Method development and validation for quantitative determination of methadone enantiomers in human plasma by liquid chromatography/tandem mass spectrometry ☆

H.R. Liang*, R.L. Foltz, M. Meng, P. Bennett

Tandem Labs, A Division of NWT Inc., 1121 East 3900 South, Salt Lake City, UT 84124, USA

Abstract

A high-throughput method for quantitative determination of methadone enantiomers in human plasma was developed and validated by liquid chromatography/tandem mass spectrometry. The effects of pH and of types and concentrations of mobile-phase modifiers on the enantioselectivity of (R)- and (S)-methadone were investigated on a Chiral-AGP column. A baseline separation of the enantiomers was achieved with a retention time of less than 5 min. Ionization suppression and other matrix effects were evaluated. Morphine, cocaine, 6-monoacetylmorphine, benzoylecgonine and ecgonine methyl ester did not interfere with the performance of the assay. The specificity, linearity, intra- and inter-assay precision and accuracy, and extraction recovery were fully evaluated. The method showed excellent reproducibility (overall coefficient of variance < 8%) and accuracy (overall bias < 2.7%) with a broad linear range. The enantiomers were stable in human plasma after five freeze-thaw cycles, under bench-top storage at room temperature (RT) for 6 h, in the extract reconstitution solution at RT for 17 h, and in processed-extracts stored at RT for 142 h. This validated LC/MS/MS assay offers high-throughput and improved specificity, sensitivity, linear range and ruggedness over previously published methods and has been successfully applied to the analysis of clinical samples.
© 2004 Published by Elsevier B.V.

Keywords: Enantiomer separation; Methadone

1. Introduction

Methadone plays important roles in the treatment of severe pain and drug addiction. (R)-Methadone is pharmacologically more active than the (S)-enantiomer [1]. Fig. 1 shows the chemical structures of (R)- and (S)-methadone and methadone-D_3 that was used as the internal standard in this study.

There have been many publications describing the determination of methadone enantiomers by liquid chromatography (LC) with UV detection [2–8], gas chromatography/mass spectrometry (GC/MS) [9,10], LC/MS [11–13] and by capillary electrophoresis [14–16]. So far, there have been no publications on the quantitative determination of

the enantiomers by LC coupled to atmospheric pressure ionization (API)-tandem MS (LC/MS/MS). The major advantages of tandem mass spectrometry are highly specific and sensitive [17–21]. The aim of the present study was to develop and validate a rapid, specific, sensitive, robust and reliable method for the quantitative determination of the methadone enantiomers in human plasma by LC/MS/MS.

2. Experimental

2.1. Reference materials, biological matrix and chemicals

(±)-Methadone and (±)-methadone-D_3 were purchased from Cerilliant as methanolic solutions (Austin, TX). Blank human plasma with K_3-EDTA as an anticoagulant was purchased from Biochemed Pharmacologicals (Winchester, VA). All chemicals were of analytical-reagent grade; hexane, methanol, isopropyl alcohol, acetonitrile, methyl *t*-butyl

---

* Corresponding author. Tel.: +1-801-293-2400;
fax: +1-801-293-2389.
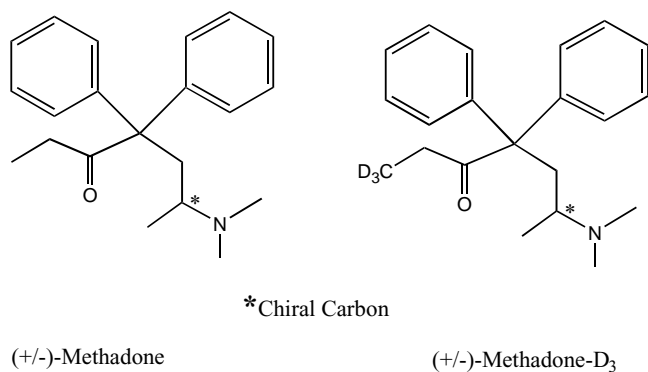*E-mail address:* hairui@tandemlabs.com (H.R. Liang).

Fig. 1. Chemical structures of chiral methadone and methadone-D_3.

ether and ethyl acetate were purchased from EM Science (Gibbstown, NJ); ammonium acetate, sodium bicarbonate from VWR Scientific Products (West Chester, PA).

2.2. Instrumentation

The following apparatus were used in this study: Beckman GS-6R Centrifuge (Beckman Instruments, Fullerton, CA); Turbo Vap 96 Evaporator (Zymark Corp., Hopkinton, MA); Orion 230A pH Meter (Orion, Boston, MA) and Tomtec Quadra 96 Model 320 used for automated sample preparation (Tomtec, Hamden, CT).

Chromatography was performed on a Shimadzu SCL-10A controller with CL-10AD pump and CTO-10A column oven (Shimadzu Scientific Instruments Inc., Columbia, MD). A Chiral-AGP (5 μm, 2.0 mm × 50 mm) was used as the chromatographic column (Analytical Sales and Services Inc., Pompton Plains, NJ). The autosampler used was a PE Series 200 Autosampler (Perkin-Elmer, Hopkinton, MA). Analyses were performed on a API 365 tandem mass spectrometer (MDS Sciex, Concord, Ontario).

2.3. Calibration standards, internal standard and quality controls (QCs)

(±)-Methadone calibration standard and QC spiking solutions were prepared in methanol/water (1/1), kept in amber glass containers and stored in a 1–8 °C refrigerator. (R)-Methadone-D_3 and (S)-methadone-D_3 working internal standards (200 ng/ml) were prepared in 500 mM sodium bicarbonate buffer (pH 11) and stored in a 1–8 °C refrigerator. Low, medium, high and dilution QCs (15.0, 393, 786 and 1960 ng/ml, respectively) were prepared in human plasma (K_3EDTA). The aliquots of the low, medium, high and dilution QC pools were kept in cryogenic vials and stored in a −20 °C freezer. The calibrators were prepared on the day of each run by spiking 50.0 μl of the appropriate spiking solution into 450 μl of blank plasma. The calibrators included seven different concentrations (5.00, 10.0, 50.0, 200, 500, 850 and 1000 ng/ml).

2.4. Sample preparation

One hundred μl of each standard, QC, QC0 (plasma blank with internal standard) and blank control sample was aliquoted into separate wells in a 96-well plate. Then 100 μl of the working internal standard solution [(R)- and (S)-methadone-D_3, 200 ng/ml] was added to each sample except blank control samples. One hundred μl of 500 mM sodium bicarbonate buffer (pH 11.0) was added to the blanks. All samples were vortex mixed and centrifuged. Four hundred μl of hexane was added to each sample and mixed 30 times using an automated Tomtec Quadra 96-well workstation. Two hundred and fifty μl of each supernatant was transferred into a clean 96-well plate. The samples were dried and reconstituted with 200 μl of 12% isopropyl alcohol in 10 mM ammonium acetate.

2.5. LC and MS conditions

The final optimized LC separation was performed on a Chiral-AGP column (5 μm, 2.0 mm × 50 mm) with a mobile phase containing 12% isopropyl alcohol in 10 mM ammonium acetate (pH unadjusted) at a flow rate of 400 μl/min and an oven temperature of 45 °C. The API 365 tandem mass spectrometer was operated in the multiple-reaction monitoring (MRM) mode under optimized conditions for detection of (R)- and (S)-methadone and methadone-D_3 positive ions formed by TurboIonSpray[TM] ionization.

2.6. Interference experiment

A solution containing morphine, cocaine, 6-monoacetylmorphine, ecgonine methyl ester, and benzoylecgonine (1000 ng/ml for each compound) was spiked into a 96-well plate. The plate was dried completely. Aliquots of low and high QC samples were added into the wells containing the interference drugs. These samples were then extracted and analyzed to determine if the presence of these compounds would interfere the determination of the methadone enantiomers.

2.7. Matrix effects

Matrix effects were evaluated by postcolumn infusion of methadone or methadone-D_3 into the MS detector and autosampler injection of extracted blank onto the analytical column [22,23].

2.8. Method validation

The quantitation range was from 5.00 to 1000 ng/ml. Two calibration curves over this range were prepared on three separate days. The curves were fit by a least-square $1/x$-weighted linear regression method. Each analytical accuracy and precision run included calibration standards in duplicate with seven different concentrations; low, medium

and high quality control samples (QCs) in replicates of six; two plasma blanks (no internal standard) and QC0s (plasma blank with internal standard). Dilution QCs were analyzed in replicates of six. The freeze-thaw stability of (R)- and (S)-methadone in human plasma was determined by subjecting low and high QC samples to five freeze-thaw cycles before processing. The room temperature (RT) matrix stability of (R)- and (S)-methadone in human plasma was determined by storing low and high QC samples for 6 h under RT and normal light conditions. The autosampler stability of (R)- and (S)-methadone was determined for an analytical run length of approximately 17 h at RT. A set of low and high QC samples was stored at RT for approximately 17 h after initiation of the run. These test QC samples were reinjected and quantitated against the original calibration curves and compared against theoretical. The processed extract stability of (R)- and (S)-methadone was determined for low, medium and high QC extracts maintained at RT and normal light conditions for approximately 142 h prior to analysis. The data were acquired by re-injecting a previously analyzed set of calibration standards and QC samples. The extraction efficiencies of (R)- and (S)-methadone were determined by comparing peak areas of the analytes extracted from plasma with those of post-extracted blanks fortified with the analytes. The experiments were performed at low, medium and high concentrations to determine that there was no concentration bias.

2.9. Data management and quantitation

(R)- and (S)-methadone and methadone-D_3 chromatographic peaks were integrated using Analyst software (version 1.2). The first eluted peak of the (±)-methadone-D_3 ((R)-methadone-D_3) was used for the integration of the (R)-methadone. The (S)-methadone was quantified using the second peak of the (±)-methadone-D_3 ((S)-methadone-D_3). Quantitation was based upon linear regression analysis of calibration curves (weighted $1/x$) using the area ratio versus theoretical concentration utilizing Watson® DMLIMS software (version 6.1.1.04).

All studies were conducted within the guidelines of the US FDA Good Laboratory Practice Regulations for Nonclinical Laboratory Studies (Title 21 CFR Part 58).

3. Results and discussion

3.1. LC/MS/MS

The product ion spectra of methadone and methadone-D3 are shown in Figs. 2 and 3. The most abundant and stable product ions for methadone, and methadone-D_3 are 265 and 268 amu, respectively. Therefore, the precursor-product ion transitions monitored were $310 \rightarrow 265$ (±0.3 amu) for (R)- and (S)-methadone, and $313 \rightarrow 268$ (±0.3 amu) for (R)- and (S)-methadone-D_3.

3.2. Optimized separation of (R)- and (S)-methadone

The nature and concentrations of organic modifiers, anionic buffers and buffer pH strongly influence the retention, enantioselectivity and intensity of chiral compounds on a Chiral-AGP column [24–26]. The effect of different organic modifiers, including methanol, isopropyl alcohol and acetonitrile, on the retention and enantioselectivity of (R)- and (S)-methadone was studied. The best sensitivity and resolution of (R)- and (S)-methadone were obtained with isopropyl alcohol. Then the effect of isopropyl alcohol concentrations was further investigated. As shown in Fig. 4, the retention and enantioselectivity of the enantiomers decreased with
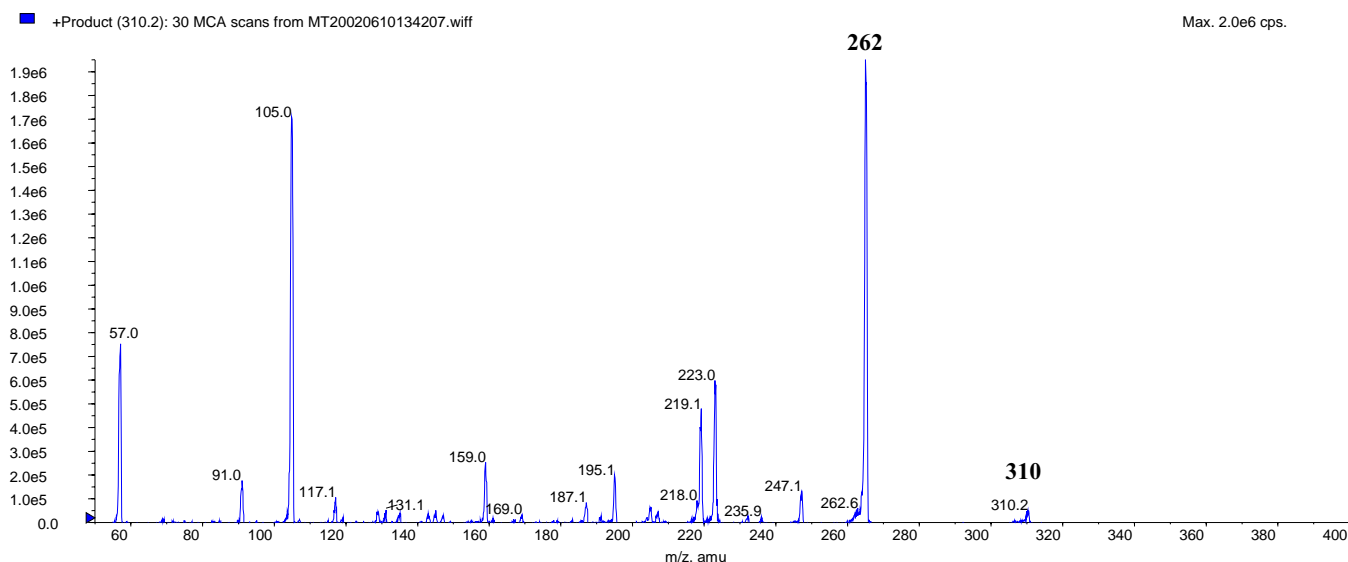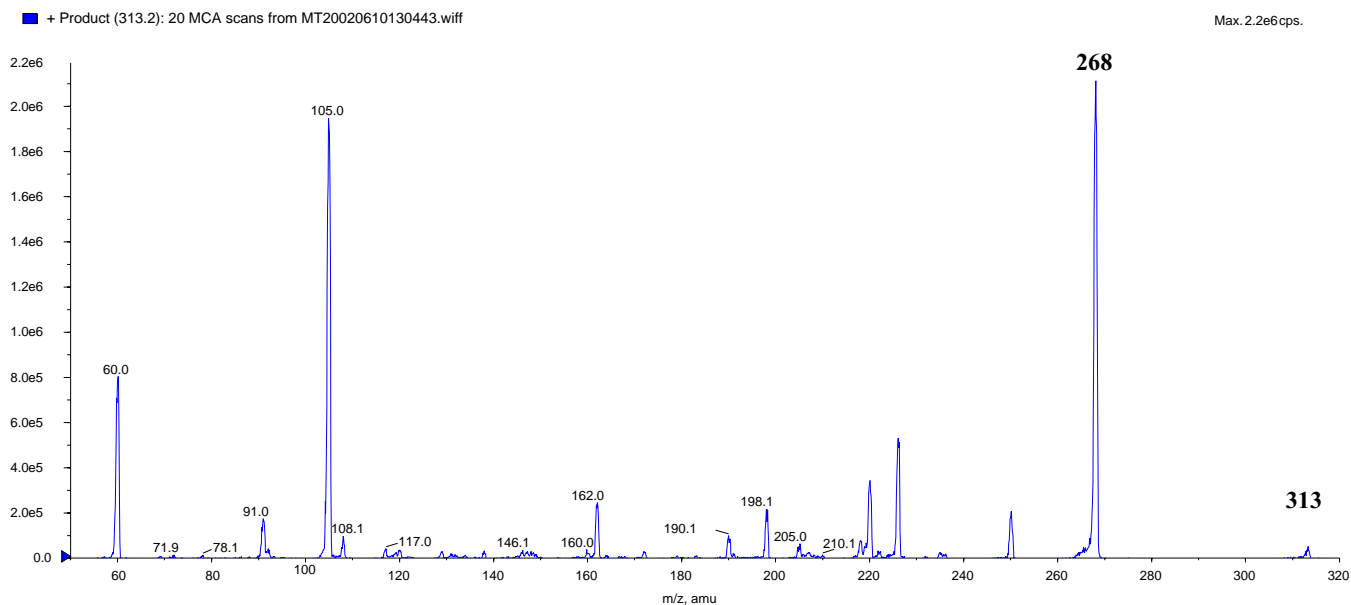

Fig. 2. Product ion spectrum of methadone.

+ Product (313.2): 20 MCA scans from MT20020610130443.wiff   Max. 2.2e6 cps.

Fig. 3. Product ion spectrum of methadone-D$_3$.

increasing the concentrations of isopropyl alcohol [27]. The effect of NH$_4$OAc concentration on the resolution of (*R*)- and (*S*)-methadone was investigated further. As you can see in Fig. 5, the higher concentration of ammonium acetate, the

worse the separation resolution; the better signal intensity; and the shorter the retention time. The resolution of the enantiomers was not sufficient using 20 or 50 mM of ammonium acetate in mobile phase although the high sensitivity and
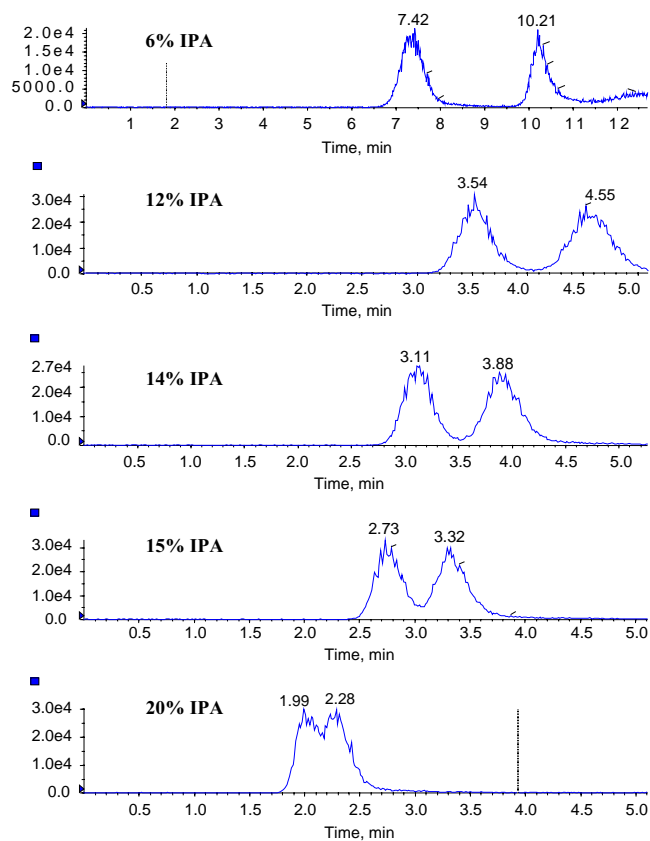
Fig. 4. Effect of concentrations of isopropyl alcohol (IPA) on the retention and enantioselectivity of (*R*)- and (*S*)-methadone.
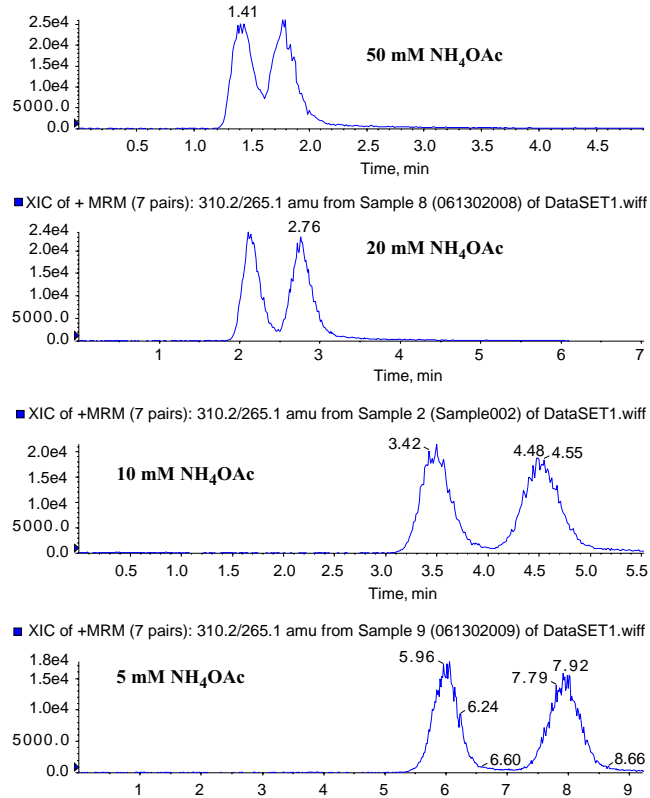
Fig. 5. Effect of concentrations of NH$_4$OAc on the retention and enantioselectivity of (*R*)- and (*S*)-methadone.

very short retention time was obtained. Ten millimolar ammonium acetate was chosen because we wanted to keep better sensitivity and shorter retention time while the separation resolution was adequate. Ten millimolar ammonium acetate in the mobile phase was not used as a buffer but as a modifier.

The effect of pH ranging from 4 to 7 on separation of the enantiomers was also investigated. The retention and enantioselectivity of (R)- and (S)-methadone increased with increasing pH. The optimized pH was from 6.8 to 7.0, which is the pH of unadjusted NH_4OAc in water. The optimized LC conditions were performed isocratically using a mobile phase consisting of 12% isopropyl alcohol in 10 mM ammonium acetate (pH unadjusted) on a Chiral-AGP column. Under these conditions, the enantiomers were baseline resolved within 5 min.

3.3. Sample extraction

The human plasma samples were extracted with different organic solvents including hexane, methyl *t*-butyl ether and ethyl acetate at different pH using different buffer solutions including phosphate buffers (pH 2 and 7), acetate buffer (pH 4.5), borate buffer (pH 8.5), and carbonate buffer (pH 11.0). Buffering plasma samples with carbonate at pH 11.0 and then extracting with hexane provided the cleanest samples, best precision, accuracy and recovery for (R)- and (S)-methadone.

3.4. Drug interference

Methadone plays important roles in the treatment of drug addiction. Therefore, it is important to evaluate if the presence of the addictive drugs and their metabolites would influence the performance of the (R)- and (S)-methadone assay. The test QC samples containing morphine, cocaine, 6-monoacetylmorphine, ecgonine methyl ester and benzoylecgonine were extracted and analyzed using the developed method. The results showed that the presence of all these compounds in human plasma did not interfere with the performance of the (R)- and (S)-methadone assay.

3.5. Method specificity

Six different individual lots of blank plasma were extracted and analyzed as blanks and as QC0s. The results

Table 2
Calibration curve parameters for (R)- and (S)-methadone

n = 3	Slope	Intercept	R-squared
(R)-Methadone			
Mean	0.005961	−0.000816	0.9973
S.D.	0.000372	0.002343	0.0025
%CV	6.2	−287.1	0.3
(S)-Methadone			
Mean	0.005838	−0.001091	0.9973
S.D.	0.000479	0.002517	0.0020
%CV	8.2	−230.7	0.2

Linear weighted $1/x$. The two curves were prepared and run on three different days.

showed that there were no measurable peak areas detected in the blanks and QC0s for the analyte or internal standard that could affect quantitation.

3.6. Matrix effects or ionization suppression

It is very important to investigate the matrix effects in order to develop a reliable and reproducible LC/MS/MS method. There have been few applications using a Chiral-AGP column in LC/MS/MS [28] even though there have been numerous applications in LC/UV. Often a mobile phase containing very high percentage of water used on a reversed-phase column can lead to matrix effects during LC/ESI-MS experiments [29]. The chiral selector in the CHIRAL-AGP phase is α1-acid glycoprotein (AGP) and the column is used in reversed-phase with a mobile phase containing very high percentage of water. We anticipated that there would be matrix effects when using a Chiral-AGP column. Ionization suppression on a Chiral-AGP column was then investigated by post-column infusion of (R)- and (S)-methadone or methadone-D_3 and on-column injection of the extracted blanks. No ionization suppression or other matrix effects were observed during the retention times of the enantiomers even though a mobile phase containing a very high percentage of water was used on the CHIRAL-AGP column. The results were further confirmed by the following experiment. (R)- and (S)-methadone and methadone-D_3 were spiked separately into human plasma and into water and extracted with exactly the same procedures. The results showed that there was no significant difference in peak areas of (R)- and (S)-methadone and methadoen-D_3 extracted

Table 1
Evaluation of ionization suppression by comparing peak areas of (R)- and (S)-methadone and methadone-D_3 extracted from human plasma and water

n = 10	(R)-Methadone	(S)-Methadone	(R)-Methadone-D_3	(S)-Methadone-D_3
Peak areas in human plasma (1000 ng/ml)				
Average	222000	234000	207000	218000
%CV	12.9	13.8	13.4	13.5
Peak areas in water (1000 ng/ml)				
Average	189400	206100	215200	224000
%CV	5.4	5.4	18.5	19.2

Table 3
Back-calculated concentrations of calibrators for (R)- and (S)-methadone

	Nominal concentration (ng/ml)						
	5.00	10.0	50.0	200	500	850	1000
(R)-Methadone							
Mean (n = 6)	5.30	9.77	48.1	204	479	867	1000
%CV	7.5	3.2	4.7	3.6	4.1	5.8	4.5
%Bias	6.0	−2.3	−3.8	2.0	−4.2	2.0	0.0
(S)-Methadone							
Mean (n = 6)	5.36	9.79	46.4	207	486	862	998
%CV	6.8	4.8	4.5	4.6	6.4	4.6	4.8
%Bias	7.2	−2.1	−7.2	3.5	−2.8	1.4	−0.2

Table 4
Intra- and inter-assay precision and accuracy for (R)- and (S)-methadone quality control (QC) samples

Number of runs: 3, nominal concentration (ng/ml)	Low QC, 15.0	Medium QC, 393	High QC, 786	Dilution QC, 1960, d.f. = 10
(R)-Methadone				
Mean observed concentration	14.6	390	778	1950
%Bias	−2.7	−0.8	−1.0	−0.5
Between run %CV	5.8	6.0	0.0	N/A
Within run %CV	6.9	3.2	3.0	3.1
(S)-Methadone				
Mean observed concentration	15.1	393	792	1960
%Bias	0.7	0.0	0.8	0.0
Between run %CV	4.7	8.0	2.4	N/A
Within run %CV	8.3	2.8	3.5	2.9

Sample numbers of each run: 6. N/A: not applicable because samples were only included in one run.

from human plasma and from water (Table 1), indicating that there were no matrix effects.

3.7. Method validation

The method exhibited linearity over the range 5–1000 ng/ml for both (R)- and (S)-methadone (Table 2). The back-calculated concentrations of calibration standards for (R)- and (S)-methadone are shown in Table 3. The accuracy and precision of the LC/MS/MS method for (R)- and

(S)-methadone in human plasma were determined by analyzing quality control samples in replicates of six on three separate days. Dilution QCs were analyzed in replicates of six on a single day. The method showed excellent reproducibility and accuracy with overall CV ≤ 8.3% and Bias ≤2.7% (Table 4). The lower limit of quantitation (LLOQ) of 5.00 ng/ml was determined using 100 µl aliquots and six different lots of human plasma. The overall CV for (R)- and (S)-methadone in the LLOQ experiment was 6.5 and 3.9%, respectively; and the overall bias was 2.2 and 1.8%.

Table 5
Freeze-thaw, benchtop storage and autosampler run stability for (R)- and (S)-methadone QC samples

Stability (n = 3)	(R)-Methadone		(S)-Methadone	
Nominal concentration (ng/ml)	Low QC 15.0	High QC 786	Low QC 15.0	High QC 786
5 cycles F/T stability				
%CV	3.4	3.1	0.4	2.4
%Theoretical	92.7	99.2	98.0	106.4
%Bias	−7.3	−0.8	−2.0	6.4
Benchtop storage stability				
%CV	5.7	2.3	5.7	1.5
%Theoretical	112.0	101.0	113.3	100.8
%Bias	12.0	1.0	13.3	0.8
Autosampler stability				
%CV	7.6	2.7	7.1	3.3
%Theoretical	95.3	98.9	102.7	103.4
%Bias	−4.7	−1.1	2.7	3.4

Table 6
(*R*)- and (*S*)-methadone extraction efficiency

$n = 3$	(*R*)-Methadone			(*S*)-Methadone		
	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC
%CV	5.5	2.9	1.8	2.7	5.5	3.5
%Mean recovery	78.8	65.5	71.6	73.6	63.5	70.6
Overall mean %CV		3.4			3.9	
Overall %mean recovery		72.0			69.2	

The results of freeze-thaw, matrix, autosampler run and processed extract stability for (*R*)- and (*S*)-methadone in human plasma are shown in Table 5. Each enantiomer was stable in human plasma after five freeze-thaw cycles, under bench-top storage at room temperature for 6 h, in the reconstitution solution at RT for 17 h, and in processed-extracts stored at RT for 142 h. The extraction efficiencies of (*R*)- and (*S*)-methadone were determined by comparing peak areas of (*R*)- and (*S*)-methadone extracted from plasma with those of post-extracted blanks fortified with (*R*)- and
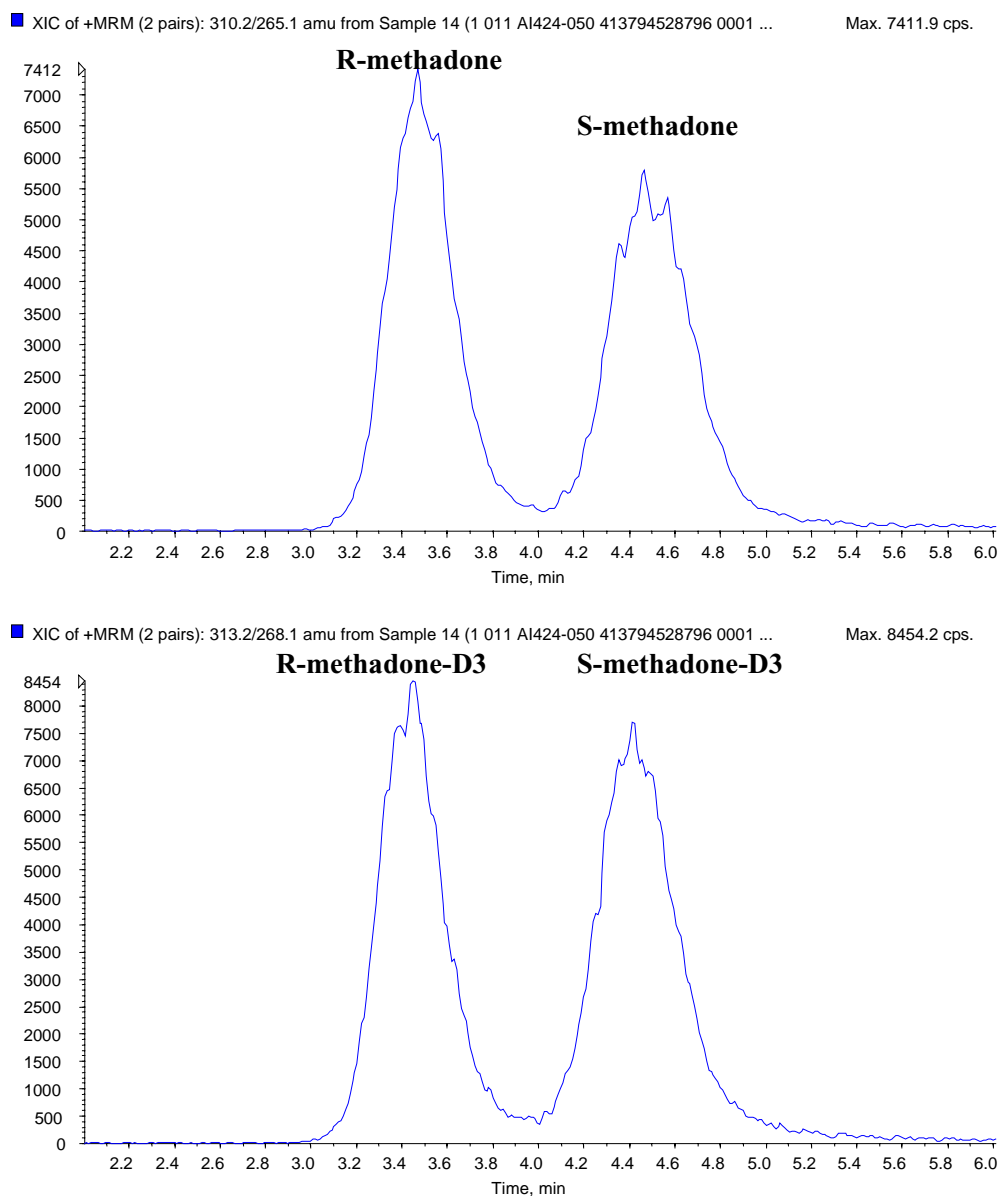

Fig. 6. Chromatogram of (*R*)- and (*S*)-methadone under optimized conditions.

(*S*)-methadone. The mean extraction efficiency (%) from low, medium and high QCs was 72.0 and 69.2% for (*R*)- and (*S*)-methadone, respectively (Table 6). Different lots of HPLC columns, LC pumps, autosamplers and mobile phase prepared on different days using different lots of isopropyl alcohol and ammonium acetate were used to evaluate the reliability and reproducibility of the assay. The results showed that the assay was reliable and reproducible under all of the different conditions evaluated.

3.8. Sample analysis

This validated method has been successfully applied to the analyses of clinical samples. Over 100 samples were extracted and analyzed within a day. A typical chromatogram of clinical samples was showed in Fig. 6.

4. Conclusions

For the first time, LC/MS/MS has been successfully applied for the quantitative determination of the methadone enantiomers in human plasma. The baseline separation of the enantiomers was achievable within 5 min using an MS compatible mobile phase and simple isocratic LC conditions. This validated LC/MS/MS assay offers high-throughput and improved specificity, sensitivity, linear range and ruggedness over previously published methods. The Chiral-AGP column has been successfully applied to the LC/MS/MS quantitation of methadone enantiomers in human plasma without ionization suppression or other matrix effects. The present study of matrix effects helps extend the application of Chiral-AGP columns in LC/MS/MS and better understand matrix effects and ionization suppression in LC/MS/MS.

References

[1] A.F. Casy, R.T. Parfitt, Opioid Analgesics, Chemistry and Receptors, Plenum Press, New York, 1986.
[2] O. Beck, L.O. Boreus, P. Lafolie, G. Jacobsson, J. Chromatogr. 570 (1991) 198.
[3] N. Schmidt, K. Brune, K.M. Williams, G. Geisslinger, Chirality 6 (1994) 492.
[4] H.R. Angelo, N. Beck, K. Kristensen, J. Chromatogr. A 666 (1994) 283.
[5] S. Rudaz, J.L. Veuthey, Chirality 11 (1999) 319.
[6] S. Rudaz, D. Ortelli, M. Gex-Fabry, J.J. Deglon, L. Balant, J.L. Veuthey, Chirality 11 (1999) 487.
[7] N. Schmidt, K. Brune, G. Geisslinger, J. Chromatogr. 583 (1992) 195.
[8] D.J. Foster, A.A. Somogyi, F. Bochner, J. Chromatogr. B 744 (2000) 165.
[9] F. Sporkert, F. Pragst, J. Chromatogr. B 746 (2000) 255.
[10] G.I. Kang, F.S. Abbott, J. Chromatogr. 231 (1982) 311.
[11] D.G. Wilkins, P.R. Nagasawa, S.P. Gygi, R.L. Foltz, D.E. Rollins, J. Anal. Toxicol. 20 (1996) 355.
[12] P. Kintz, H.P. Eser, A. Tracqui, M. Moeller, V. Cirimele, P. Mangin, J. Forensic Sci. 42 (1997) 291.
[13] P. Kintz, A. Tracqui, C. Marzullo, A. Darreye, F. Tremeau, P. Greth, B. Ludes, Ther. Drug Monit. 20 (1998) 35.
[14] S. Cherkaoui, S. Rudaz, E. Varesio, J.L. Veuthey, Electrophoresis 22 (2001) 3308.
[15] S. Rudaz, S. Cherkaoui, J.Y. Gauvrit, P. Lanteri, J.L. Veuthey, Electrophoresis 22 (2001) 3316.
[16] S. Rudaz, E. Calleri, L. Geiser, S. Cherkaoui, J. Prat, J.L. Veuthey, Electrophoresis 24 (2003) 2633.
[17] W. Muck, Pharmazie 54 (1999) 639.
[18] L. Yang, M. Amad, W.M. Winnik, A.E. Schoen, H. Schweingruber, I. Mylchreest, P.J. Rudewicz, Rapid. Commun. Mass Spectrom. 21 (2002) 2060.
[19] J.M. Onorato, J.D. Henion, P.M. Lefebvre, J.P. Kiplinger, Anal. Chem. 73 (2001) 119.
[20] M.S. Lee, E.H. Kerns, Mass Spectrom. Rev. 18 (1999) 187.
[21] P.R. Tiller, J. Cunniff, A.P. Land, J. Schwartz, I. Jardine, M. Wakefield, L. Lopez, J.F. Newton, R.D. Burton, B.M. Folk, D.L. Buhrman, P. Price, D. Wu, J. Chromatogr. A 771 (1997) 119.
[22] C. Miller-Stein, R. Bonfiglio, T.V. Olah, R.C. King, Am. Pharm. Rev. (2000) 54.
[23] R.C. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, T.V. Olah, J. Am. Soc. Mass Spectrom. 11 (2000) 942.
[24] E. Arvidsson, S.O. Jansson, G. Schill, J. Chromatogr. 591 (1992) 55.
[25] J. Hermansson, A. Grahn, J. Chromatogr. A 694 (1995) 57.
[26] S.R. Narayanan, J. Pharm. Biomed. Anal. 10 (1992) 251.
[27] M. Enquist, J. Hermansson, J. Chromatogr. 519 (1990) 271.
[28] S.A. Wood, A.H. Parton, R.J. Simmonds, D. Stevenson, Chirality 8 (1996) 264.
[29] R. Bakhtiar, F.L.S. Tse, Rapid Commun. Mass Spectrom. 14 (2000) 1128.