Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 853 (2007) 70–79
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Development and validation of bioanalytical methods for Imidafenacin (KRP-197/ONO-8025) and its metabolites in human plasma by liquid chromatography–tandem mass spectrometry

Yuichi Masuda*, Norihiro Kanayama, Shigeru Manita, Satoshi Ohmori, Tsuyoshi Ooie

Research Center, Kyorin Pharmaceutical Co. Ltd., 1848 Nogi, Nogi-machi, Shimotsuga-gun, Tochigi 329-0114, Japan

Received 17 October 2006; accepted 23 February 2007
Available online 14 March 2007

Abstract

Imidafenacin (KRP-197/ONO-8025, IM), 4-(2-methyl-1*H*-imidazol-1-yl)-2,2-diphenylbutanamide, is a new antimuscarinic agent currently under application for the indication of treatment of overactive bladder in Japan. We developed and validated the sensitive and selective bioanalytical methods for the extremely low levels of IM and its metabolite, M-2 (Method 1), M-4 (Method 2) and M-9 (Method 3) in human plasma using liquid chromatography–tandem mass spectrometry (LC–MS/MS). In each method, plasma sample was extracted by solid phase extraction, separated on a semi-micro high performance liquid chromatography column and detected by tandem mass spectrometer with an atmospheric pressure chemical ionization or ionspray interface. Selected reaction monitoring mode was used for quantification. Each method was found to have acceptable accuracy, precision, stability, selectivity and linearity over the concentration range of 10–500 pg/mL for IM and M-2, 10–1000 pg/mL for M-4 and 50–5000 pg/mL for M-9. Using these analytical methods, concentration profiles of IM and its metabolites in human plasma were successfully determined even in the low pg/mL levels after oral administration of IM at the therapeutic dosage of 0.1 mg.

Keywords: Imidafenacin; KRP-197; ONO-8025; Metabolites; Human-plasma; LC–MS/MS

1. Introduction

Micturition is mediated through the actions of several neurotransmitters affecting tone of the smooth muscle in the urinary bladder. Acetylcholine (ACh), in particular, plays an important role in contracting the reservoir and relaxing the outlet through activation of ACh receptors [1]. Compounds with high affinity for the muscarinic ACh receptors, such as propiverine, tolterodine, oxybutynin, darifenacin and solifenacin have been used in the management of overactive bladder (OAB) [2,3].

Imidafenacin (KRP-197/ONO-8025, IM), 4-(2-methyl-1*H*-imidazol-1-yl)-2,2-diphenylbutanamide (Fig. 1), is an antagonist for muscarinic ACh receptor currently under application for the indication of treatment of OAB in Japan, and is scheduled for launch into the market before long. IM has high affinity for M_1 and M_3 receptor subtypes [4–6]. Therefore, IM inhibits bladder contraction by mediating antagonism to M_3 receptor and regulates ACh release by inhibiting prejunctional facilitatory M_1 subtype [7–10].

Administration of IM (0.1 mg twice daily) improved symptoms of OAB with good tolerability and safety in Phase III clinical trial conducted in Japan. Because its therapeutic dosage is about 10–1000 times lower compared with those of other anticholinergics [11], the quantification methods with selectivity and sensitivity sufficient for determining the extremely low level (pg/mL) of concentrations of IM and three main metabolites; M-2, M-4 and M-9 in human plasma are indispensable in order to consider the efficacy and safety of these compounds after administration of IM in humans.

In this study, we developed and validated the highly selective and sensitive analytical methods for determination of IM, M-2, M-4 and M-9 in human plasma using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Then, the methods were applied to determination of the concentrations at pg/mL

* Corresponding author. Tel.: +81 280 57 1551; fax: +81 280 57 2336.
E-mail address: yuuchi.masuda@mb.kyorin-pharm.co.jp (Y. Masuda).

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.02.047

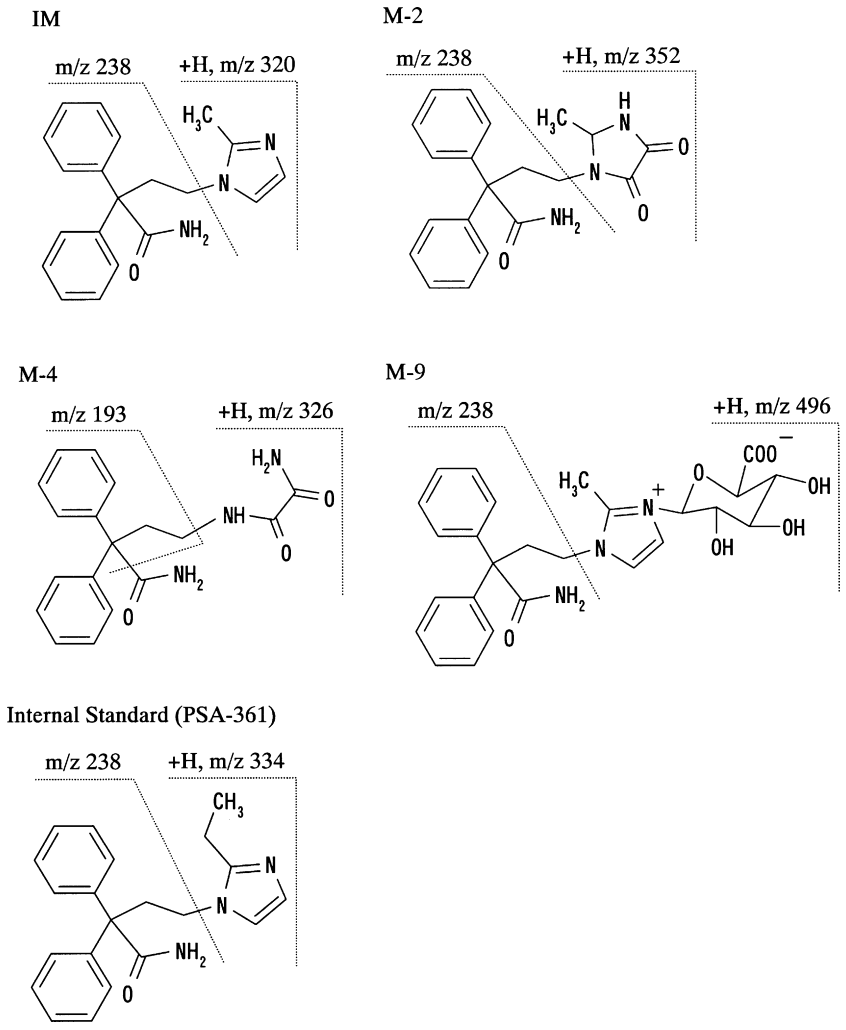

Fig. 1. Chemical structures of IM, its metabolites M-2, M-4, M-9 and the internal standard PSA-361. Broken lines indicate precursor and product ions obtained by collision-induced dissociation in LC–MS/MS system.

levels of IM and its metabolites in human plasma after oral administration of IM at the therapeutic dosage of 0.1 mg in healthy Japanese male volunteers.

2. Experimental

2.1. Materials

IM, M-2, M-4, M-9 and the internal standard (IS, PSA-361, Fig. 1) were synthesized at Kyorin Pharmaceutical Co. Ltd. Acetonitrile, methanol and distilled water were of high performance liquid chromatography (HPLC) grade. All other reagents were of analytical grade. Human control plasma was collected from healthy male volunteers or was purchased from Interstate Blood Bank (Memphis, TN, USA). Human control plasma for selectivity test was collected individually from six healthy male volunteers.

2.2. Instrumentation

LC–MS/MS instruments were separately applied to the three analytical methods for determination of IM and M-2 (Method 1), M-4 (Method 2) and M-9 (Method 3) as listed in Table 1. The triple-stage quadrupole mass spectrometers, API 300, API 4000 and API 3000 (Applied Biosystems, Foster City, CA, USA) were used as MS/MS units for Methods 1, 2 and 3, respectively, with an atmospheric pressure chemical ionization (APCI) interface: Heated-Nebulizer (Method 1) or an ionspray interface: TurboIonSpray (Methods 2 and 3). Agilent 1100 series (Agilent Technologies, Palo Alto, CA, USA) was used as an HPLC unit for the three methods.

2.3. Chromatographic conditions

For determination of IM and M-2 (Method 1), a semi-micro HPLC column, CAPCELLPAK C_{18} UG120 (5 μm, 2.0 mm i.d. × 150 mm, Shiseido, Tokyo, Japan) was used with a guard column, CAPCELLPAK C_{18} UG120 (5 μm, 2.0 mm i.d. × 10 mm, Shiseido, Tokyo, Japan). Mobile phase was the mixture of distilled water containing 0.1% formic acid and acetonitrile in the volumetric ratio of 75:25 and 65:35 for IM and M-2, respectively. The flow rate was 0.2 mL/min and the column temperature was maintained at 25 °C.

Table 1
Extraction procedures and LC–MS/MS conditions for determination of IM and M-2 (Method 1), M-4 (Method 2) and M-9 (Method 3)

Procedures and conditions	Method 1 IM, M-2	Method 2 M-4	Method 3 M-9
Extraction procedures			
Sample volume (mL)	1	0.2	0.2
Solid-phase extraction cartridge	ISOLUTE C18 (EC)	OASIS HLB	OASIS HLB
Analytical column	CAPCELLPAK C_{18} UG120	XTerra MS C_{18}	TSKgel ODS-80Ts
Mobile phase	Isocratic mixture of distilled water containing 0.1% formic acid and acetonitrile, 75:25 (IM), 65:35 (M-2)	Sequential mixture of distilled water containing 0.5% formic acid and acetonitrile containing 0.5% formic acid	Sequential mixture of distilled water containing 0.1% formic acid and acetonitrile
LC–MS/MS conditions			
Instruments	API 300	API 4000	API 3000
Interfaces	Heated-nebulizer	TurboIonSpray	TurboIonSpray
Polarization	Positive	Positive	Positive
Corona discharge needle current	2 μA	–	–
Ionspray voltage	–	5.5 kV	5.5 kV
Nebulizer/ionspray temperature (°C)	450	400	500
Orifice voltage (V)	10	41 (M-4), 61 (IS)	41 (M-9), 36 (IS)
Focusing ring voltage (V)	150	–	200 (M-9), 150 (IS)
Collision energy (eV)	22.5	29 (M-4), 23 (IS)	39 (M-9), 23 (IS)

For determination of M-4 (Method 2), a semi-micro HPLC column, XTerra MS C_{18} (5 μm, 2.1 mm i.d. × 50 mm, Waters, Milford, MA, USA) was used. As the mobile phase, distilled water containing 0.5% formic acid (A) and acetonitrile containing 0.5% formic acid (B) consisted of a starting composition of A/B (80:20) for 8 min, and changed to A/B (5:95) in 0.2 min according to a linear gradient mode and maintained at A/B (5:95) for 2 min. The flow rate was 0.2 mL/min and the column temperature was maintained at 40 °C.

For determination of M-9 (Method 3), a semi-micro HPLC column, TSKgel ODS-80Ts (5 μm, 2.0 mm i.d. × 150 mm, Tosoh, Tokyo, Japan) was used with a guard column, Develosil ODS-UG-S (5 μm, 1.5 mm i.d. × 10 mm, Nomura Chemical, Aichi, Japan). As the mobile phase, distilled water containing 0.1% formic acid (A) and acetonitrile (B) consisted of a starting composition of A/B (73:27) for 8 min, and changed to A/B (5:95) in 1 min according to a linear gradient mode. The flow rate was 0.2 mL/min and the column temperature was maintained at 25 °C.

2.4. Mass spectrometric conditions

Mass spectrometers were operated in the positive mode for all analytes. Quantification was performed using selected reaction monitoring of the transition from *m/z* 320 to 238 for IM, from *m/z* 352 to 238 for M-2, from *m/z* 326 to 193 for M-4, from *m/z* 496 to 238 for M-9 and from *m/z* 334 to 238 for IS (Fig. 1). Nitrogen was used as collision gas. Zero air was used as nebulizer, auxiliary and turbo gas. Mass spectrometric conditions for each determination method are listed in Table 1.

2.5. Preparation of standard stock solutions

Standard stock solutions of IM, M-2, M-4 and M-9 were prepared with methanol at the concentrations of 10 μg/mL for IM and M-2, and 100 μg/mL for M-4 and M-9, by weighing separately for standard samples and quality-control (QC) samples. Stock solution of IS was prepared with methanol at the concentration of 10 or 100 μg/mL. Stock solutions were stored in a refrigerator (below 10 °C) for appropriate period assured for each analyte.

2.6. Preparation of standard and QC samples

For IM, M-2 and M-9, their standard stock solutions were sequentially diluted with methanol. A portion (appropriate volume) of diluted solutions were placed in the test tubes and concentrated to dryness under nitrogen at 40 °C. The residues were reconstituted in the control plasma to prepare the standard samples and the QC samples at the concentrations ranged from 10 to 500 pg/mL for IM and M-2, and from 50 to 5000 pg/mL for M-9. For M-4, standard stock solution was sequentially diluted with distilled water. A portion (0.1 mL) of diluted solutions were mixed with 0.2 mL of the control plasma to prepare the standard samples as the actual concentrations ranged from 10 to 1000 pg/mL in plasma. Another portion (0.1 mL) of diluted solutions were mixed with 9.9 mL of the control plasma to prepare the QC samples at the concentrations ranged from 10 to 1000 pg/mL.

2.7. Extraction procedure

Three separate extraction procedures were applied for determination of IM and M-2 (Method 1), M-4 (Method 2) and M-9 (Method 3) as listed in Table 1.

2.7.1. Method 1

Simultaneous extraction of IM and M-2 required 1 mL of human plasma. A solid-phase extraction cartridge ISOLUTE C_{18} (EC) (100 mg/1 mL, International Sorbent Technology,

Mid Glamorgan, UK) was activated with methanol and distilled water. Then, 1 mL of the plasma sample was mixed with 0.1 mL of IS solution (20 ng/mL, diluted with distilled water) and the mixture was applied into the activated ISOLUTE C18 (EC). After washing twice with 1 mL of distilled water, 1 mL of methanol was added to the ISOLUTE C18 (EC) to elute the intended compounds. The eluate was concentrated to dryness under nitrogen at 40 °C. The residue was reconstituted in 0.1 mL of distilled water containing 0.1% formic acid–acetonitrile (65:35, v/v) and filtrated with a Chromatodisc (pore size 0.45 μm, GL Sciences). Finally, 20 μL of the filtrate was injected into the LC–MS/MS system separately for IM and M-2 determination with different composition of mobile phase for each analyte.

2.7.2. Method 2

Extraction of M-4 required 0.2 mL of human plasma. A solid-phase extraction cartridge OASIS HLB (30 mg/1 mL, Waters, Milford, MA, USA) was activated with methanol and distilled water. Then, 0.2 mL of the plasma sample was mixed with 0.1 mL of IS solution (1 ng/mL, diluted in distilled water) and 0.8 mL of distilled water. In the case of the standard samples, 0.3 mL of the standard samples prepared in Section 2.6 was mixed with 0.1 mL of IS solution and 0.7 mL of distilled water. This mixture was applied to the activated OASIS HLB. After washing with 1 mL of distilled water and 1 mL of methanol–distilled water (40:60, v/v), the washing solution was removed by centrifugation of OASIS HLB. Then, 1 mL of methanol was added to the OASIS HLB to elute the intended compound. The eluate was collected by centrifugation and was concentrated to dryness under nitrogen at 40 °C. The residue was reconstituted in 0.1 mL of the mixture of distilled water containing 0.5% formic acid and acetonitrile containing 0.5% formic acid (80:20, v/v) and filtrated with a Chromatodisc (pore size 0.45 μm, GL Sciences). Finally, 20 μL of the filtrate was injected into the LC–MS/MS system.

2.7.3. Method 3

Extraction of M-9 required 0.2 mL of human plasma. OASIS HLB (30 mg/1 mL) was placed in a high-throughput solid-phase extraction system SPE215 (Gilson, Middleton, WI, USA). First, 0.2 mL of the plasma sample was mixed with 0.1 mL of IS solution (1 ng/mL, diluted in distilled water containing 0.1% formic acid) and 0.7 mL of distilled water containing 0.1% formic acid. Second, OASIS HLB was activated with methanol and 0.1% formic acid. Third, the plasma sample processed at the first step was applied into the activated OASIS HLB and then, OASIS HLB was washed four times with 1 mL of 5% methanol. Finally, 1 mL of methanol was added to the OASIS HLB to elute the intended compound. After removing from the SPE215 system, the eluate was concentrated to dryness under nitrogen at 40 °C. The residue was reconstituted in 0.1 mL of distilled water containing 0.1% formic acid–acetonitrile (73:27, v/v), and then 20 μL of the resultant solution was injected into the LC–MS/MS system.

3. Results

3.1. Development of analytical methods of IM and its metabolites

To evaluate the pharmacokinetic profiles of IM and three metabolites in human plasma, we developed and validated the analytical methods in this study. Because plasma concentrations of IM and the metabolites were extremely low after oral administration of IM at the therapeutic dosage of 0.1 mg twice daily, we developed three separate analytical methods to detect each analyte with sufficient sensitivity. IM and M-2 were simultaneously extracted and eluted on individual HPLC conditions by changing the composition of mobile phase for detecting both analytes sensitively (Method 1). HPLC elution profile of M-4 was so distinct from other analytes that the analytical method of M-4 was separately developed (Method 2). M-9 was a glucuronic acid conjugate of IM, identified in the human plasma in the early clinical phase. Though M-9 was eluted separately from IM on the HPLC chromatogram, degradation to a protonated molecular ion of IM was observed with APCI used in Method 1. Consequently, the analytical method for M-9 was developed separately using stable ionspray ionization (Method 3). These methods had good selectivity and sensitivity for determination of each analyte in human plasma. Validation of these analytical methods was carried out as described below.

3.2. Method validation

Validation of the analytical methods was carried out in accordance with the “FDA Guidance for Industry, Bioanalytical Method Validation” [12].

3.2.1. Selectivity

Selectivity was evaluated by comparing LC–MS/MS chromatograms of the control plasma obtained from six individuals with that of plasma spiked with IM, M-2, M-4, M-9 and IS in each analytical method. There were no peaks interfering with IM and M-2 (Method 1, Fig. 2), M-4 (Method 2, Fig. 3) and M-9 (Method 3, Fig. 4).

3.2.2. Extraction recovery

Extraction recovery was evaluated by comparing peak areas of the extracted standard plasma sample spiked with IM, M-2, M-4, M-9 and IS with the mean peak areas spiked after-extraction samples at the same concentrations. Each analyte was sufficiently extracted with recovery of 83.1% or more (Table 2). IS was also sufficiently extracted with recovery of 80.7% or more.

3.2.3. Linearity of calibration curve

Calibration curves were constructed by plotting the peak area ratios (analyte/IS) of plasma standard versus nominal concentration. Weighted (1/concentration) least squares linear regression was employed. IM, M-2, M-4 and M-9 had good linearity in the concentration ranges from 10 to 500, from

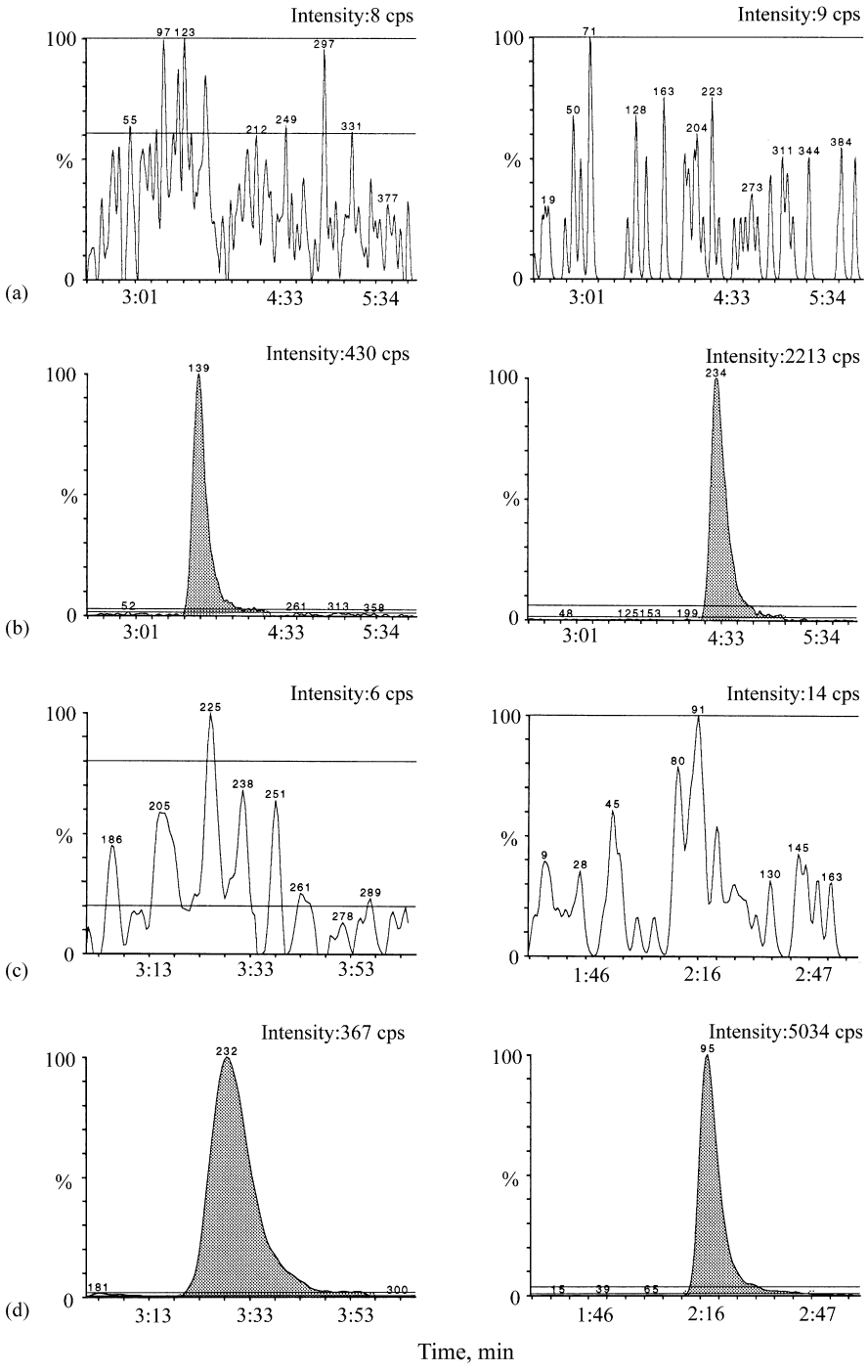

Fig. 2. Representative LC–MS/MS chromatograms of human plasma analyzed by Method 1. (a): Control plasma during determination of IM, (b): plasma spiked with IM (200 pg/mL) and IS during determination of IM, (c): control plasma during determination of M-2, (d): plasma spiked with M-2 and IS during determination of M-2. Left panels monitored IM or M-2 and right panels monitored IS.

10 to 500, from 10 to 1000 and from 50 to 5000 pg/mL, respectively. The typical equations of the calibration curves were as follows: $y = -1.4480 \times 10^{-3} + 9.063 \times 10^{-4}x$ for IM, $y = -1.7696 \times 10^{-4} + 5.901 \times 10^{-4}x$ for M-2, $y = 7.93 \times 10^{-6} + 1.93 \times 10^{-4}x$ for M-4, $y = 6.29 \times 10^{-4} + 1.22 \times 10^{-4}x$ for M-9. The correlation coefficients (r) of IM, M-2, M-4 and M-9 with 1/concentration weighting were 0.9996, 0.9975, 0.9997 and 0.9998, respectively.

3.2.4. Reproducibility and the lower limit of quantification

The reproducibility of the method was examined using the QC samples. The accuracy and precision were calculated from the five measurements conducted within a day (intra-day reproducibility) and from the two measurements conducted on three separate days (inter-day reproducibility). Results in the intra-day and inter-day reproducibility tests are shown in Table 3.

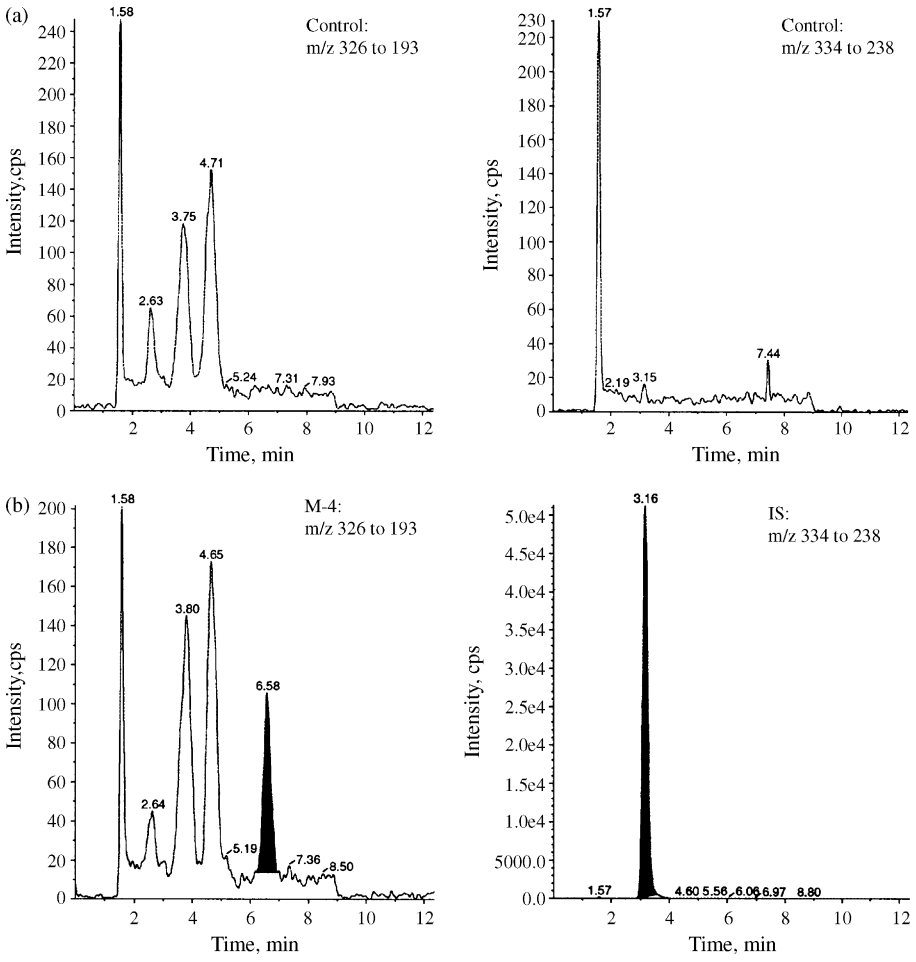

Fig. 3. Representative LC–MS/MS chromatograms of human plasma analyzed by Method 2. (a): Control plasma, (b): plasma spiked with M-4 (10 pg/mL) and IS.

The accuracy and precision of all analytes were within ±15.8% and 12.3% at the lowest concentrations, and within ±12.6% and 12.5% at higher concentrations. From the results of reproducibility tests, the lower limits of quantification (LLOQ) in these methods were 10 pg/mL for IM, M-2 and M-4, and 50 pg/mL for M-9; and the dynamic concentration ranges were 10–500 pg/mL for IM, M-2, 10–1000 pg/mL for M-4, and 50–5000 pg/mL for M-9.

Table 2
Extraction recoveries of IM, M-2, M-4 and M-9 from plasma samples in the solid-phase extraction process

Method	Nominal Concentration (pg/mL)	Analyte	Recovery (mean ± SD, %)
1	400	IM	95.2 ± 2.4
		M-2	96.6 ± 10.4
		IS	100.7 ± 2.5
2	50	M-4	87.6 ± 2.9
		IS	87.6 ± 0.8
	500	M-4	90.7 ± 1.1
		IS	84.5 ± 0.7
3	400	M-9	90.7 ± 1.0
		IS	80.7 ± 1.2
	4000	M-9	92.6 ± 3.5
		IS	83.1 ± 1.9

3.2.5. Stability

Stabilities of IM, M-2, M-4, M-9 and IS in the standard stock solutions are shown in Table 4. Residual ratio was 79.6% or more for all analytes after storage for 24 h at room temperature (ca. 25 °C), and 96.9% or more for IM and M-2 after storage for 10 weeks, for M-4 after storage for 3 months, and for M-9 after storage for 1 month at refrigerating temperature (below 10 °C).

Stabilities of IM, M-2, M-4 and M-9 in the plasma samples and the pretreated samples are shown in Table 5. Residual ratios were 92.0% or more for IM and 89.6% or more for M-2 after storage for 24 h at room temperature (ca. 25 °C), after storage for 12 months at freezing temperature (set at −20 °C), after three cycles of freezing and thawing and 24 h after pretreatment at room temperature (ca. 25 °C). Residual ratios were 91.5% or more for M-4 after storage for 24 h at room temperature (ca. 25 °C), after storage for 11 weeks at freezing temperature (set to −30 °C), after three cycles of freezing and thawing and 48 h after pretreatment at room temperature (ca. 25 °C). Residual ratios were 96.8% or more for M-9 after storage for 24 h at room temperature (ca. 25 °C), after storage for 3 months at freezing temperature (set at −40 °C), after three cycles of freezing and

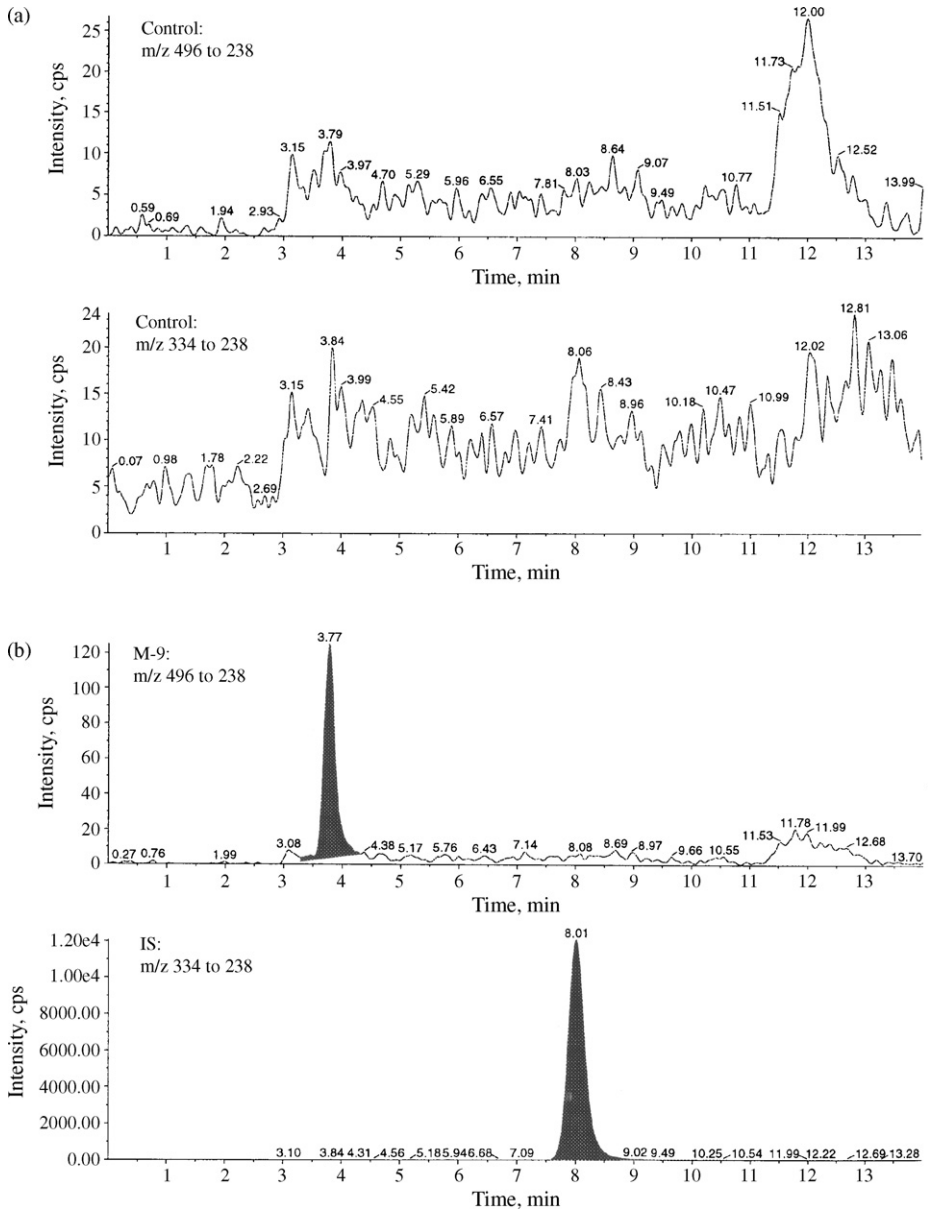

Fig. 4. Representative LC–MS/MS chromatograms of human plasma analyzed by Method 3. (a): Control plasma, (b): plasma spiked with M-9 (50 pg/mL) and IS.

thawing and at 48 h after pretreatment at room temperature (ca. 25 °C).

These results demonstrate that the standard stock solutions can be stored in a refrigerator (below 10 °C) for at least 1 month, and plasma samples can be handled at room temperature (ca. 25 °C) for at least 24 h, and can be stored in a freezer (set at −40 °C) for at least 11 weeks, and these samples can be handled at room temperature (ca. 25 °C) after pretreatment within 24 h.

3.3. Application of analytical methods

The aforementioned analytical methods were applied to the study of pharmacokinetic profiles of IM, M-2, M-4 and M-9 after oral administration of 0.1 mg IM to 12 healthy Japanese male volunteers. Plasma samples were withdrawn and stored in a freezer set at −40 °C until analysis. QC samples were simultaneously determined in duplicate in the following three concentrations: within three-fold of LLOQ, in the midrange, and near the upper limit of the dynamic range.

The concentration profiles of IM, M-2, M-4 and M-9 in human plasma determined by these analytical methods are shown in Fig. 5. There were no peaks preventing determination of these analytes on the chromatograms of actual plasma samples. The QC samples ranged within 15% of the nominal concentrations, which met the acceptance criteria of the "FDA Guidance for Industry, Bioanalytical Method Validation" [12]. After oral administration of 0.1 mg of IM, the maximum concentrations of IM, M-2, M-4 and M-9 reached 471, 97.9, 162 and 504 pg/mL, respectively, in healthy Japanese male volunteers. These analytical methods can be successfully applied to the analysis of the concentration profiles of IM and metabo-

Table 3
Accuracy and precision in the intra-day and inter-day reproducibility tests

Analytes	Intra-day reproducibility			Inter-day reproducibility		
Concentration added (pg/mL)	Concentration found (pg/mL)	Accuracy (%)	Precision (%)	Concentration found (pg/mL)	Accuracy (%)	Precision (%)
IM						
10	9.6 ± 1.0	−4.0	10.7	9.12 ± 0.23	−8.8	2.5
20	18.6 ± 1.4	−7.2	7.8	17.8 ± 0.5	−11.0	2.8
100	94.5 ± 4.0	−5.5	4.3	96.3 ± 1.5	−3.7	1.6
500	476 ± 21	−4.9	4.5	470 ± 14	−6.0	3.0
M-2						
10	9.0 ± 1.0	−9.9	11.3	9.72 ± 0.35	−2.8	3.6
20	17.9 ± 2.3	−10.3	12.5	19.1 ± 1.5	−4.5	7.9
100	92.8 ± 9.9	−7.2	10.7	89.7 ± 1.3	−10.3	1.4
500	437 ± 47	−12.6	10.8	462 ± 27	−7.6	5.8
M-4						
10	8.42 ± 0.29	−15.8	3.4	9.80 ± 1.21	−2.0	12.3
100	88.3 ± 2.4	−11.7	2.7	100 ± 9	0.0	9.0
1000	933 ± 29	−6.7	3.1	1000 ± 90	0.0	9.0
M-9						
50	53.4 ± 2.88	6.8	5.4	54.8 ± 6.30	9.6	11.5
100	96.9 ± 7.56	−3.1	7.8	102 ± 10.3	2.0	10.1
500	493 ± 46.5	−1.4	9.4	502 ± 15.5	0.4	3.1
4000	3790 ± 227	−5.3	6.0	3950 ± 281	−1.3	7.1
5000	4920 ± 225	−1.6	4.6	5310 ± 298	6.2	5.6

Found concentration in the intra-day reproducibility test represents the mean ± SD of five measurements conducted within a day.
Found concentration in the inter-day reproducibility test represents the mean ± SD of two measurements conducted on 3 separate days.

lites in human plasma after oral administration of IM at the therapeutic dosage of 0.1 mg.

4. Discussion

After oral administration of IM at the dose of 0.1 mg to healthy male subject, parent IM and three metabolites are observed in plasma. M-9 was an *N*-glucuronic acid conjugate of IM, identified first in the early clinical phase and its determination method was developed immediately for evaluation of safety. At LC–MS/MS detection, a part of M-9 was degraded by APCI and detected as the IM peak under the HPLC conditions for IM determination. However, we considered overestimation of IM would not occur in the present study, because (1) M-9 was stable in the solid phase extraction process [13], (2) stability of M-9 was confirmed under the various storage conditions in the

Table 4
Stabilities of IM, M-2, M-4, M-9 and IS in the standard stock solutions

Analytes/conditions	Period	Nominal concentration (μg/mL)	Residual ratio[a] (%)
IM			
Room temperature (ca. 25 °C)	24 h	10	116.8 ± 2.5
Refrigerating (below 10 °C)	10 weeks	10	103.4 ± 2.6
M-2			
Room temperature (ca. 25 °C)	24 h	10	79.6 ± 7.4
Refrigerating (below 10 °C)	10 weeks	10	104.9 ± 4.8
M-4			
Room temperature (ca. 25 °C)	24 h	100	100.4 ± 0.5
Refrigerating (below 10 °C)	3 months	100	96.9 ± 6.1
M-9			
Room temperature (ca. 25 °C)	24 h	101	103.5 ± 9.0
Refrigerating (below 10 °C)	1 month	101	102.0 ± 8.5
IS			
Room temperature (ca. 25 °C)	24 h	10	104.4 ± 0.9
	24 h	100	99.5 ± 1.3
Refrigerating (below 10 °C)	10 weeks	10	101.7 ± 6.0
	3 months	100	103.7 ± 1.3[a]

[a] Calculated as (observed peak area/mean peak area of freshly prepared sample) × 100. Each value represents the mean ± SD of 3 measurements.

Table 5
Stabilities of IM, M-2, M-4 and M-9 in the plasma samples and the pretreated samples

Analytes			Low concentration		High concentration	
Samples	Conditions	Period	Added[a] (pg/mL)	Residual ratio [b](%)	Added[a] (pg/mL)	Residual ratio[b] (%)
	IM					
Plasma	Room temperature (ca. 25 °C)	24 h	50	107.0 ± 6.2	500	112.3 ± 1.8
Plasma	Freezing (set at −20 °C)	12 months	50	92.0 ± 3.1	400	94.3 ± 3.8
Plasma	Freezing and thawing	3 cycle	50	103.5 ± 4.5	500	103.8 ± 0.4
Pretreated	Room temperature (ca. 25 °C)	24 h	50	97.3 ± 1.1	500	102.7 ± 0.7
	M-2					
Plasma	Room temperature (ca. 25 °C)	24 h	50	108.1 ± 8.6	500	114.2 ± 4.1
Plasma	Freezing (set at −20 °C)	12 months	50	89.9 ± 22.0	400	105.3 ± 8.9
Plasma	Freezing and thawing	3 cycle	50	101.0 ± 8.8	500	108.8 ± 11.3
Pretreated	Room temperature (ca. 25 °C)	24 h	50	91.0 ± 4.1	500	89.6 ± 0.6
	M-4					
Plasma	Room temperature (ca. 25 °C)	24 h	20	103.5 ± 9.1	800	103.9 ± 2.2
Plasma	Freezing (set at −30 °C)	11 weeks	20	105.5 ± 16.8	800	100.7 ± 3.4
Plasma	Freezing and thawing	3 cycle	20	91.5 ± 1.7	800	97.4 ± 1.2
Pretreated	Room temperature (ca. 25 °C)	48 h	20	109.0 ± 5.0	800	98.1 ± 0.7
	M-9					
Plasma	Room temperature (ca. 25 °C)	24 h	400	106.2 ± 0.9	4000	108.0 ± 2.0
Plasma	Freezing (set at −40 °C)	3 months	400	101.4 ± 2.9	4000	103.3 ± 2.3
Plasma	Freezing and thawing	3 cycle	400	103.3 ± 3.8	4000	109.7 ± 5.0
Pretreated	Room temperature (ca. 25 °C)	48 h	400	98.7 ± 3.2	4000	96.8 ± 1.4

[a] Nominal concentration.
[b] Calculated as (observed concentration/mean initial concentration) × 100. Each value represents the mean ± SD of 3 measurements.

validation study (see Tables 4 and 5) and (3) the peaks of IM and M-9 were clearly resolved in the HPLC chromatogram.

Since API-300, a MS/MS unit in Method 1, is known to have cross-talk phenomenon, and since IM and M-2 have the same product ion, quantification bias would be apprehensive. However, all the analytes were clearly resolved by HPLC prior to MS/MS analysis and had the separate retention times in each method. Therefore, we considered the influence of cross-talk would be avoided.

When the structural analog is used as the internal standard, quantification bias could be observed from inconsistent matrix effect originated from multiple lot of plasma. In order to avoid the concern, matrix was removed by HPLC using long column (150-mm length of analytical column connected with 10-mm guard column) in Methods 1 and 3. In Method 2 using short column (50-mm length), matrix effect was evaluated by using QC samples constructed with 6 lot of individual plasma. As a result, matrix effect was not observed (see Table 6). In determination of the analytes in the pharmacokinetic plasma sample, multiple lot of human plasma were used for constructing calibration curve and QC samples; however, determinations were successfully completed in compliance with the routine drug analysis criterion in the FDA guidance. Accordingly, we considered matrix effect originated from different sources of plasma would not cause quantification bias in the present study.

Table 6
Influence of biological matrices on the determination of M-4 in human plasma

Concentration added (pg/mL)	Lot no. of control plasma	Concentration found (pg/mL)	Mean concentration (pg/mL)	Precision (%)	Accuracy (%)
50.0	1	50.3	49.7 ± 3.6	7.2	0.6
	2	51.2			2.4
	3	50.5			1.0
	4	50.5			1.0
	5	53.1			6.2
	6	42.8			−14.4
500	1	503	494 ± 39	7.9	0.6
	2	494			−1.2
	3	507			1.4
	4	487			−2.6
	5	547			9.4
	6	426			−14.8

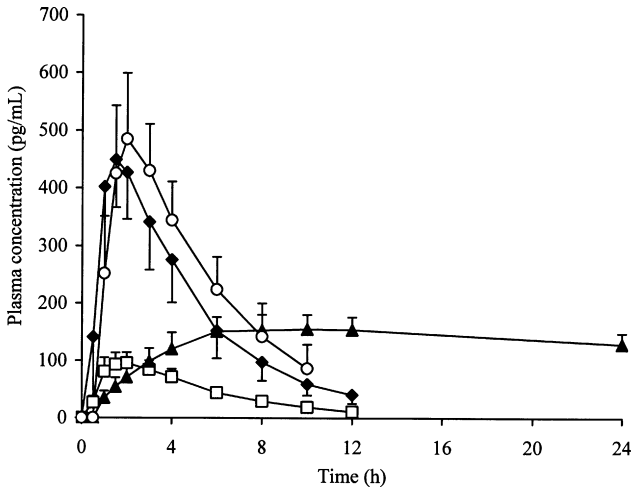

Fig. 5. Concentration profiles of IM (diamond), M-2 (square), M-4 (triangle) and M-9 (circle) in plasma after oral administration of 0.1 mg of IM to fasted healthy male volunteers. Each point represents the mean ± SD of 12 individuals.

From the result of pharmacokinetic study, plasma IM level was sufficient for exhibiting anitmuscarinic activity of IM. In contrast, no clinically significant pharmacological activities have been identified in the three metabolites including M-4. In addition, plasma levels of IM and the three metabolites in humans were much lower than those in the animals in the preceding toxicological studies, supporting safety of these analytes in humans.

5. Conclusions

We developed and validated the three bioanalytical methods for IM and its metabolites, M-2, M-4 and M-9 in human plasma with sufficient sensitivity and selectivity by using LC–MS/MS method. There were no peaks interfering with IM, M-2, M-4, M-9 and IS on the chromatograms of the control plasma. These analytes and IS were sufficiently recovered from the plasma sample by the solid-phase extraction process. Calibration curves had good linearity. From the results of accuracy and precision in the intra-day and inter-day reproducibility tests, LLOQ were 10 pg/mL for IM, M-2 and M-4, and 50 pg/mL for M-9. The analytes in the standard stock solutions and plasma samples remained stable during pretreatment at room temperature, and after storage in refrigerating or freezing conditions. By using these analytical methods, concentration profiles of IM and metabolites in human plasma were successfully determined after oral administration of IM at the therapeutic dosage of 0.1 mg twice daily in healthy male volunteers, indicating these analytical methods would provide the drug-monitoring methods for IM in the clinical settings.

Acknowledgement

The authors thank Ms. Ikuyo Takemoto for preparing the manuscript.

References

[1] K.E. Andersson, M. Yoshida, Eur. Urology 43 (2003) 1.
[2] C.R. Chapple, T. Yamanishi, R. Chess-Williams, Urology 60 (Suppl. 5A) (2002) 82.
[3] D. Robinson, L. Cardozo, Int. J. Clin. Pract. 59 (2005) 1229.
[4] F. Kobayashi, Y. Yageta, M. Segawa, S. Matsuzawa, Arzneim. Forsch. /Drug Res. 57 (2007) 92.
[5] H. Miyachi, H. Kiyota, M. Segawa, Bio. Med. Chem. Lett. 9 (1999) 3003.
[6] H. Miyachi, H. Kiyota, H. Uchiki, M. Segawa, Bio. Med. Chem. 7 (1999) 1151.
[7] M. Matsui, D. Motomura, H. Karasawa, T. Fujikawa, J. Jiang, Y. Komiya, S. Takahashi, M.M. Taketo, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 9579.
[8] C.P. Nelson, P. Gupta, C.M. Napier, S.R. Nahorski, R.A. Challiss, J. Pharmacol. Exp. Ther. 310 (2004) 1255.
[9] G.T. Somogyi, W.C. de Groat, J. Auton, Nerv. Syst. 37 (1992) 89.
[10] A.S. Braverman, I.J. Kohn, G.R. Luthin, M.R. Ruggieri, Am. J. Physiol. 274 (1998) R517.
[11] A. Sahai, M.S. Khan, M. Arya, J. John, R. Singh, H.R.H. Patel, Expert Opin. Pharmacother. 7 (2006) 509.
[12] U.S. Food and Drug Administration, 2001. Guidance for Industry, Bioanalytical Method Validation, U.S. Food and Drug Administration, Center for Drug Evaluation and Research, (http://www.fda.gov/cder/guidance/index.htm).
[13] E.M. Hawes, Drug Metab. Dispos. 26 (1998) 830.