Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
31 (2003) 341–349
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Sensitive determination of oxybutynin and desethyloxybutynin in dog plasma by LC–ESI/MS/MS

Hohyun Kim [a,b,*], Sang Beom Han [a,b]

[a] *Department of Pharmacokinetics, Seoul Medical Science Institute, Seoul Clinical Laboratories (SCL), 7-14 Dongbinggo-dong, Yongsan-gu, Seoul 140-809, South Korea*
[b] *Department of Drug Development Service, BioCore Co. Ltd, Seoul 137-130, South Korea*

Received 2 August 2002; received in revised form 25 November 2002; accepted 25 November 2002

Abstract

A sensitive and selective liquid chromatographic method coupled with tandem mass spectrometry (LC–MS/MS) was developed for the quantification of oxybutynin and desethyloxybutynin in dog plasma. Diazepam was used as internal standard, with plasma sample extracted using *n*-hexane and back-extracted using hydrochloric acid. A centrifuged lower layer (aqueous layer) was injected into a C_{18} XTerra MS column (2.1×30 mm^2) with 3.5 μm particle size. The analytical column lasted for at least 500 injections. The mobile phase was composed of 90% methanol, with flow rate at 200 μl/min. The mass spectrometer was operated in positive ion mode using electrospray ionization. Nitrogen was used as the nebulizer gas and argon was used as the collision gas. Using MS/MS with multiple reaction monitoring (MRM) mode, oxybutynin and desethyloxybutynin were detected without severe interferences from plasma matrix. Oxybutynin produced a protonated precursor ion ($[M+H]^+$) at m/z 358 and a corresponding product ion at m/z 142. Desethyloxybutynin produced a protonated precursor ion ($[M+H]^+$) at m/z 330 and a corresponding product ion at m/z 96. And internal standard (diazepam) produced a protonated precursor ion ($[M+H]^+$) at m/z 285 and a corresponding product ion at m/z 193. Detection of oxybutynin and desethyloxybutynin in dog plasma were accurate and precise, with detection limit at 0.1 ng/ml. This method has been successfully applied to a study of oxybutynin and desethyloxybutynin in dog plasma.

Keywords: Tandem mass spectrometry; Liquid chromatography; Multiple reaction monitoring; Oxybutynin; Desethyloxybutynin; Diazepam

1. Introduction

Oxybutynin (α-cyclohexyl-α-hydroxybenzenacetic acid 4-(diethylamino)-2-butynyl ester) is commonly prescribed to treat symptoms of urge incontinence, urgency, and frequency arising from overactivity of the detrusor muscle (overactive bladder) [1]. Anticholinergic (antimuscarinic) agents, such as oxybutynin hydrochloride, inhibit the binding of acetylcholine to the cholinergic receptor and suppress involuntary bladder contractions. The primary adverse events asso-

* Corresponding author. Fax: +82-2-3461-0590.
E-mail address: novakims@yahoo.com (H. Kim).

0731-7085/03/$ - see front matter
doi:10.1016/S0731-7085(02)00644-1

ciated with immediate-release oxybutynin hydrochloride are anticholinergic in nature and may limit the drug's therapeutic applications [2]. Of the anticholinergic effects, dry mouth is among the most commonly cited reasons patients either discontinue oxybutynin treatment or have to titrate to a lower dose [2,3]. Its metabolite, desethyloxybutynin, has demonstrated equipotent anticholinergic activity in vitro [2,4]. Desethyloxybutynin is a major and active metabolite of oxybutynin that is used in the treatment of urinary incontinence. The plasma concentration-time profile of desethyloxybutynin parallels that oxybutynin, while the maximum plasma concentration (C_{max}) and the area under the concentration–time curve value of desethyloxybutynin is about 10 times higher than that of the precursor drug [4–6].

Previous studies have reported several different methods for the qualitative and quantitative detection of oxybutynin and desethyloxybutynin in human plasma and pharmaceutical formulations, e.g., gas chromatography with mass spectrometric detection (GC-MS) [7–11], high performance liquid chromatography (HPLC) with diode array detection (DAD) [12], HPLC with electrochemical detection (ECD) [13], ion pair liquid chromatography [14], and radioreceptor assay [15]. However, these published methods are not ideal for pharmacokinetics work, because they are time-consuming, i.e., derivatization step, arduous sample preparation, and long chromatographic run times. Likewise, they need a relatively large amount of sample to reach a low detection limit. In addition, detection of oxybutynin and desethyloxybutynin using liquid chromatography—electrospray ionization tandem mass spectrometry (LC–ESI/MS/MS) has yet to be reported.

Therefore, this study established a novel quantification method for detecting oxybutynin and desethyloxybutynin in dog plasma using LC–ESI/MS/MS. This method is not only selective and reliable; it is also faster and simpler compared to other recently reported methods. Likewise, this method has been successfully applied to preliminary pharmacokinetic studies to determine the concentration of oxybutynin and desethyloxybutynin in dog plasma.

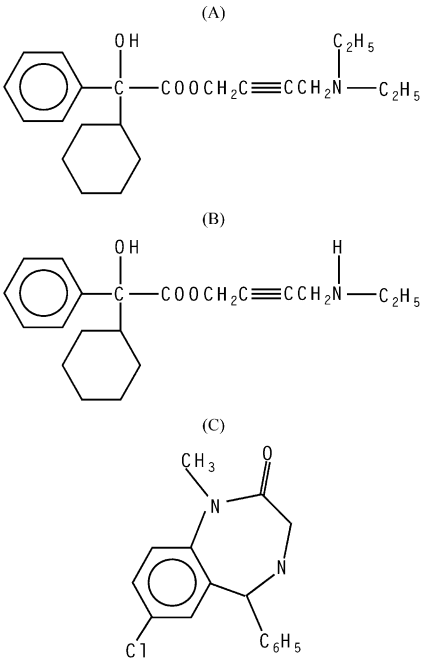

Fig. 1. Structures of (A) oxybutynin, (B) desethyloxybutynin and (C) diazepam (Internal standard).

2. Experimental

2.1. Chemicals and solutions

Oxybutynin and desethyloxybutynin were obtained from AmorePacific Co., Ltd (Kyunggi-Do, South Korea). HPLC grade methanol, acetonitrile, *n*-hexane, and water were purchased from Fisher Scientific (Fair Lawn, NJ), while diazepam (internal standard) and hydrochloric acid were purchased from Sigma-Aldrich Co. (St. Louis, MO). Fig. 1 shows the structures of oxybutynin, desethyloxybutynin and diazepam (internal standard).

To prepare standard stock solutions, appropriate amount of oxybutynin, desethyloxybutynin, and diazepam were weighted accurately and dissolved separately in 50 ml water. From these stock solutions, working standard solutions of oxybuty-

nin and other analytes were prepared through sequential dilutions with water to produce varying concentrations (0.1–200 ng/ml).

2.2. LC–MS/MS conditions

Tandem mass spectrometry (MS/MS) was performed on the Quattro micro triple quadruple mass spectrometer equipped with electrospray ion source (Micromass Co., Manchester, UK). Twenty microliters of sample were delivered into the ESI source using the (liquid chromatograph, LC and autosampler, Model Waters HT 2795, Waters Co., Milford, MA) with C_{18} XTerra MS column (2.1 × 30 mm^2, 3.5 μm particle size). The mobile phase used after degassing was composed of 90% methanol, with total running time of 2.0 min and flow rate of 200 μl/min.

The following instrument settings were used: electrospray interface maintained at 150 °C with a nitrogen nebulization; nitrogen flow of 550 l/h. In detecting oxybutynin using the multiple reaction monitoring (MRM) scan mode with positive ion detection, the following parameter settings were used: cone voltage at 32 V; extractor at 2 V; RF lens at 0.2 V; source temperature at 120 °C; collision cell entrance potential at 2.0 V; collision energy at 25 eV; collision cell exit potential at 20 V; multiplier at 650 V; and dwell time of 0.25 s. In detecting desethyloxybutynin using the MRM scan mode with positive ion detection, the following parameter settings were used: cone voltage at 28 V; collision energy at 17 eV; and dwell time of 0.25 s. On the other hand, the detection of internal standard (diazepam) using MRM scan mode with positive ion detection had the following parameter settings: cone voltage at 42 V; collision energy at 30 eV; and dwell time of 0.25 s.

Mass calibration was performed through the infusion of a 10^{-4} M polyethylene glycols 1000 (PEG 1000) solution into the ionspray source. The peak widths of precursor and product ions were maintained at ~0.7 u at half-height in the MRM mode.

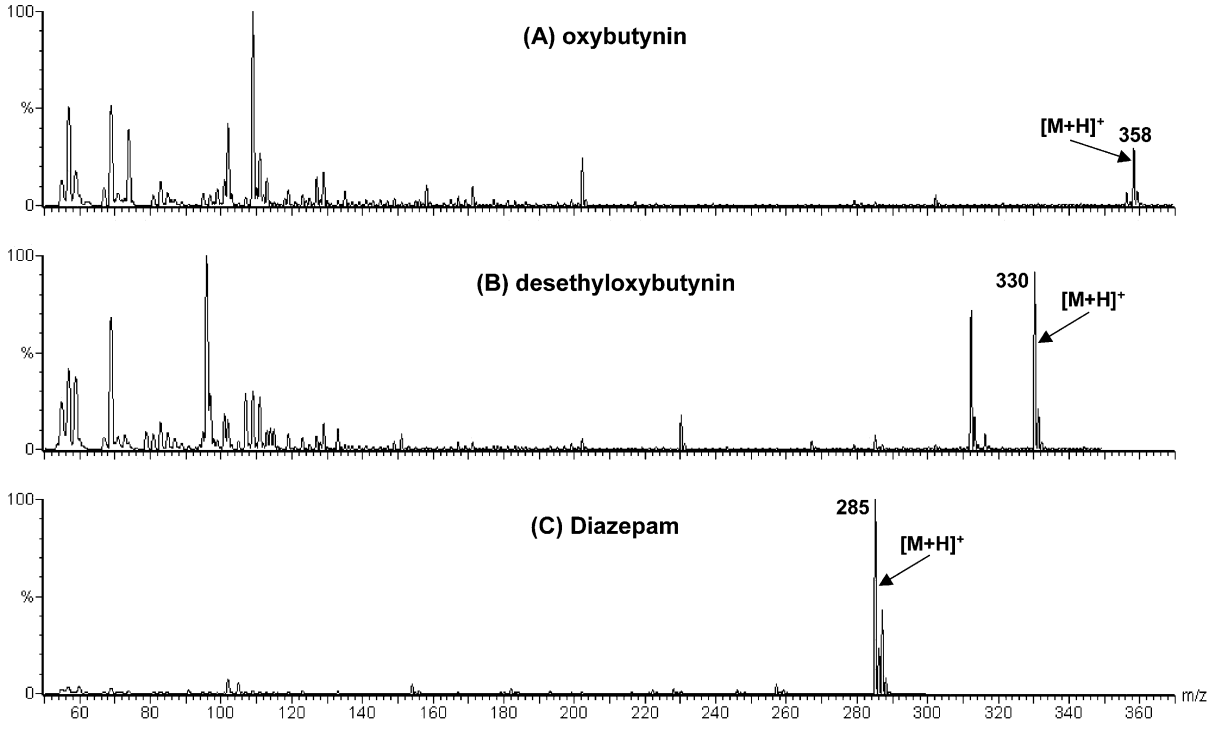

Fig. 2. Full scan first quadrupole spectrum of (A) oxybutynin, (B) desethyloxybutynin and (C) diazepam (internal standard).

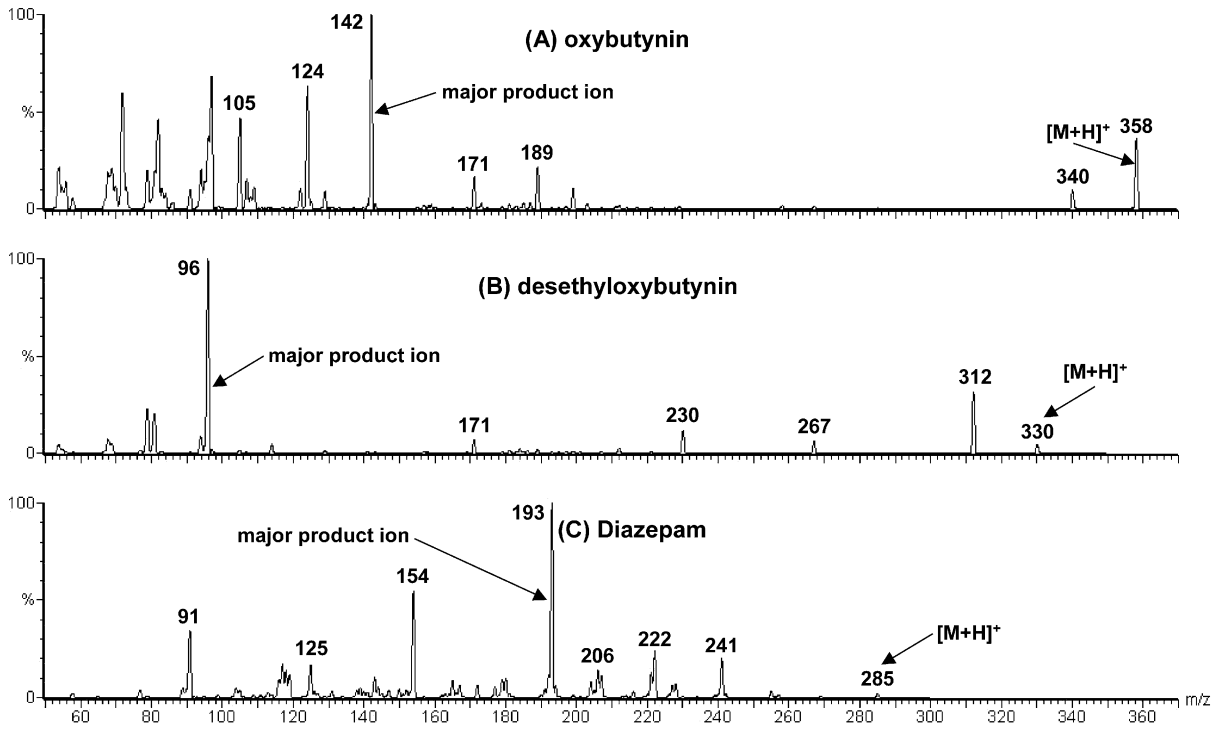

Fig. 3. Product ion spectrum of (A) oxybutynin, (B) desethyloxybutynin and (C) diazepam (internal standard).

2.3. Sample preparation

One milliliter plasma specimens were pipetted into conical glass tubes and spiked with 0.1 ml of 1 μg/ml internal standard solution. After adding 0.5 ml of acetonitrile, the plasma was vortex-mixed for 10 s and added 3 ml *n*-hexane. The sample was then shacked for 10 min. The two phases were separated through centrifugation at 2000 × *g* for 5 min. The upper organic layer (*n*-hexane layer) was transferred into another conical glass tube and added 0.3-ml hydrochloric acid (0.1 M). The sample was then shacked for 10 min. The two phases were separated through centrifugation at 2000 × *g* for 5 min. The lower aqueous layer was injected into the LC–MS/MS.

2.4. Validation procedures

To assess the intraday precision and accuracy of the method, five replicates of plasma standards at four concentrations (0.5, 2.0, 10, and 50 ng/ml) were analyzed. Similarly, five replicates of the quality control samples at varying concentrations of 0.5, 2.0, 10, and 50 ng/ml were analyzed to determine the initial interday precision and accuracy. The accuracy was expressed as (mean observed concentration)/(spiked concentration) × 100%, with the precision expressed using the relative standard deviation (RSD).

For the plasma QC samples, 400 μl of the appropriate QC working solution (0.02, 0.05, 0.1, 0.2, 0.5, 2, 5, 10, and 20 mg/ml) was added to 50 ml polypropylene tubes containing 39.6 ml dog control plasma to yield QC concentration of 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, and 200 ng/ml. The QC samples were used to construct the calibration curve and intra–inter day validation. The bulk QC plasma samples were then vortex-mixed, with 1.5-ml aliquots transferred into 2-ml microcentrifuge tubes and capped and stored at −70 °C. All the standard plasma contained oxybutynin

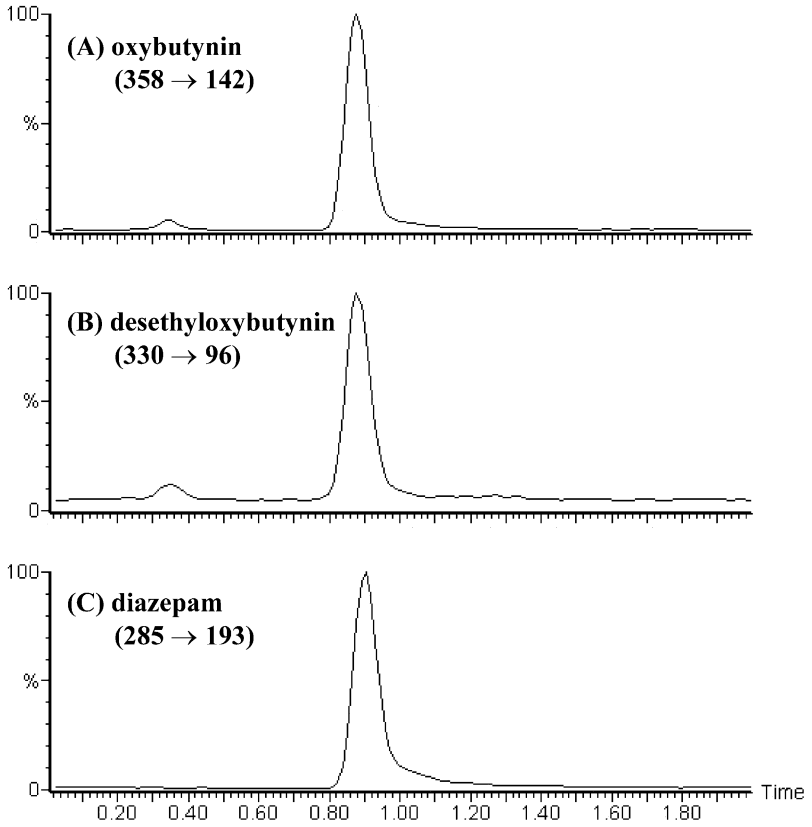

Fig. 4. LC–MS/MS chromatograms of (A) oxybutynin, (B) desethyloxybutynin and (C) diazepam in dog plasma.

and desethyloxybutynin in the 1:1 concentration ratio.

2.5. Preliminary pharmacokinetic study

A single 5 mg dose of oxybutynin was administered orally to five dogs that were advised about the nature and purpose of the study. The dogs possessed good health and have not taken any medication for at least 2 weeks prior to the study. The group consisted of healthy males with a mean weight of 27.6 ± 3.3 kg. Blood samples were taken 0, 0.5, 1, 2, 3, 4, 6, and 8 hr after ingestion using heparin vacutainer collection tubes. Dog plasma was obtained through centrifugation at $2000 \times g$ for 10 min. Plasma specimens were then stored at -70 °C prior to analysis. Also, dog blank plasma was obtained from Seoul Medical Science Institute. Seoul Clinical Laboratories (SCL).

3. Results and discussion

Currently, oxybutynin and desethyloxybutynin are assayed in dog (or human) plasma and pharmaceutical formulations using GC-MS [7,8],

Table 1
Analyte/Internal standard response and precision for the matrix effect test

No	Different source Analyte/IS response	Single source Analyte/IS response
1	1.80E−01	1.86E−01
2	1.86E−01	1.96E−01
3	1.94E−01	1.97E−01
4	1.89E−01	1.89E−01
5	1.92E−01	1.83E−01
Mean	1.88E−01	1.90E−01
SD	5.50E−03	6.14E−03
% RSD	2.92	3.23

Table 2
Intraday precision and accuracy of measurement of oxybutynin

Oxybutynin Nominal concentration (ng/ml)	Oxybutynin (Mean ± SD)[a] Calculated concentration (ng/ml)	Accuracy (%)	Precision (% RSD)
0.50	0.53 ± 0.01	105.2	2.55
2.00	1.83 ± 0.05	91.4	2.88
10.00	9.68 ± 0.18	96.8	1.82
50.00	47.48 ± 0.68	95.0	1.43

[a] Five replicates at each concentration level ($n = 5$).

Table 3
Intraday precision and accuracy of measurement of desethyloxybutynin

Desethyloxybutynin Nominal concentration (ng/ml)	Desethyloxybutynin (Mean ± SD)[a] Calculated concentration (ng/ml)	Accuracy (%)	Precision (% RSD)
0.50	0.48 ± 0.02	95.2	3.52
2.00	2.09 ± 0.05	104.3	2.41
10.00	10.53 ± 0.41	105.3	3.87
50.00	46.47 ± 1.08	92.9	2.32

[a] Five replicates at each concentration level ($n = 5$).

Table 4
Interday precision and accuracy of measurement of oxybutynin

Oxybutynin Nominal concentration (ng/ml)	Oxybutynin (Mean ± SD)[a] Calculated concentration (ng/ml)	Accuracy (%)	Precision (% RSD)
0.50	0.53 ± 0.02	106.4	3.09
2.00	1.84 ± 0.03	91.9	1.86
10.00	9.81 ± 0.15	98.1	1.55
50.00	47.17 ± 1.24	94.3	2.62

[a] Five replicates at each concentration level ($n = 5$).

Table 5
Interday precision and accuracy of measurement of desethyloxybutynin

Desethyloxybutynin Nominal concentration (ng/ml)	Desethyloxybutynin (Mean ± SD)[a] Calculated concentration (ng/ml)	Accuracy (%)	Precision (% RSD)
0.50	0.52 ± 0.03	104.0	5.77
2.00	2.11 ± 0.15	105.7	7.09
10.00	10.26 ± 0.35	102.6	3.44
50.00	46.23 ± 1.83	92.5	3.97

[a] Five replicates at each concentration level ($n = 5$).

HPLC–DAD [12], and HPLC–ECD [13]. However, these methods are time-consuming, i.e., derivatization step, arduous sample preparation, and long chromatographic run times. Likewise, they need a relatively large amount of sample to reach a low detection limit. Therefore, the study established a highly sensitive and selective method for detecting oxybutynin and desethyloxybutynin in dog plasma using LC–MS/MS for pharmacokinetics studies.

Under electrospray ionization condition, oxybutynin, desethyloxybutynin and diazepam (internal standard) exhibit favorable sensitivity in positive ion detection mode. Fig. 2(A) shows the full scan first quadrupole positive ion spectrum of oxybutynin, Fig. 2(B) shows the full scan first quadrupole positive ion spectrum of desethyloxybutynin, while Fig. 2(C) shows the full scan first quadrupole positive ion spectrum of internal standard. These formed protonated precursor molecules $[M+H]^+$ as major ion peaks. Oxybutynin produced a protonated precursor molecule ($[M+H]^+$) at m/z 358 with a major product ion at m/z 142, desethyloxybutynin produced a protonated precursor molecule ($[M+H]^+$) at m/z 330 with a major product ion at m/z 96. On the other hand, diazepam (internal standard) produced a protonated precursor molecule ($[M+H]^+$) at m/z 285 with a major product ion at m/z 193. Fig. 3(A)–(C) show the product ion spectrum of $[M+H]^+$ for oxybutynin, desethyloxybutynin and internal standard. MRM mode was used for quantification, thereby obtaining a very high sensitivity. Using MS/MS with MRM mode, oxybutynin, desethyloxybutynin and internal standard were detected without severe interferences from plasma matrix (Fig. 4(A)–(C)).

For the chromatographic analysis and electrospray ionization of oxybutynin and desethyloxybutynin, initially attempted to develop a reversed phase chromatographic system with methanol or acetonitrile. Methanol was used instead of acetonitrile, since methanol has better sensitivity. The amount of methanol in mobile phase was then optimized at 90%. Likewise, the pH of the mobile phase was optimized at 6–7. Using 90% methanol without modifier showed high sensitivity than 90% methanol with modifier (ammonium acetate or formic acid). However, the chromatogram of oxybutynin and desethyloxybutynin showed high sensitivity under these conditions. Fig. 4(A)–(C) show LC–MS/MS chromatograms of oxybutynin, desethyloxybutynin and internal standard.

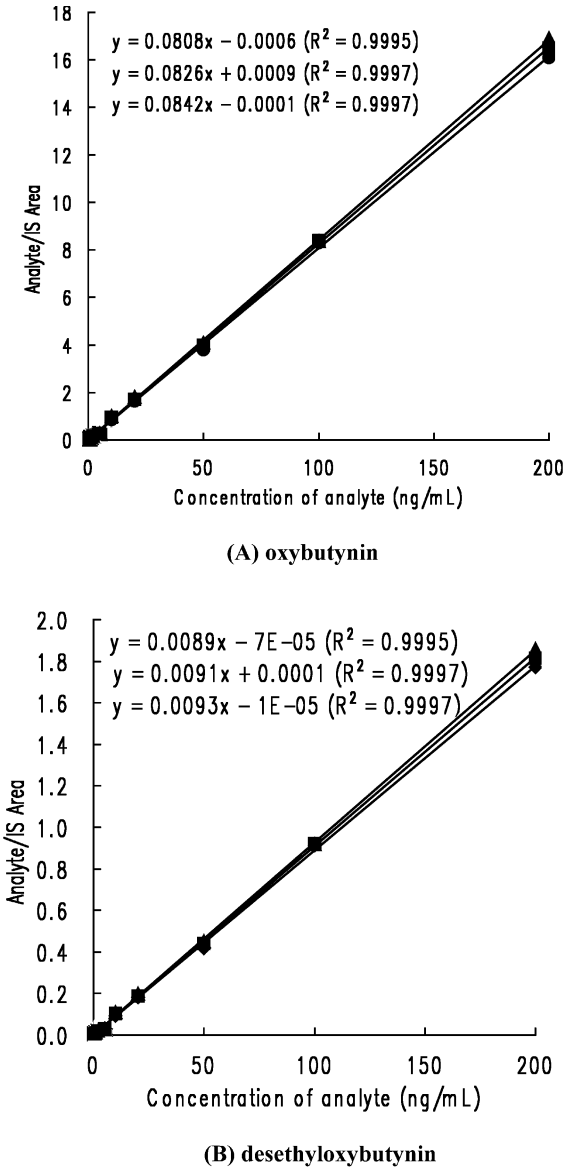

Fig. 5. Standard calibration curves of (A) oxybutynin and (B) desethyloxybutynin in dog plasma.

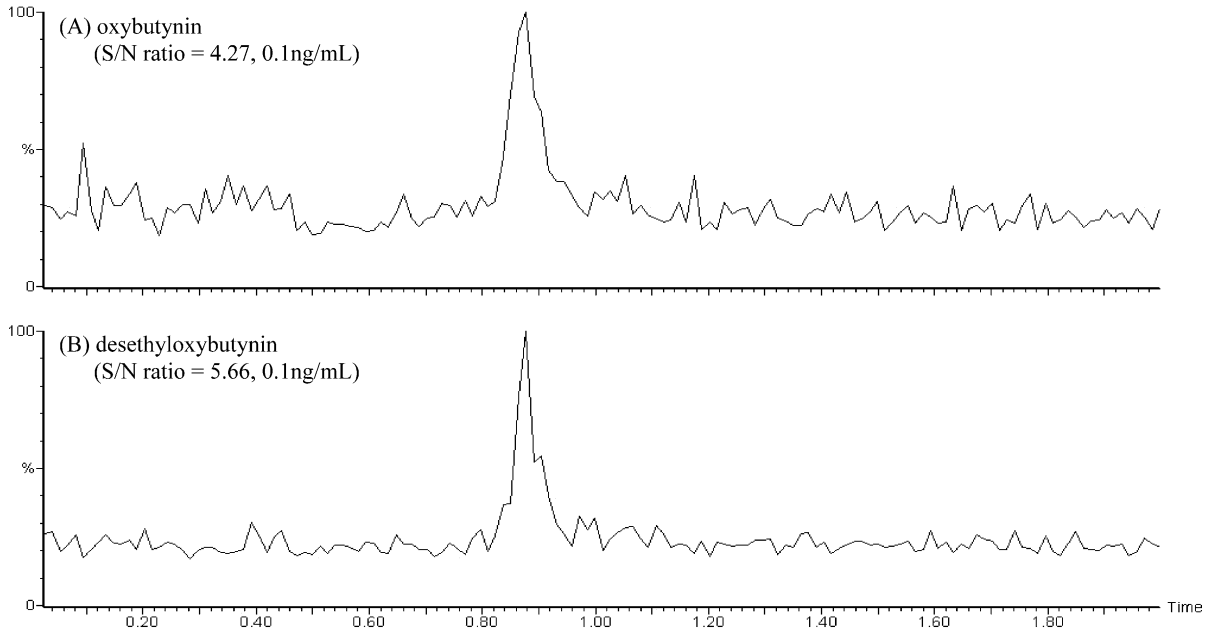

Fig. 6. Chromatogram of limit of detection of (A) oxybutynin and (B) desethyloxybutynin in dog plasma.

In addition, the matrix effect was evaluated. The chromatographic conditions may cause co-elution with a number of endogenous interferences that are not detected by MS/MS. However, they may potentially affect the slightly high ionization efficiency of analytes. To solve the problem, plasma samples from different sources (subjects) ($n = 5$) were extracted and then spiked with the same amount of oxybutynin. The MS/MS responses and precision were then compared, with the same analysis repeated ($n = 5$) after spiking a single source of matrix. If the responses and precision vary among different sources and within a single source, then the matrix effect exists. Otherwise, there is no matrix effect with similar responses and precision. The study was not able to detect a matrix effect (Table 1).

The intraday precision expressed as % RSD was measured as 1.43–3.87% for 0.5, 2, 10, and 50 ng/ml standard concentrations, with five replicates at each concentration level. The intraday accuracy expressed as a percentage of nominal values was measured as 91.4–105.2% for four standard concentrations, with five replicates at each concentration level. Table 2 and Table 3 show intraday precision and accuracy of measurement of oxybutynin and desethyloxybutynin. The interday precision was measured as 1.55–7.09% for four standard concentrations, with five replicates at each concentration level. Conversely, the interday accuracy was measured as 91.9–106.4% for four standard concentrations, with five replicates at each concentration level. Table 4 and Table 5 show interday precision and accuracy of measurement of oxybutynin and desethyloxybutynin.

Standard calibration curves were constructed on different working days (3 days) using the same biological matrix (Fig. 5(A) and (B)). The response was linear throughout the concentration range of the study, with the coefficient of determination (r^2) greater than 0.9995 for all cases. Based on a signal-to-noise level (S/N) of 3, the detection limit for oxybutynin and desethyloxybutynin were found to be 0.1 ng/ml upon injection of 20 μl of the sample into the LC–MS/MS system (Fig. 6).

Finally, determining the concentration of oxybutynin and desethyloxybutynin in dog plasma was applied to preliminary pharmacokinetic stu-

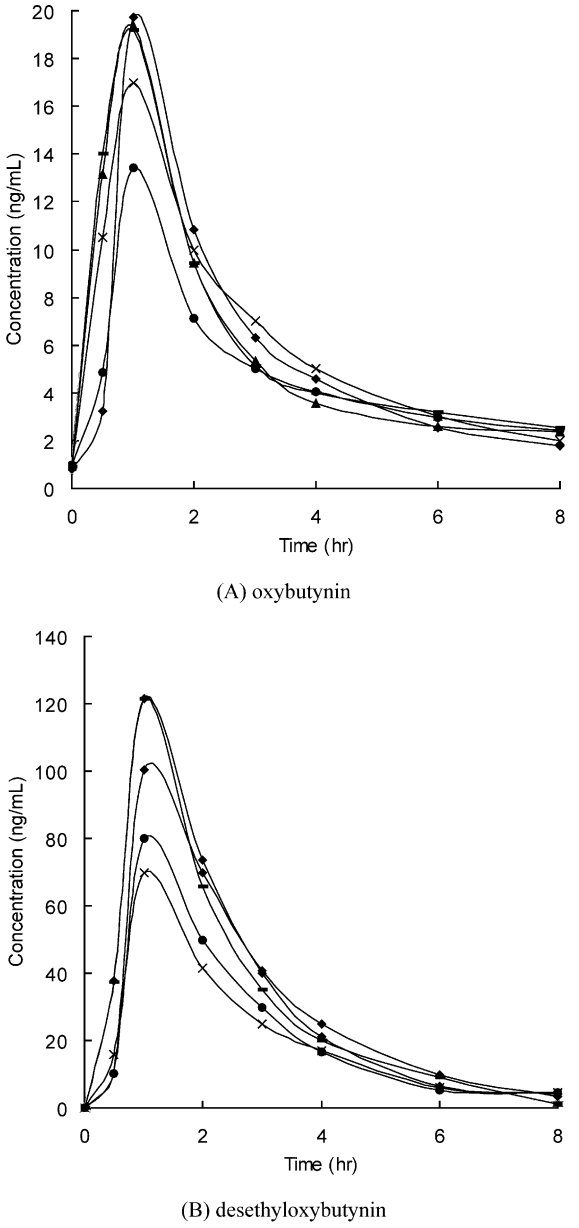

Fig. 7. Plasma concentration of (A) oxybutynin and (B) desethyloxybutynin in dog plasma-time curve.

dies. Fig. 7 shows the concentration of oxybutynin and desethyloxybutynin in dog plasma—time curve after administration of a 5 mg single dose of oxybutynin. Fig. 7 indicate that the proposed method has been successfully applied to pharmacokinetic studies to determine the concentration of oxybutynin and desethyloxybutynin in dog plasma.

References

[1] IMS America, National Prescription Audit Plus Database, Plymouth Meeting, PA, (1999).
[2] Y.E. Yarker, K.L. Goa, A. Fitton, Drug Aging 6 (1995) 243–262.
[3] B. Jacquetin, Acta Obstet. Gynecol. Scand. 76 (1997) 47.
[4] K. Waldeck, B. Larsson, K.E. Andersson, J. Urol. 157 (1997) 1093–1097.
[5] E. Lukkari, P. Taavisainen, A. Juhakoshi, O. Pelkonen, Pharmacol. Toxicol. 82 (1998) 161–166.
[6] E. Lukkari, T. Hakonen, P.J. Neuvonen, Eur. J. Clin. Pharmacol. 53 (1998) 351–354.
[7] E. Lukkari, A. Juhakoski, K. Aranko, P.J. Neuvonen, Eur. J. Clin. Pharmacol. 52 (1997) 403–406.
[8] E. Lukkari, K. Aranko, A. Juhakoski, T. Hakonen, P.J. Neuvonen, Pharmacol. Toxicol. 81 (1997) 31–34.
[9] K.S. Patrick, J.S. Markowitz, E.J. Jarvi, A.B. Straughn, M.C. Meyer, J. Chromatogr. 487 (1989) 91–98.
[10] B. Lindeke, G. Hallstrom, C. Johansson, O. Ericsson, L.I. Olsson, S. Stromberg, Biomed. Mass Spectrom. 8 (1981) 506–513.
[11] B. Lindeke, H. Brotell, B. Karlen, G. Rietz, A. Vietorisz, Acta Pharm. Suec. 18 (1981) 25–34.
[12] R. Massoud, G. Federici, S. Casciani, S.M.D. Stasi, G. Fucci, A. Giannantoni, C. Cortese, J. Chromatogr. B 734 (1999) 163–167.
[13] J. Douchamps, F. Derenne, A. Stockis, D. Gangji, M. Juvent, A. Herchuelz, Eur. J. Clin. Pharmacol. 35 (1988) 515–520.
[14] J.A. De Schutter, P. De Moerloose, J. Chromatogr. 450 (1988) 337–342.
[15] L. Aaltonen, H. Allonen, E. Iisalo, A. Juhakoski, T. Kleimola, R. Sellman, Acta Pharmacol. Toxicol. 55 (1984) 100–103.