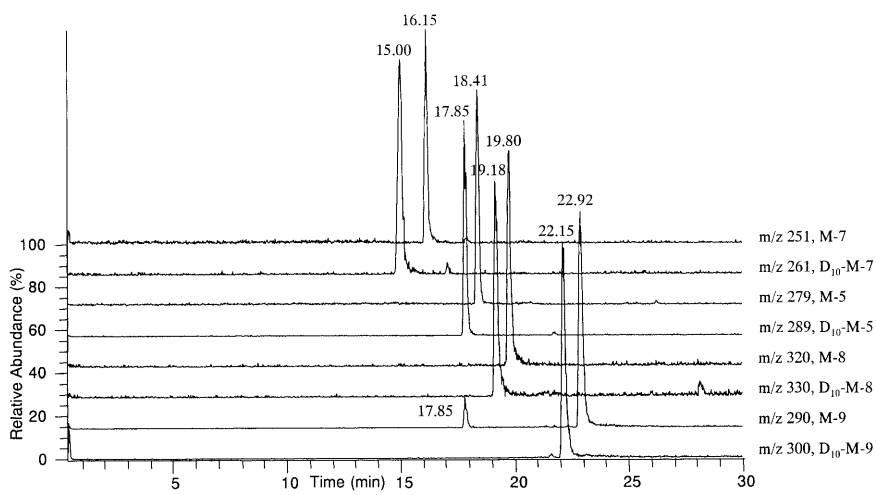

Fig. 4. RP-HPLC separation of pibutidine metabolites and their deuterium-labeled analogues. Column: Capcell PAK C_{18} UG120 S5, 150 × 2.0 mm (Shiseido, Tokyo, Japan); Mobile phase: ammonium acetate (20 mM, pH 7.0)-methanol-acetonitrile, 0-5 min, 90:10:0, v/v/v → linear gradient → 20–30 min, 30:30:40, v/v/v.

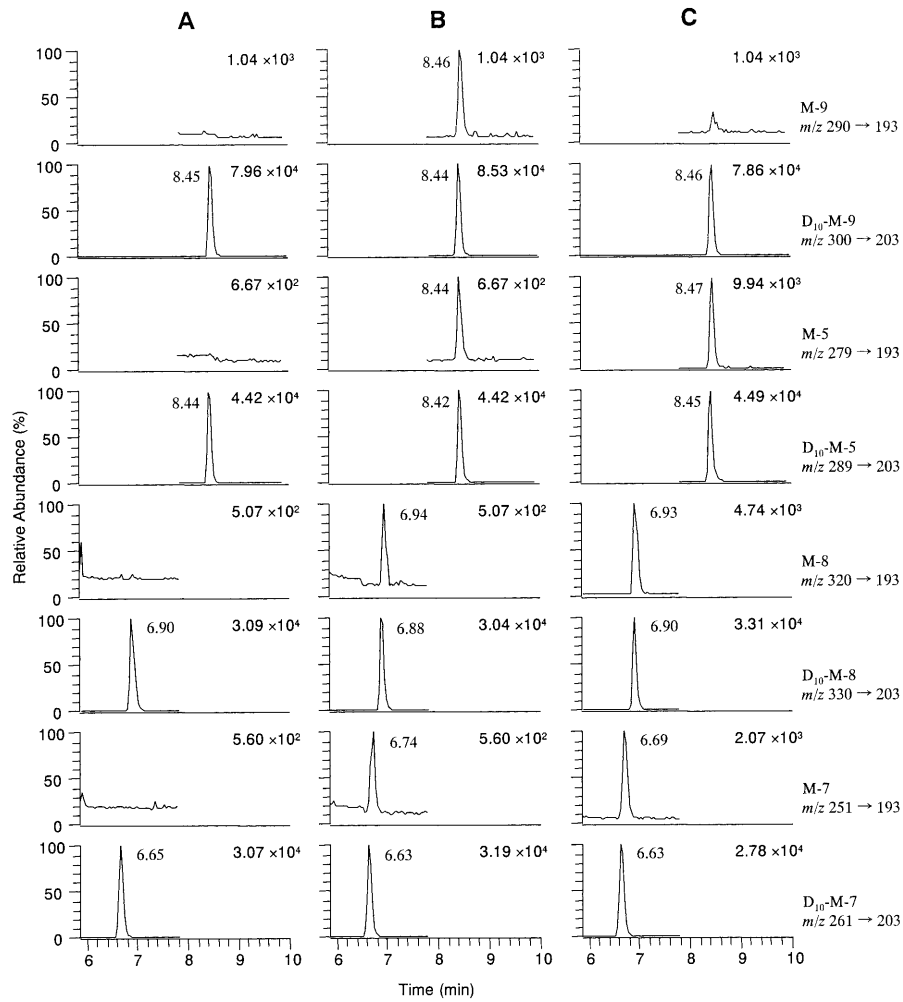

Fig. 5. Representative LC-MS/MS chromatograms of plasma samples obtained by selected reaction monitoring; A, blank plasma; B, blank plasma spiked with standards at 0.1 ng ml^{-1}; C, plasma at 2 h after a 20-mg dose of pibutidine hydrochloride (assayed levels: 1.50 ng ml^{-1} for M-5, 0.46 ng ml^{-1} for M-7, 0.79 ng ml^{-1} for M-8, and below the LLOQ for M-9). The numbers in the upper right-hand corner of the chromatograms indicate the peak height expressed in arbitrary units.

3.5. Assay validation

High assay specificity and shorter run time were achieved with use of gradient elution on HPLC separation with a short analytical column in addition to SRM detection, in which only the transitions of the precursor ions to their respective product ions were monitored. Despite the non-specific sample preparation, none of the blank plasma samples obtained from six volunteers were found to contain endogenous compounds that could interfere with the assay: virtually no interference was observed in the chromatograms, as illustrated for a blank plasma sample in Fig. 5A. For six daily runs, the relationships between the peak area ratios of analytes to respective internal standards and the concentrations of analytes were linear over the concentration range of 0.1-25.6 ng ml^{-1} as the HCl salts in human plasma (Table 1): linear regression analyses yielded correlation coefficients (r) of the calibration curves above 0.996 for all analytes. The differences between the nom-

Table 1
Linearity and reproducibility of calibration curves for pibutidine metabolites in human plasma

Nominal concentration (ng base ml^{-1})	Back-calculated concentration/nominal concentration (%)						Mean	RSD (%)
	Day-1	Day-2	Day-3	Day-4	Day-5	Day-6		
(A) M-5[a]								
0.0887	99.2	99.3	103.4	100.1	99.1	98.9		
0.3547	102.8	101.7	90.4	98.4	103.7	107.6		
1.419	102.0	104.9	103.9	105.0	101.1	99.8		
5.676	100.4	98.6	103.2	99.8	100.4	98.6		
22.70	96.2	96.3	101.9	97.3	96.3	96.4		
Slope	0.145517	0.140734	0.138726	0.143123	0.144371	0.152117	0.144098	3.2
Intercept	0.000359	0.000723	0.001684	0.000073	0.000367	−0.002793		
r	0.999594	0.999375	0.997811	0.999505	0.999569	0.998942		
(B) M-7[b]								
0.0881	99.1	100.3	98.0	102.0	99.5	104.0		
0.3523	103.2	97.9	113.3	93.4	101.7	89.1		
1.409	105.0	106.2	101.8	106.9	100.6	104.4		
5.637	93.9	95.0	99.4	100.5	99.4	106.0		
22.55	100.3	102.0	92.2	99.1	98.8	100.6		
Slope	0.148606	0.149888	0.153366	0.147513	0.148461	0.150011	0.149641	1.4
Intercept	0.000167	0.001093	−0.001510	0.002637	0.000556	0.001877		
r	0.998879	0.998948	0.996718	0.998603	0.999925	0.996875		
(C) M-8[c]								
0.0902	104.1	100.7	102.5	102.4	100.2	99.9		
0.3608	89.7	94.8	90.8	90.7	98.0	100.3		
1.443	107.1	113.3	110.6	107.4	101.1	103.7		
5.773	99.2	95.5	102.7	100.5	102.6	99.0		
23.09	103.7	99.7	97.6	102.1	98.4	97.5		
Slope	0.154426	0.165840	0.164942	0.168394	0.165161	0.181419	0.166697	5.2
Intercept	0.003700	−0.003213	0.001152	−0.000433	−0.004502	−0.004528		
r	0.997156	0.997112	0.996805	0.997510	0.999781	0.999701		
(D) M-9[d]								
0.0893	101.2	103.6	100.2	100.1	101.5	100.6		
0.3570	95.1	88.9	98.6	99.5	95.4	97.7		
1.428	100.5	103.6	102.3	101.1	100.0	101.5		
5.712	96.1	100.0	99.4	97.8	102.6	97.8		
22.85	109.6	108.6	99.6	101.7	101.2	102.9		
Slope	0.137916	0.137392	0.142620	0.143921	0.139240	0.149004	0.141682	3.1
Intercept	−0.001271	0.000287	0.000925	−0.000843	0.001181	0.000040		
r	0.998165	0.996325	0.999885	0.999866	0.999518	0.999686		

[a] Weighing factor: 1.003; Molecular weight: 314.81 (HCl salt), 278.35 (free base).
[b] Weighing factor: 1.009; Molecular weight: 286.76 (HCl salt), 250.30 (free base).
[c] Weighing factor: 1.005; Molecular weight: 355.86 (HCl salt), 319.40 (free base).
[d] Weighing factor: 1.005; Molecular weight: 325.84 (HCl salt), 289.38 (free base).

Table 2
Precision and accuracy of the LC-MS/MS method for determination of pibutidine metabolites

Compound	Nominal concentration (ng base ml^{-1})	Within run[a]		Between run[b]	
		Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
M-5	0.0887	6.2	100.2	12.3	106.1
	1.419	4.3	105.7	3.6	103.4
	22.70	1.7	102.1	2.1	97.9
M-7	0.0881	15.7	100.0	16.9	101.7
	1.409	3.4	102.4	4.6	101.9
	22.55	2.4	105.2	3.7	99.0
M-8	0.0902	4.1	108.6	12.8	97.7
	1.443	2.6	104.0	4.3	103.4
	23.09	3.2	100.1	3.5	99.3
M-9	0.0893	7.7	103.0	13.3	99.6
	1.428	4.0	105.0	2.8	102.6
	22.85	2.6	108.3	4.1	103.7

[a] Six replicates at each level.
[b] Based on 6 runs, with duplicates at each level in each run.

inal standard concentration and the concentration back-calculated from the linear regression lines were less than 15% for each point on the calibration curves, and the RSD of the slopes of the calibration lines ranged from 1.4 to 5.2% for all the analytes. Representative chromatograms of a plasma sample spiked at the LLOQ level (0.1 ng ml^{-1} as HCl salts) are presented in Fig. 5B. The signal-to-noise ratios were greater than two orders of magnitude for all analytes. The precision and accuracy at LLOQ were respectively less than 17% and within 100 $\pm$ 9% of theoretical concentrations; these values were acceptable. The intra- and inter-day precision with QC samples at the highest and middle concentrations were below 5% for all analytes. Accuracy was between 97.9 and 108.3% for all compounds (Table 2). The overall means of extraction recovery were 88.5% for M-5, 86.9% for M-7, 90.0% for M-8 and 91.5% for M-9. No difference was observed in signal intensity between the standards and those that were added to blank plasma that had been processed. This result suggested that virtually no ion suppression by matrix materials occurred under the assay conditions. A summary of the stability of pibutidine metabolites in human plasma is shown in Table 3. No deterioration was observed for any analytes of interest after repeated freezing and thawing of plasma samples (three cycles), nor was there a lowering in their concentrations during storage for 3 days at approximately 4°C after pretreatment. For up to 5 months of frozen storage, the analytes were fairly stable; the decreases were less than 4% of theoretically calculated concentration, except for the LLOQ samples, for which precision and accuracy were rather poor.

3.6. Clinical application

The LC-MS/MS quantitative method was used to determine the concentrations of metabolites in plasma following oral administration of 20 mg pibutidine hydrochloride. Chromatograms obtained from plasma at 2 h after administration are shown in Fig. 5C; all of the four metabolites were detectable. However, the concentrations of M-9 were below the LLOQ at all time points. The plasma concentration-time profiles for the metabolites are shown in Fig. 6 together with data for unchanged drug [13] at 1, 2, 4, 6 and 12 h after administration. Areas under the plasma concentration-time curves ($AUC_{0-12\ hr}$) for M-5, M-7 and M-8 were approximately 38, 14 and 15% of that for pibutidine, respectively, after single oral

Table 3
Stability of pibutidine metabolites

Compound	Nominal concentration (ng base ml^{-1})	Before storage[a]		Freeze-thaw 3 cycles[a]		Processed sample[a]		5 months at −20°C[a]	
		Percentage of theoretical value	RSD (%)	Percentage of theoretical value	RSD (%)	Percentage of theoretical value	RSD (%)	Percentage of theoretical value	RSD (%)
M-5	0.0887	99.3	7.7	107.8	3.0	102.5	8.3	94.0	10.2
	1.419	106.3	5.3	98.6	4.9	105.5	5.9	97.3	3.0
	22.70	102.9	1.4	96.2	1.0	102.0	2.3	96.3	2.5
M-7	0.0881	101.4	19.6	100.5	18.5	103.8	13.0	83.8	18.7
	1.409	100.2	1.2	100.1	2.0	107.7	3.7	101.0	3.4
	22.55	105.0	3.1	99.5	1.9	104.1	2.3	99.8	2.5
M-8	0.0902	109.3	5.2	115.5	11.2	106.7	14.4	86.3	19.7
	1.443	103.4	3.2	100.8	4.3	106.0	3.2	100.3	3.7
	23.09	98.2	1.4	99.8	5.1	103.1	4.4	97.0	1.3
M-9	0.0893	101.2	9.4	97.9	7.0	102.3	6.1	97.8	7.3
	1.428	105.8	4.6	96.4	4.1	106.0	3.0	96.8	2.2
	22.85	107.9	2.1	102.4	0.3	109.4	1.6	97.9	2.2

[a] Four replicates at each level.

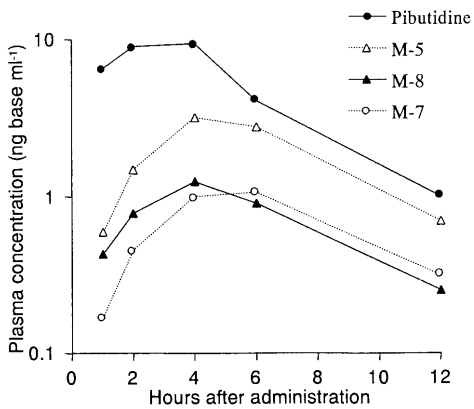

Fig. 6. Plasma concentration–time curves of pibutidine and its metabolites after oral administration of pibutidine hydrochloride (20 mg) to healthy subjects.

administration of 20 mg pibutidine hydrochloride, and these metabolites appeared to be eliminated from plasma with a profile similar to that for the unchanged compound from 6 through 12 h. Based on these results, general pharmacological studies [14] and single-dose toxicity studies [15] of these compounds were performed. On the other hand, since plasma concentrations of M-9, the metabolite with H_2-receptor antagonistic activity, were below the LLOQ for all of the samples, as mentioned above, it appeared that M-9 make almost no contribution to the pharmacological activity of pibutidine.

4. Conclusions

Methods for the identification and quantification of pibutidine metabolites in human plasma were developed using LC-MS/MS methodology. The two-stage CID method was useful for screening these metabolites at nanogram levels in plasma. As a result, pibutidine and its four metabolites were identified in plasma. A highly selective and sensitive method was subsequently developed and validated for the simultaneous determination of the significant metabolites of pibutidine, M-5, M-7, M-8 and M-9, with HPLC conditions yielding a shorter run time to increase sample throughput. This assay method has been effectively used for the analysis of samples in a clinical study trial.

References

[1] Y. Isobe, H. Kijima, C. Ito, H. Nagai, M. Muramatsu, I. Arai, K. Fukushima, Jpn. Pharmacol. Ther. 27 (1999) 261–271.
[2] M. Muramatsu, H. Hirose-Kijima, H. Aihara, S. Otomo, Jpn. J. Pharmacol. 57 (1991) 13–23.
[3] Y. Kiuchi, Y. Isobe, H. Kijima, T. Saitoh, S. Kajita, S. Yasuda, et al., Jpn. Pharmacol. Ther. 27 (1999) 289–293.
[4] M. Arai, Y. Nagase, T. Sasaki, S. Jingu, J. Yamaguchi, S. Kawahara, et al., Jpn. Pharmacol. Ther. 27 (1999) 619–642.
[5] M. Arai, Y. Nagase, S. Jingu, M. Akimoto, S. Higuchi, Jpn. Pharmacol. Ther. 27 (1999) 605–618.
[6] K. Kato, S. Jingu, N. Ogawa, S. Higuchi, Rapid Commun. Mass Spectrom. 13 (1999) 1626–1632.
[7] K. Kato, S. Jingu, N. Ogawa, S. Higuchi, J. Chromatgr. B 740 (2000) 187–193.
[8] B.K. Matuszewski, C.M. Chavez-Eng, M.L. Constanzer, J. Chromatogr. B 716 (1998) 195–208.
[9] N.E. Tayar, H. van de Waterbeemd, M. Gryllaki, B. Testa, W. Trager, Int. J. Pharm. 19 (1984) 271–281.
[10] H. Shibasaki, T. Furuta, Y. Kasuya, J. Chromatogr. B 692 (1997) 7–14.
[11] N. Tanaka, E.R. Thornton, J. Am. Chem. Soc. 98 (1976) 1617–1619.
[12] E.S.P. Bouvier, D.M. Martin, P.C. Iraneta, M. Capparella, Y-F. Cheng, D.J. Phillips, LC/GC 15 (1997) 152–158.
[13] J. Azuma, T. Aoki, Y. Seto, Jpn. Phamacol. Ther. 27 (1999) 973–990.
[14] Y. Isobe, H. Kijima, K. Fukushima, H. Iizuka, S. Unagami, K. Yamaguchi, et al., Jpn. Pharmacol. Ther. 27 (1999) 543–561.
[15] S. Nakagawa, M. Sugiura, M. Murakami, H. Yamada, I. Nakamura, M. Okamoto, M. Kimura, Jpn. Pharmacol. Ther. 27 (1999) 315–329.