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Simultaneous determination of a novel M_3 muscarinic receptor antagonist and its active 5-OH metabolite in human plasma using liquid chromatography/tandem mass spectrometry

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Abstract

A sensitive, specific, and robust liquid chromatography (LC)/mass spectrometry (MS)/MS method has been developed and validated for a novel M_3 muscarinic receptor antagonist (***I***) and its active 5-OH metabolite (***II***) in human plasma. The assay involves a two-step liquid–liquid extraction of the compounds from human plasma, high performance liquid chromatography (HPLC) separation, and MS/MS for the detection of the analytes. The method provides a linear response from a quantitation limit of 0.05–20 ng/ml for ***I*** and 0.1–20 ng/ml for ***II*** using 1 ml of plasma. The mean absolute recovery was 85.4% for ***I*** and 80.8% for ***II***, respectively. The intra-assay accuracy of ***I*** and ***II*** averaged from 95.0 to 105.3% with coefficient of variation (CV) values $\leq 6.5\%$ over the standard curve range. The stability study showed that ***I*** and ***II*** are stable in the plasma matrix over a period of 11 months at -70 °C. The accuracy, ruggedness, and reproducibility of this method were demonstrated by analyzing over 5000 plasma samples in clinical pharmacokinetics studies over a 6-month period.

Keywords: Muscarinic receptor; LC/MS/MS; Liquid–liquid extraction

1. Introduction

2(*R*)-*N*-[1-(6-Aminopyridin-2-ylmethyl)piperdin-4-yl]-2-[(1*R*)-3,3-difluorocyclopentyl]-2-hydroxy-2-phenylacetamide (***I***, Fig. 1) is a novel M_3 selective muscarinic receptor antagonist, which was hypothesized to have utility for the treatment of urinary tract disorders, irritable bowel syndrome and respiratory disorders [1–3]. No analytical method has been published for the quantification of this compound and its active 5-OH metabolite (***II***, Fig. 1) in biological fluids. One major challenge of this method is that we need to achieve base-line chromatographic separation of the pharmacologically active 5-OH metabolite from the

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inactive 3-OH metabolite without compromising sensitivity and analysis time. The 3-OH metabolite of ***I*** chromatographically elutes very closely to, and also mass spectrometrically overlaps all the possible detecting channels with the 5-OH metabolite. Compound stability issue and autosampler carry-over effect are other challenges to overcome in this method. We have developed successfully a very sensitive liquid chromatography (LC)/tandem mass spectrometry (MS/MS) method with a limit of quantitation (LOQ) of 0.05 ng/ml for ***I***, and 0.1 ng/ml for its 5-OH metabolite.

2. Experimental

2.1. Material

I was obtained from Merck Research Laboratories (West Point, PA). ***II*** (dihydrochloride salt) and the internal standard (***III***, Fig. 1), a chemical analogue of ***I*** without two fluorine atoms on the cyclopentyl ring were obtained from Banyu Pharmaceuticals Co. Ltd (Japan). High performance liquid chromatography (HPLC) grade methanol and acetonitrile were from Fisher (Fair Lawn, NJ, USA), methyl-*t*-butyl ether and isopropanol were from Burdick & Jackson (Muskegon, MI, USA). Spectrophotometric grade trifluoroacetic acid (TFA) was obtained from Aldrich (St. Louis, MO, USA). Formic acid (99%) and ammonium acetate were purchased from Sigma (St. Louis, MO, USA). Heparinized control human plasma was purchased from Sera-Tec Biologicals (New Brunswick, NJ, USA).

2.2. Apparatus

Chromatographic separation was performed using a Perkin–Elmer (Norwalk, CT, USA) Series 200 autosampler and a quaternary pump, and a Jones Chromatography (Lakewood, CO, USA) column heater. A PE Sciex (Thornhill, Canada) API 365 or API3000 LC/MS/MS with a Turbo ionspray source (TIS) was used to monitor the analytes. Data were processed using PE Sciex MacQuan software (Version 1.5) on a Macintosh Power PC 9500 computer. A Zymark (Hopkinton, MA, USA) Turbo-Vap evaporator was used in the method.

2.3. Chromatographic conditions

The chromatographic separation was performed on a Discovery C18 analytical column (150 × 2 mm, 5 μm) purchased from Supelco (Bellefonte, PA, USA). The mobile phase was a mixture of methanol and 10 mM pH 5.5 ammonium acetate buffer (45:55, v/v). The flow rate was 0.2 ml/min and the column temperature was kept at 40 °C. In order to keep the sample stable throughout the injection period, the autosampler sample tray was kept at 4 °C.

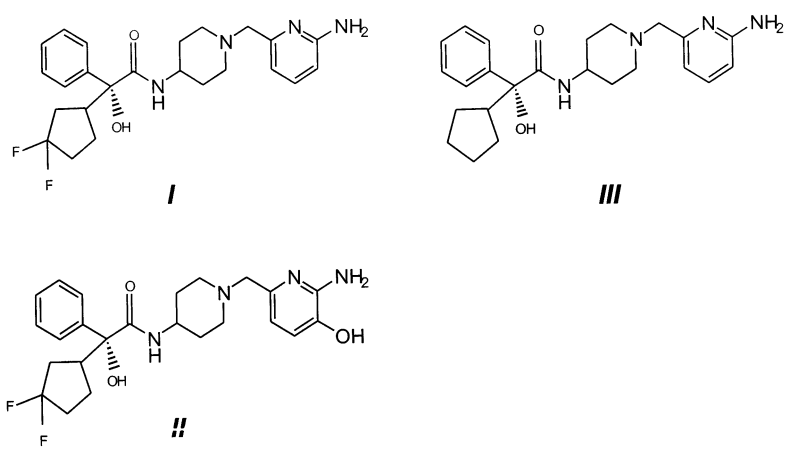

Fig. 1. Structure of ***I***, ***II*** and ***III*** (internal standard).

2.4. Standard solutions

A stock solution of ***I*** was prepared at 100 μg/ml in methanol–water (50:50, v/v). This solution was further diluted to a series of working standard solutions with concentrations of 1, 2, 10, 40, 100, 200 and 400 ng/ml. All solutions were stored at −70 °C.

II is not stable in methanol–water solution. The stock solution was prepared at 100 μg/ml (free base) in methanol:0.1% formic acid (50:50, v/v). This solution was further diluted to give a series of working standards with concentrations of 2, 4, 10, 30, 100, 200 and 400 ng/ml in methanol:0.1% formic acid (50:50, v/v). All solutions were stored at −70 °C. Due to the stability issue, all working standard solutions were prepared from the primary stock solution every 6 weeks or shorter.

Internal standard (***III***) working solution was prepared at 30 ng/ml in methanol–water (50:50, v/v). This solution was stored at −70 °C.

Plasma standards were prepared by adding 50 μl of each working standard to 1 ml of human control plasma. The resulting plasma standard concentrations ranged from 0.05 to 20.0 ng/ml for ***I***, and 0.1 to 20.0 ng/ml for ***II***.

Quality control (QC) stock solutions for both ***I*** and ***II*** were prepared separately at a concentration of 100 μg/ml in methanol–water (50:50, v/v) and methanol: 0.1% formic acid (50:50, v/v), respectively. These stock solutions were diluted with methanol–water (50:50, v/v) to generate working QC stock solution. Plasma QC samples were prepared by adding the appropriate volume of working QC stock solution into control human plasma to yield final concentrations of 0.15, 3.0 and 15.0 ng/ml for ***I***, and 0.3, 3.0 and 15.0 ng/ml for ***II***. All QC samples were stored at −70 °C.

2.5. Sample preparation

The frozen plasma samples were thawed at room temperature, vortexed and centrifuged at 3000 rpm for 5 min prior to preparation. One millimeter of plasma was aliquoted into a 14-ml polypropylene culture tube, followed by 50 μl of working internal standard and 0.5 ml of 0.1 M ammonium acetate buffer (pH 5.5). After vortexing, 5 ml of methyl-*t*-butyl ether was added to the tube and the tube was capped and rotated for 15 min at 60 rpm, and centrifuged for 5 min at 3000 rpm. After freezing the aqueous layer in a dry-ice/acetone bath, the organic layer was transferred into a 12-ml polypropylene conical tube containing 100 μl of 0.2% TFA. The conical tube was rotated another 15 min and centrifuged for 5 min at 3000 rpm. After freezing the aqueous layer in a dry ice/acetone bath, the organic phase was discarded. The tube with remaining aqueous was put in the Turbo-Vap under nitrogen gas at 40 °C for 3 min to remove residual organic solvent.

2.6. Quantification

In each analytical run, the standard curve was constructed from the peak area ratios of ***I*** and ***II*** to the internal standard versus the nominal concentration of the standards, respectively. Unknown plasma sample concentrations were calculated from the equation ($y = mx + b$) as determined by the weighted ($1/x$) linear regression of the standard curve.

3. Results and discussion

3.1. Mass spectrometry conditions

3.1.1. Ion source

Both TIS and atmospheric pressure chemical ionization (APCI) were evaluated. In APCI mode, the sensitivity for both compounds was poor compared with that in TIS mode. Since our goal was to achieve maximum sensitivity, TIS was selected as the source in our method.

3.1.2. Ion detecting channels

The analytes were detected in positive ionization mode by monitoring their precursor–product combination in multiple reaction monitoring (MRM) mode. The detecting channels were m/z 445 → m/z 190 for ***I***, m/z 461 → m/z 123 for ***II*** and m/z 409 → m/z 391 for the internal standard (***III***). The product scan spectra for the protonated molecule $[M + H]^+$ of the ***I***, ***II*** and ***III*** are shown in Fig. 2.

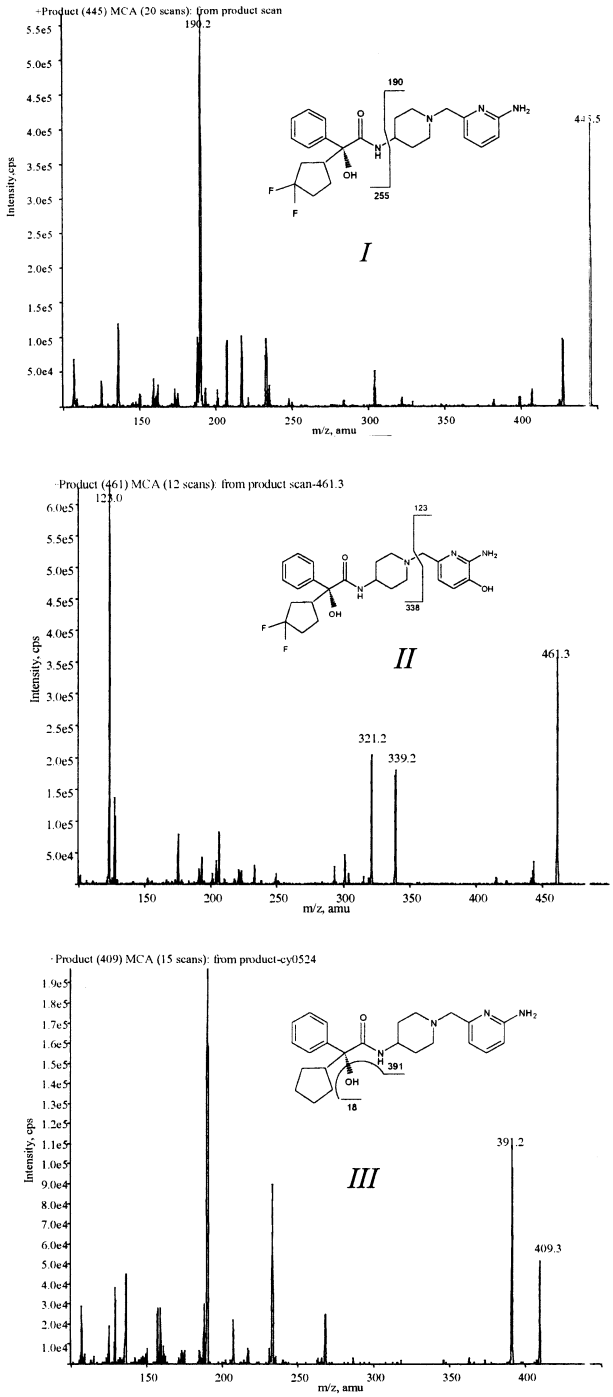

Fig. 2. Product scan spectra of compounds ***I***, ***II*** and ***III*** (internal standard).

3.1.3. Plasma matrix effect

Since plasma is a complex biological matrix, any other endogenous compounds which co-elute with the analytes might cause ion suppression or enhancement, which in turn makes the quantification of the analytes inaccurate[4,5]. Plasma matrix effect was tested by comparing the chromatographic peak areas from neat standards and blank plasma extracts spiked with neat standards at concentrations of 0.2, 1.0 and 10.0 ng/ml for ***I***, ***II*** and internal standard. Result showed that the peak area ratio of spiked plasma sample to their corresponding neat solution were from 98.5 to 104.5% for both compounds and internal standard. Therefore, matrix effect from plasma extract is insignificant.

3.1.4. Effect of co-eluting metabolite on *I*

It was observed that compound ***I*** and the 3-OH metabolite eluted at the same retention time. Although the metabolite was not found in the detecting channel of ***I***, it might compete with ***I*** in the ionization process and cause ion suppression or enhancement. To evaluate the possibility of such an adverse effect, two groups of samples were prepared. One sample group contains a high concentration of the 3-OH metabolite and a low concentration of ***I***, the other sample group contains ***I*** and the 3-OH metabolite at low concentration. By injecting these two groups of samples the LC/MS/MS system, we found no difference for ***I*** in these two groups in terms of peak area, so ion suppression or enhancement effects are negligible.

3.2. Optimization of chromatographic conditions

Since compound ***II*** and the 3-OH metabolite share exactly the same mass spectrometric detecting channels, the two compounds require baseline separation in order to be accurately quantified. The challenge here is that the two compounds are a pair of isomers, which are difficult to separate, while at the same time we could not compromise sensitivity by using a longer column to elute the compound at a very long retention time. Many different columns with different stationary phases

and particles sizes, different chromatographic conditions were tested, including Aquasil C18, Valupak Cyano, Haisil TS C18, Micro Chiral-AGP, Intersil ODS2, Symmetry® Shield RP8 as well as Discovery C18 column, and the separation results were not satisfactory. Finally, we found Supelco Discovery C18 column provided the best separation within a reasonable time frame, under 8 min.

The pH of the mobile phase also played an important role in the separation of these two compounds and their detectability. When the pH of the buffer in mobile phase was close to neutral, the two metabolites were separated easily, but the detectability of the compounds was very poor. When the pH was decreased, the separation of the two compounds also decreased rapidly. At pH 5 or below, the two compounds eluted at the same retention time. The best results were obtained at pH 5.5, which provided baseline separation of the two compounds, while the observed sensitivity loss was minimal. Representative chromatograms of ***I***, ***II*** and ***III*** in a plasma sample are shown in Fig. 3.

3.3. Sensitivity and linearity

Sensitivity, or LOQ, is defined as the lowest concentration on the standard curve that can be measured with acceptable precision ($\leq 15\%$) and accuracy ($\leq 15\%$ of the nominal value). In this method, the LOQ for ***I*** and ***II*** were 0.05 and 0.1 ng/ml, respectively. The linear range for ***I*** was 0.05–20 ng/ml and for ***II***, 0.1–20 ng/ml in plasma.

3.4. Specificity

Human control plasma samples from more than ten different lots were extracted and analyzed to assess the specificity of the method. The chromatographic conditions specified in this assay were found to be selective for ***I***, ***II*** and the internal standard (***III***). None of the control plasma samples had any detectable interference at the retention times of ***I***, ***II*** or internal standard (***III***). Representative chromatograms of control plasma are shown in Fig. 4.

3.5. Recovery

Extraction recovery of the assay was determined by comparing the chromatographic peak areas of the analyte spiked into the blank plasma before and after the extraction at three different concentrations (0.2, 1, and 10 ng/ml). The overall recovery for ***I*** was 85.4%, and for ***II*** was 80.8%. Recovery of internal standard from human control plasma was 93.2%.

3.6. Accuracy and precision

Intraday variability of the assay was assessed by preparing and analyzing replicates of five standard curves. Precision and accuracy data are shown in Table 1. The peak area ratios are used for the calculation of the coefficients of variations (CV). For ***I***, the CV values were $\leqslant 6.5\%$, and the accuracies were from 95.0 to 105.3% across the standard curve concentration range. For compound ***II***, the CV values were $\leqslant 6.1\%$, and the accuracies were from 98.0 to 105.0%.

Precision and accuracy for intraday QC samples were assessed by the determination of accuracy of the mean found concentrations as compared with the nominal concentrations and CV of those concentrations. Accuracy ranged from 101 to 110% for ***I*** and 109 to 113% for ***II***. Precision was < 6 and $< 5\%$ for ***I*** and ***II***, respectively.

Interday accuracy and precision were evaluated using the standard curve data from ten different days. Interday accuracy ($n = 10$) was from 94.0 to 107.2% with CV $\leq 6.8\%$ for ***I***, and accuracy from 98.0 to 101.9% with CV $\leq 5.0\%$ for ***II***, at concentrations across the standard curve range. These data are summarized in Table 2.

3.7. Sample stability

Stability was studied using neat standard solution, three freeze–thaw cycles in plasma, 24-h plasma extract in autosampler, and long-term storage in plasma at -70 °C.

Table 1
Intraday precision and accuracy for the determination of compounds ***I*** and ***II*** in plasma

I				***II***			
Nominal standard concentrations (ng/ml)	Mean assayed concentrations (ng/ml) ($n = 5$)	Accuracy (%)[a]	Coefficient of variation (%)	Nominal standard concentrations (ng/ml)	Mean assayed concentrations (ng/ml) ($n = 5$)	Accuracy (%)[a]	Coefficient of variation (%)
0.05	0.051	102.0	6.5	0.10	0.10	100.0	3.2
0.10	0.099	99.0	5.2	0.20	0.20	100.0	4.7
0.50	0.475	95.0	2.4	0.50	0.49	98.0	3.1
2.00	2.106	105.3	1.1	1.50	1.52	101.3	1.6
5.00	5.035	100.7	1.6	5.00	5.25	105.0	2.8
10.00	9.672	96.7	1.1	10.00	9.81	98.1	2.1
20.00	20.211	101.1	2.1	20.00	19.94	99.7	6.1

The calibration equation for ***I***: $y = 0.007 + 0.510x$, $r^2 = 0.9994$. ***II***: $y = -0.007 + 0.233x$, $r^2 = 0.9992$.

[a] Expressed as (mean observed concentration/nominal concentration) $\times$ 100.

Table 2
Interday precision and accuracy for the determination of compounds ***I*** and ***II*** in human plasma

I				***II***			
Nominal standard concentrations (ng/ml)	Mean assayed concentrations (ng/ml) ($n = 10$)	Accuracy (%)[a]	Coefficient of variation (%)	Nominal standard concentrations (ng/ml)	Mean assayed concentrations (ng/ml) ($n = 10$)	Accuracy (%)[a]	Coefficient of variation (%)
0.05	0.047	94.0	6.8	0.10	0.100	100.0	5.0
0.10	0.100	100.0	2.4	0.20	0.196	98.0	2.7
0.50	0.513	102.6	3.0	0.50	0.499	99.8	2.9
2.00	2.143	107.2	3.3	1.50	1.524	101.6	1.8
5.00	4.867	97.3	4.2	5.00	5.096	101.9	2.2
10.00	9.983	99.8	1.8	10.00	10.139	101.4	1.9
20.00	19.999	100.0	1.8	20.00	19.747	98.7	1.2

[a] Expressed as (mean observed concentration/nominal concentration) × 100.

3.7.1. Neat solution stability

It was noticed that compound ***II*** is unstable in methanol–water or acetonitrile–water. Neat solutions of compound ***II*** in methanol–water (50:50, v/v) at different concentration levels were injected in duplicate into the LC/MS/MS system immediately after preparation and re-injected about 5 h later. The results showed that the response of ***II*** decreased by more than 30% at concentration of 15.0 ng/ml or lower. Acidic solvents, such as MeOH/0.1% formic acid (50/50, v/v), could keep the stock solution of ***II*** stable up to 6 weeks at −70 °C. Our recommendation is that working standard solutions should be re-prepared every 6 weeks or sooner to ensure standard solution stability, considering exposure of the solutions to room temperature during sample analysis.

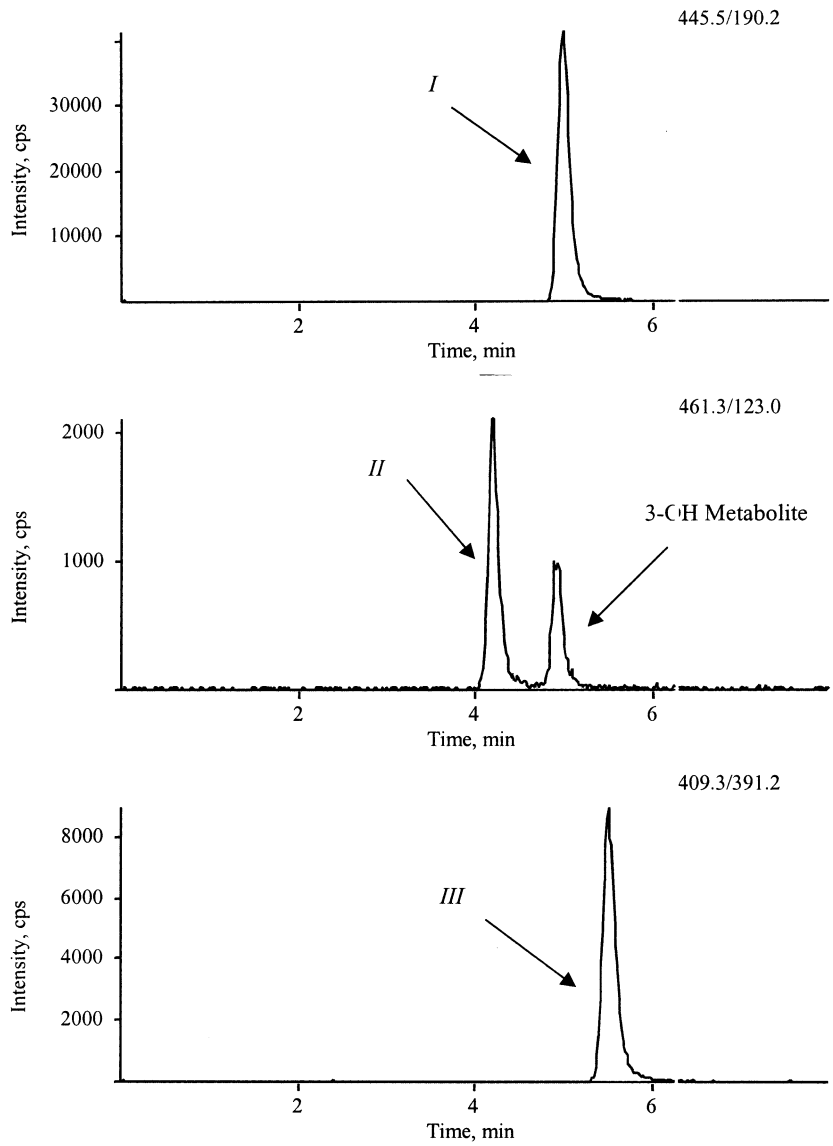

Fig. 3. Representative chromatograms for plasma sample (***I***, 3.287; ***II***, 0.15 ng/ml) spiked with internal standard (***III***, 1.5 ng/ml).

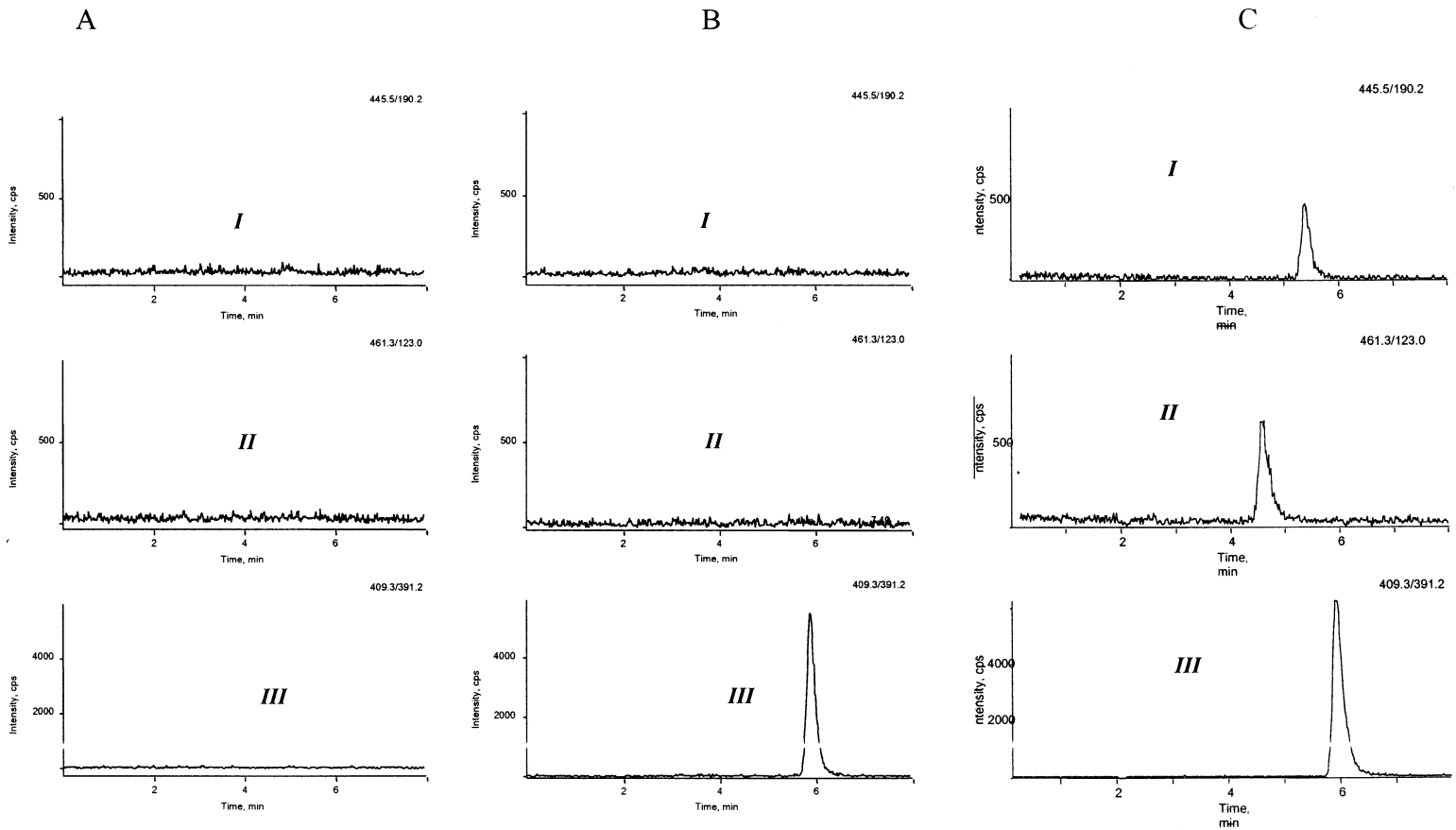

Fig. 4. Representative chromatograms. (A) Blank control plasma; (B) control plasma spiked with internal standard (***III***, 1.5 ng/ml); (C) control plasma spiked with 0.05 ng/ml ***I*** (LOQ), 0.1 ng/ml of ***II*** (LOQ) and internal standard (***III***, 1.5 ng/ml).

Table 3
Freeze–thaw stability of plasma quality controls (ng/ml)

	I			*II*		
	Low	Medium	High	Low	Medium	High
Initial concentrations	0.156	3.309	15.180	0.34	3.29	16.80
1	0.159	3.387	15.864	0.37	3.58	18.27
2	0.161	3.258	15.555	0.36	3.42	17.89
3	0.155	3.338	15.271	0.37	3.33	17.59
Mean	0.158	3.328	15.563	0.37	3.44	17.92
S.D.	0.003	0.065	0.297	0.01	0.13	0.34
CV (%)	1.9	2.0	1.9	1.6	3.7	1.9
Accuracy (%)[a]	101.3	100.6	102.5	107.8	104.6	106.7

[a] Accuracy = (mean found concentration/initial concentration) × 100.

Compound ***I*** was stable in either MeOH/H_2O or MeOH/0.1% formic acid (50/50, v/v) at room temperature.

3.7.2. Plasma sample freeze–thaw stability

Freeze–thaw stability was evaluated by three cycles of a freeze–thaw procedure using QC samples. In each cycle, the QC samples ($n = 3$ at each concentration level) were thawed to room temperature and kept at room temperature for at least 4 h and then refrozen to storage temperature, −70 °C. The concentrations of these QC samples were determined and compared with the initial values of QCs at day 0. The results, listed in Table 3, indicated that both ***I*** and ***II*** are stable in plasma after three freeze–thaw cycles.

3.7.3. Extracted plasma sample autosampler stability

Autosampler stability was tested in order to ensure the extracted plasma sample remained stable throughout the injection period, or in the event the need to re-inject the extracted samples occasionally should arise due to instrument failure. This was performed by re-injecting the extracted samples 24 h after the first injection. The extracted samples were kept in the autosampler tray at 4 °C. Comparison of the back calculated concentrations of these two injections at each concentration level for both compounds showed that both ***I*** and ***II*** were stable over 24 h at 4 °C in the autosampler tray.

3.7.4. Long-term stability of frozen plasma at −70 °C

Long-term stability was evaluated by analyzing the plasma QC samples after storage at −70 °C for up to 11 months. The results showed that both ***I*** and ***II*** were stable in the plasma matrix at −70 °C for at least 11 months.

3.8. Autosampler carry-over effect

Compounds ***I*** and ***II*** both in neat solution and extracted plasma sample exhibited significant carry-over in the autosampler when using MeOH/H_2O (50:50, v/v) as the needle wash solvent. Our study showed that the combination of acetonitrile/isopropanol/0.1% TFA (40:35:25, v/v/v) can clean the needle between each injection completely. No carry-over was detected after using this needle wash solution.

3.9. Application of the method and ruggedness

This method has been applied successfully to the analysis of plasma samples from clinical studies. Over a period of 6 months in five clinical studies, more than 5000 plasma samples were analyzed, and the method showed its ruggedness by consistently producing satisfactory results. Of 90 runs performed for clinical sample analysis, only two runs were failed due to sample preparation error. QC samples were used to monitor the daily performance of the method. The CV values

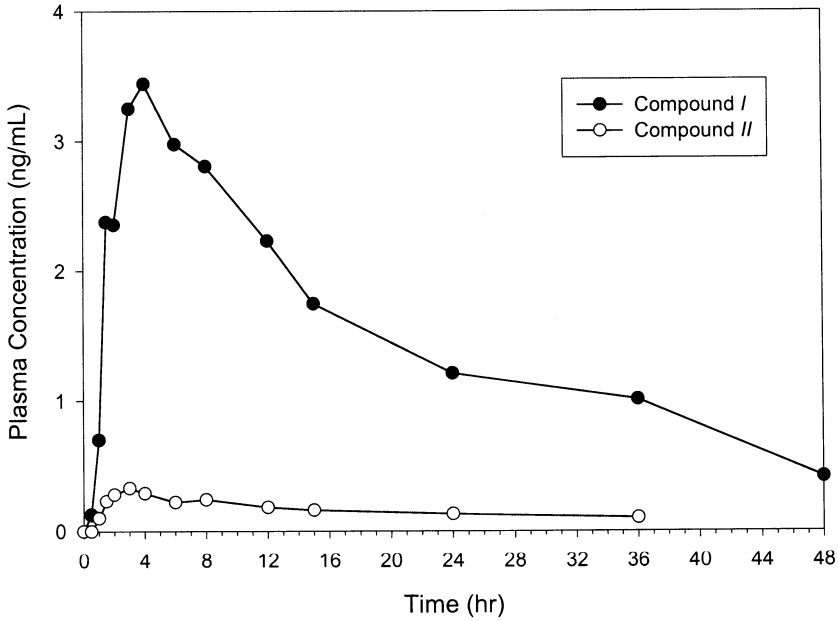

Fig. 5. Plasma concentrations of compound ***I*** and 5-OH metabolite (***II***) following a single oral dose of 2 mg in a healthy subject.

for quality control samples from all the assay runs over 6 months were less than 3.5 and 5.3% for ***I*** and ***II***, respectively. The average accuracy of the QC samples was 100.6% for ***I***, and 102.8% for ***II***. Fig. 5 is an example of plasma concentrations of ***I*** and ***II*** following oral administration of a single dose of 2 mg to one subject.

4. Conclusions

The study presented describes the development of a validated LC/MS/MS method for the simultaneous determination of ***I*** and its active 5-OH metabolite in human plasma. The method has been found to be sensitive, selective, accurate, rugged and reproducible. The usefulness of this method in routine analysis was demonstrated successfully by the analysis of large numbers of samples from clinical studies.

References

[1] M. Mitsuya, K. Kobayashi, K. Kawakami, A. Satoh, Y. Ogino, T. Kakikawa, N. Ohtake, T. Kimura, H. Hirose, A. Sato, T. Numazawa, T. Hasegawa, K. Noguchi, T. Mase, A potent, long-acting, orally active (2*R*)-2-[(1*R*)-3,3-difluorocyclopentyl]-2-hydroxy-2-phenylacetamide: a novel muscarinic M_3 receptor antagonist with high selectivity for M-3 over M-2 receptors, J. Med. Chem. 43 (26) (2000) 5017–5029.

[2] H. Hirose, T. Kimura, T. Fujikawa, T. Numazawa, I. Aoki, A. Sato, K. Sasaki, A. Nagumo, M. Nishikibe, T. Mase, K. Noguchi, Pharmacological characterization of J-104135 (L-753099), A novel muscarinic M3 receptor antagonist with high selectivity over M2 receptors, Presented at The Ninth International Muscarine Symposium, Houston, USA, October 31–November 4, 2000.

[3] H. Hirose, I. Aoki, T. Kimura, T. Fujikawa, T. Numazawa, K. Sasaki, A. Sato, T. Hasegawa, M. Nishikibe, N. Ohtake, T. Mase, K. Noguchi, Pharmacological Properties of [*R*-(*R**,*R**)-*N*-[1-96-amino-2-2-pyridinyl)methyl]-4 - piperidiny] - α - (3,3 - difluorocyclopentyl) - α - hydroxyphenylacetamide: a novel muscarinic antagonist with M_2-sparing antagonistic activity, J. Pharmacol. Exp. Ther. 297 (2) (2001) 790–797.

[4] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Matrix effect in quantitative LC/MS/MS analyses of biological fluids: a method for determination of finasteride in human plasma at picogram per milliliter concentrations, Anal. Chem. 70 (1998) 882.

[5] I. Fu, E.J. Woolf, B.K. Matuszewski, Effect of the sample matrix on the determination of indinavir in human urine by HPLC with turbo ion spray tandem mass spectrometric detection, J. Pharm. Biomed. Anal. 18 (1998) 347.