Journal of Chromatography B, 889–890 (2012) 44–49

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Development and validation of a sensitive LC–MS/MS method for the simultaneous quantitation of theophylline and its metabolites in rat plasma

Jung-woo Chae [a], Dong-hyun Kim [a], Byung-yo Lee [a], Eun jung Kim [b], Kwang-il Kwon [a,*]

[a] College of Pharmacy, Chungnam National University, Daejeon 305-764, Republic of Korea
[b] Division of Pharmacological Research, National Institute of Food and Drug Evaluation, Korea Food & Drug Evaluation, Republic of Korea

ARTICLE INFO

Article history:
Received 19 October 2011
Accepted 25 January 2012
Available online 2 February 2012

Keywords:
Theophylline
1-Methyl xanthine
3-Methyl xanthine
1,3-Dimethyl uric acid
1-Methyl uric acid
LC–MS/MS

ABSTRACT

A rapid, specific, and reliable LC–MS/MS-based bioanalytical method was developed and validated in rat plasma for the simultaneous quantitation of theophylline and its four metabolites: 1,3-dimethyluric acid (1,3-DMU), 3-methylxanthine (3-MX), 1-methylxanthine (1-MX), and 1-methyluric acid (1-MU). Chromatographic separation of these analytes was achieved on a Gemini C18 column (50 mm × 4.60 mm, 5 μm) using reversed phase chromatography. The analytes were monitored by electrospray ionization in negative ion multiple reaction monitoring mode. Modification of collision energies was performed in parallel with chromatographic separation to further eliminate interference peaks. The method was validated from 0.05 to 30 μg/mL for 1-MX, 1,3-DMU, 1-MU, and theophylline and from 0.1 to 30 μg/mL for 3-MX using 0.2 mL of plasma sample. The intra- and inter-day precision and accuracy of the quality control samples at low, medium, and high concentration levels exhibited relative standard deviations (RSD) of less than 13% and with relative error (RE) values of −8.8% to 9.7%. The method was successfully applied for the quantitation of theophylline and its metabolite in rat plasma samples.

1. Introduction

Theophylline is a methylxanthine that acts as a bronchial smooth muscle relaxant and a suppressor of the non-bronchodilator response of airways. The bronchodilation mechanism of theophylline is through the inhibition of phosphodiesterase by an increase in cAMP levels. The morbidity and mortality of theophylline toxicity is such that monitoring the plasma concentrations of theophylline has been recommended to reduce its side effects. Theophylline is reportedly metabolized by multiple forms of cytochrome P450 in human, rabbit, and rat liver microsomes [1]. These metabolites are 1-methylxanthine (1-MX), 3-methylxanthine (3-MX), 1-methyluric acid (1-MU), and 1,3-dimethyluric acid (1,3-DMU) [2].

Several high-performance liquid chromatography (HPLC)-based bioanalytical methods have been published for the quantitation of theophylline [3–5]. Most of these published methods suffer from long HPLC run times (over 60 min) and high detection limits (greater than 300 ng/mL for theophylline metabolites). In recent years, liquid chromatography–tandem mass spectrometry (LC–MS/MS)-based bioanalytical methods have also been used to quantitate theophylline in biological fluids. The use of MS has decreased detection limits to as low as 63 ng/mL for theophylline metabolites. Run times were reduced to under 10 min using LC–MS/MS methods [6–13]. However, all of these methods involved multistep extractions, large volumes of organic solvent, and required intensive labor to prepare the sample. No published reports currently describe the use of protein precipitation concerning theophylline and its four main metabolites as a means of sample preparation from biological systems.

In this study, protein precipitation was employed to shorten processing time and reduce labor. Hence a simple, sensitive, and robust method, suitable for pharmacokinetic research and routine TDM of theophylline and its metabolites, was developed. The present study was undertaken to develop and validate a simple, high-throughput, sensitive, and convenient bioanalytical assay for the simultaneous quantitation of theophylline, 1-MX, 3-MX, 1,3-DMU, and 1-MU in rat plasma by LC–MS/MS.

2. Experimental

2.1. Chemical, reagents, materials, and apparatus

Theophylline, 1-MX, 3-MX, 1,3-DMU, 1-MU, and the internal standard (IS) chlorzoxazone were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC-grade methanol was obtained from Merck (Darmstadt, Germany). A PURELAB Ultra system from ELGA

* Corresponding author. Tel.: +82 42 821 5937; fax: +82 42 823 6781.
E-mail address: kwon@cnu.ac.kr (K.-i. Kwon).

1570-0232/$ – see front matter © 2012 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2012.01.028

(Marlow, UK) was used in the laboratory to produce deionized water.

2.2. Standard stock solutions and working solution preparation

Standard stock solutions containing 1 mg/mL concentrations of free-form theophylline, 1-MX, 3-MX, 1,3-DMU, 1-MU, and the IS were made up in methanol. All standard stock solutions were stored at −20 °C. A series of working solutions was obtained by diluting appropriate amounts of these standards with 100% methanol to six concentration levels. Working solutions were stored at 4 °C away from light.

2.3. Calibration standards and quality control sample preparation

The IS was prepared in methanol at 5 μg/mL. 20 μL of each standard solution (theophylline and four metabolites) was added to 100 μL of drug-free rat plasma, and IS (200 μL for precipitation) was inserted. And the tube was vortexed for 5 min. The precipitate was removed by centrifugation at 15,000 rpm for 10 min. The supernatant was transferred and evaporated to dryness under a stream of air at 40 °C. The dried extract was resuspended in 200 μL of methanol for the injection to LC–MS/MS. The resulting plasma concentrations were 0.05, 0.5, 3, 7.5, 15, and 30 μg/mL for 1-MX, 1,3-DMU, 1-MU, and theophylline and 0.1, 0.5, 3, 7.5, 15, 30 μg/mL for 3-MX.

Quality control (QC) samples were made up in blank rat plasma at three levels: low (0.2 μg/mL), medium (7.5 μg/mL), and high (30 μg/mL) for 1-MX, 1,3-DMU, 1-MU, and theophylline, and low (0.5 μg/mL), medium (7.5 μg/mL), and high (30 μg/mL) for 3-MX. All QC samples were stored at −70 °C.

For redissolution of the dried residue, we tested different solvents with methanol and various percentages of water in methanol as components. Redissolution of theophylline, its metabolites and IS was best achieved in 100% methanol, which offered superior peak shape.

2.4. Sample preparation

Frozen plasma samples were thawed at room temperature. One hundred microliters of methanol and 200 μL methanol containing IS at concentration 5 μg/mL were added to 100 μL plasma, and the tube was vortexed for 5 min. The precipitate was removed by centrifugation at 15,000 rpm for 10 min. The supernatant was transferred and evaporated to dryness under a stream of air at 40 °C. The dried extract was resuspended in 200 μL of methanol for the injection to LC–MS/MS system. The supernatant was transferred into an injection vial and 10 μL of this solution was injected into a LC–MS/MS for quantitative analysis.

2.5. Liquid chromatography/mass spectrometry

LC was performed on an Agilent 1100 HPLC system (Agilent Technologies, Inc., Santa Clara, CA, USA) and separation was carried out at 25 °C on a Gemini C18 column (50 mm × 4.60 mm, 5 μm; Phenomenex, Torrance, CA, USA). The mobile phase consisted of 0.05% acetic acid in 10:90 (v/v) water:methanol at a flow rate of 250 μL/min. Separations were conducted under isocratic conditions. The HPLC system was coupled to a PE SCIEX API 2000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) in multiple reaction monitoring (MRM) mode. An electrospray interface was used in negative mode. The instrument parameters for monitoring theophylline, 1-MX, 3-MX, 1,3-DMU, 1-MU, and the IS during method validation and sample analysis were as follows: TurboIonSpray (TIS) temperature, 350 °C; exhausting gas, 45 psi; nebulizing gas, 95 psi; curtain gas, 50 psi; declustering potentials (DP), −51 V, −67 V, −53 V, −63 V, −62 V, and −44 V; entrance potential (EP), −9 V; collision energies (CE), −27 eV, −24 eV, −26 eV, −31 eV, −19 eV, and −26 eV; collision cell exit potential (CXP), −4 V for theophylline, 1-MX, 3-MX, 1,3-DMU, 1-MU, and the IS. The use of different collision energies significantly reduces background noise and interference peaks without sacrificing sensitivity. The following precursors to product ion transitions were used in the SRM of theophylline, 1-MX, 3-MX, 1,3-DMU, 1-MU, and the IS, respectively, m/z 179 → 164, m/z 165 → 108, m/z 165 → 122, m/z 195 → 110, m/z 181 → 138, and m/z 168 → 132 with dwell times of 250 ms. The mass spectrometer was operated at unit mass resolution for both the first and the third quadrupoles.

2.6. Method validation procedure

The method was validated with respect to selectivity, linearity, accuracy, precision, percent recovery, matrix effect, and stability.

Calibration curves were constructed between 100 and 30,000 ng/mL for 3-MX and from 50 to 30,000 ng/mL for theophylline, 1-MX, 1,3-DMU, and 1-MU by determining the best fit of peak area ratios of analyte to the IS (y) as a function of nominal concentration (x). The data were fitted to the equation $y = bx + a$ using a $1/x^2$ weighted least squares regression. Concentrations of QC samples and plasma samples were calculated in accordance with the calibration curves. Intra-day and inter-day precision and accuracy were evaluated by assaying six replicates of each spiked QC sample at the low, middle, and high concentrations on 5 separate days. The precision is expressed as a relative standard deviation (RSD). Accuracy was calculated as the percent error in the calculated mean concentration relative to the nominal concentrations (RE) [14]. For the assay to be considered acceptable, the precision and accuracy at each QC level was required to be within 15%. Absolute recoveries at low, middle, and high plasma concentrations were determined in triplicate by comparing the peak area of analyte in spiked post-protein precipitated plasma with the corresponding concentration in the spiked sample. Matrix effects were investigated by comparing the deproteinized samples of pooled blank plasma from six different drug-free rats spiked with low, middle, and high concentrations of QC with the direct injection of mobile phase spiked with the analytes. Stability under the experimental conditions was investigated at low and high levels of QC. Short-term, post-extraction, freeze–thaw, and long-term stabilities were assessed [14]. The short-term stored stabilities of analytes after being processed were evaluated by testing their stabilities after being protein precipitated and stored for 6 h at room temperature. The long-term stability was examined for 20 days at −70 °C. Freeze–thaw stability testing was determined after freezing at −70 °C and thawing to room temperature three times.

3. Results and discussion

3.1. Mass spectra

Precursor ions for theophylline, 1-MX, 3-MX, 1, 3-DMU, 1-MU, and their corresponding ions were identified and quantitated from spectra obtained from the injection of standard solutions into a mass spectrometer with an electrospray ionization source. The system was operated in negative ionization mode with nitrogen collision gas in Q2 of a MS/MS system. Theophylline, 1-MX, 3-MX, 1,3-DMU, 1-MU, and the IS produced deprotonated ions at m/z 179, 165, 165, 195, 181, and 168, respectively. Product ions were scanned in Q3 following collisions with nitrogen in Q2 at m/z 164, 108, 122, 110, 138, and 132 for theophylline, 1-MX, 3-MX, 1,3-DMU, 1-MU, and IS, respectively (Fig. 1).

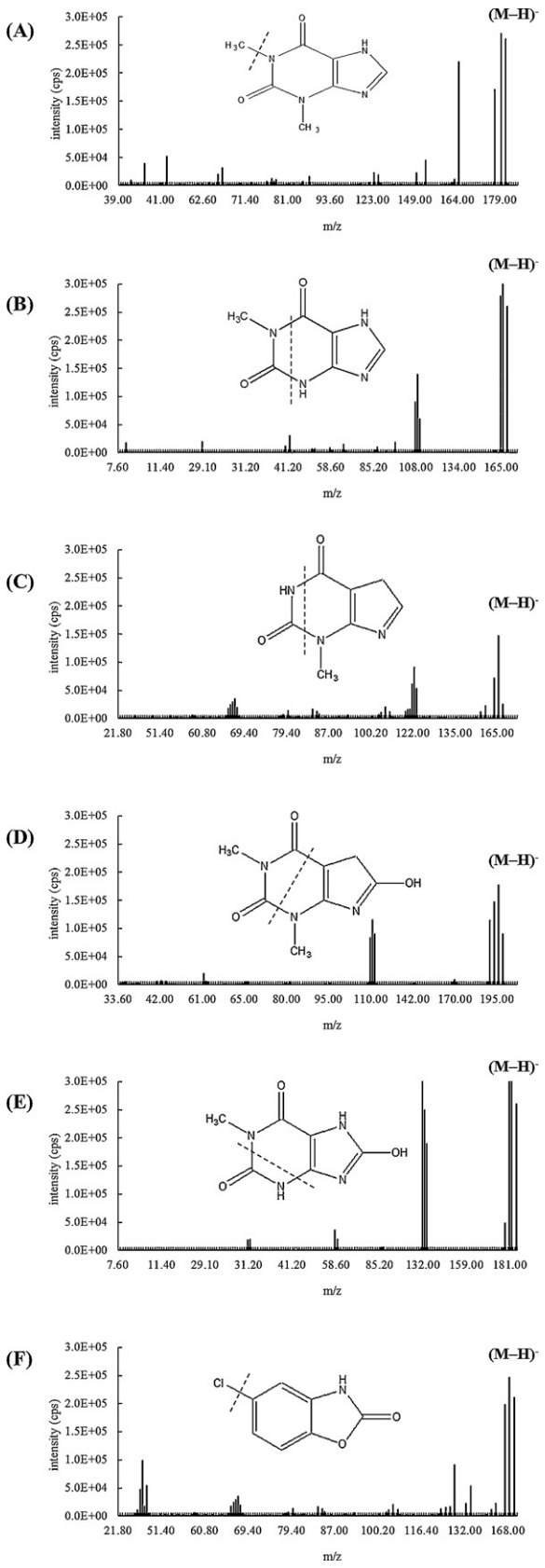

Fig. 1. Tandem mass spectra show ions from (A) theophylline, (B) 1-MX, (C) 3-MX, (D) 1,3-DMU, (E) 1-MU, and (F) chlorzoxazone (internal standard, IS) using electrospray ionization in negative ion mode.

3.2. Chromatography

Significant peak tailing was observed for theophylline and its metabolites when using an acetonitrile mobile phase. Therefore, several combinations of methanol and water were evaluated to sufficiently resolve each compound while minimizing both noise and peak tailing effects. The inclusion of 0.05% acetic acid in the mobile phase improved the peak shape. The optimal mobile phase was identified as a 90:10 (v/v) mixture of MeOH and water with 0.05% acetic acid. The retention times of theophylline, 1-MX, 3-MX, 1,3-DMU, 1-MU, and the IS in rat plasma were approximately 1.7 min and the total run time for each sample was about 5 min. Ion chromatograms from a blank sample (non-spiked blank plasma), a zero sample (spiked with the IS), a midrange concentration of five analytes (7.5 μg/mL), and a rat plasma sample (collected 1.5 h after administration of 10 mg/kg theophylline in oral) showed no significant interference peaks at the retention times of theophylline, 1-MX, 3-MX, 1,3-DMU, 1-MU, and the IS (Fig. 2).

The measured concentrations in rat plasma administered at 10 mg/kg theophylline were 10.20 μg/mL for theophylline, 1.14 μg/mL for 1-MX, 0 μg/mL for 3-MX, 5.05 μg/mL for 1,3-DMU and 2.31 μg/mL for 1-MU.

3.3. Calibration curves

The calibration curves were linear from 100 to 30,000 ng/mL for 3-MX and from 50 to 30,000 ng/mL for 1-MX, 1,3-DMU, 1-MU, and theophylline. Typical regression equations were the following: theophylline, $y = 0.0035x - 0.0127$ ($r^2 = 0.9911$); 1-MX, $y = 0.0029x - 0.0251$ ($r^2 = 0.9947$); 3-MX, $y = 0.0007x - 0.0039$ ($r^2 = 0.9979$); 1,3-DMU, $y = 0.0008x - 0.0017$ ($r^2 = 0.9994$); 1-MU, $y = 0.0023x + 0.0061$ ($r^2 = 0.9918$). LLOQs were 50 ng/mL (theophylline and for 1-MX, 1,3-DMU, 1-MU) and 100 ng/mL (3-MX), respectively.

3.4. Precision and accuracy

Intra-day and inter-day precision and accuracy data are shown in Table 1. The accuracy [calculated as the percent error in the calculated mean concentration relative to the nominal concentrations (RE)] of theophylline analyses ranged from −7.6% to 9.7% with coefficients of variation (%CV) of 4.7% to 9.6% and 3.8% to 7.2% for intra- and inter-day precision, respectively. The accuracy of 1-MX analyses ranged from −8.8% to 9.6%, with a %CV of 6.4% to 9.0%, and 4.7% to 9.2% for intra- and inter-day precision, respectively. The accuracy of 3-MX determination ranged from 5.7% to 8.2%, with a CV% of 3.0% to 11.4% and 2.7% to 10.9% for intra- and inter-day precision, respectively. The accuracy of 1,3-DMU analyses ranged from −6.7% to 9.1%, with a %CV between 6.8% and 8.3% and 7.4 to 9.3% for intra- and inter-day precision, respectively. The accuracy of 1-MU analyses ranged from −5.1% to 9.2%, with a %CV of 9.1% to2.7% and 6.7% to 12.4% for intra- and inter-day precision, respectively. These results indicate acceptable precision and accuracy for the present method.

3.5. Matrix effects and percent recovery

When analyzing supernatants from protein-precipitated plasma samples, salt and endogenous materials can cause ion suppression or enhancement that may result in greater variations than in solid phase extracts or liquid–liquid extracts [15]. Prudent assessment of these matrix effects constitutes an important and necessary part of the validation for quantitative LC–MS/MS methods that support pharmacokinetic studies in biological matrices [16].

Matrix effects and percent recoveries of theophylline and its metabolites are shown in Table 2. For all samples, including

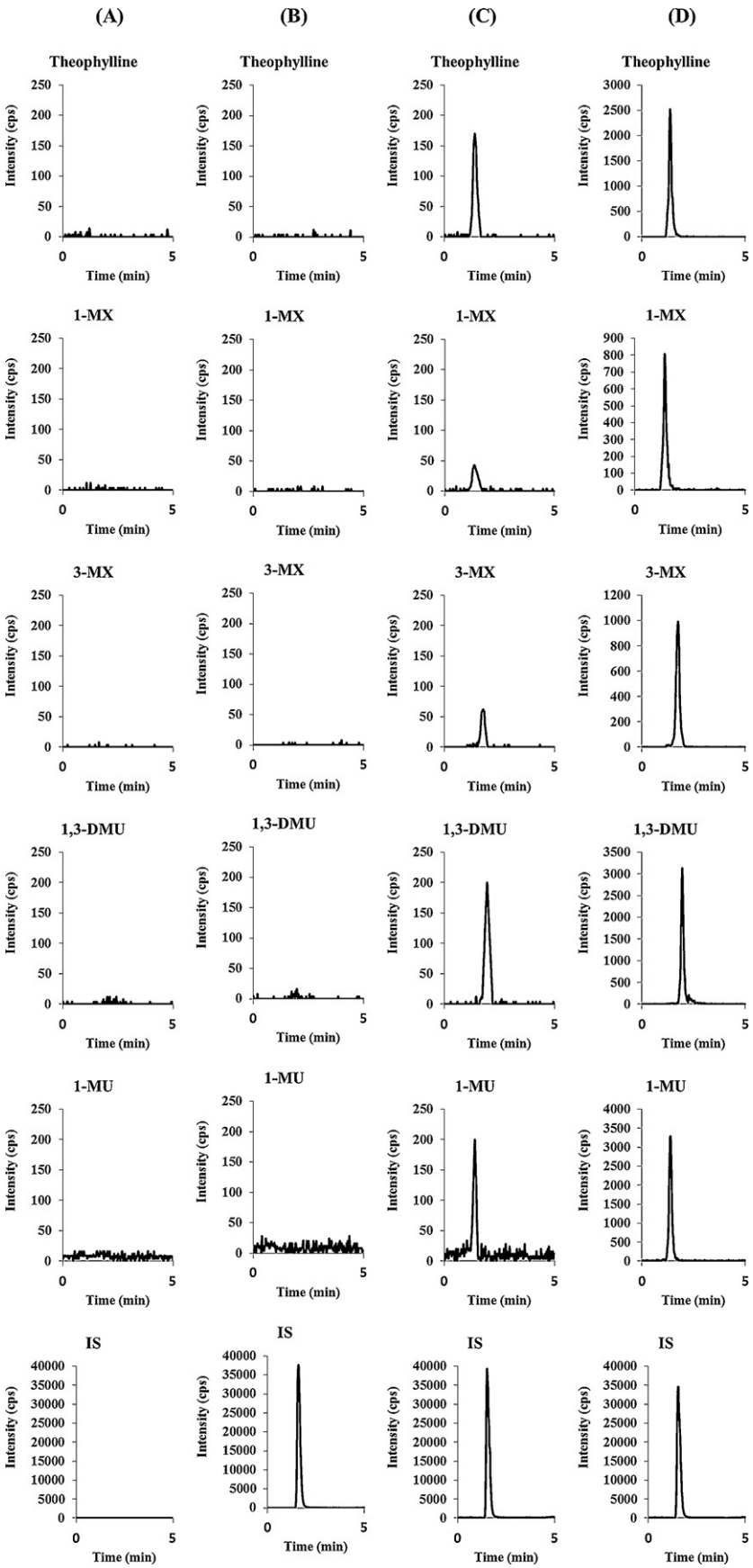

Fig. 2. Representative SRM chromatograms of the theophylline, 1-MX, 3-MX, 1,3-DMU, 1-MU, and the IS in (A) SD rat blank plasma, (B) SD rat blank plasma spiked with the IS, (C) SD rat blank plasma with five mid-concentration analytes with the IS, and (D) plasma sample collected 1.5 h after administration of 10 mg/kg theophylline in oral.

Table 1
The accuracy and precision of intra- and inter-day assays ($n = 5$).

	Nominal concentration (μg/mL)	Mean calculated concentration (μg/mL)	RSD (%)	RE (%)
Intra-day				
	0.20	0.22	9.6	9.7
Theophylline	7.50	8.12	4.7	8.2
	30.00	27.72	4.8	−7.6
	0.20	0.22	6.4	8.1
1-MX	7.50	8.22	7.2	9.6
	30.00	31.38	9.0	4.6
	0.50	0.53	11.4	5.9
3-MX	7.50	8.12	4.1	8.2
	30.00	31.71	3.0	5.7
	0.20	0.22	7.0	7.7
1,3-DMU	7.50	7.97	6.8	6.3
	30.00	31.47	8.3	4.9
	0.20	0.19	12.7	−5.1
1-MU	7.50	8.09	9.1	7.9
	30.00	32.58	11.2	8.6
Inter-day				
	0.20	0.22	6.4	8.6
Theophylline	7.50	8.18	7.2	9.1
	30.00	32.52	3.8	8.4
	0.20	0.22	6.9	8.6
1-MX	7.50	6.84	4.7	−8.8
	30.00	32.79	9.2	9.3
	0.50	0.53	2.7	6.9
3-MX	7.50	7.96	10.9	6.1
	30.00	31.71	9.5	5.7
	0.20	0.19	8.1	−6.7
1,3-DMU	7.50	8.18	7.4	9.1
	30.00	32.49	9.3	8.3
	0.20	0.22	12.4	8.8
1-MU	7.50	8.05	6.7	7.3
	30.00	32.76	8.2	9.2

theophylline, its metabolites, and IS, neither matrix effects nor the percent loss exceeded ±20%. Therefore, no significant matrix effects or interference from endogenous compounds were present in rat plasma.

3.6. Stability

A summary of assay stability under various conditions is presented in Table 3. The mean integrated peak areas of the LQC

Table 2
Matrix effects and percent recoveries of theophylline, 1-MX, 3-MX, 1,3-DMU, and 1-MU in rat plasma ($n = 6$).

Concentration (μg/mL)	Matrix effect (mean % ± SD)	Recovery (mean % ± SD)
Theophylline		
0.20	96.31 ± 1.12	94.21 ± 1.01
7.50	97.66 ± 1.54	101.11 ± 2.61
30.00	94.11 ± 1.40	97.51 ± 3.19
1-MX		
0.20	97.64 ± 1.41	95.44 ± 2.22
7.50	89.63 ± 2.27	98.78 ± 5.14
30.00	91.21 ± 3.31	97.61 ± 1.27
3-MX		
0.50	87.62 ± 2.57	103.96 ± 1.99
7.50	96.27 ± 1.54	96.51 ± 3.41
30.00	94.63 ± 2.98	91.49 ± 4.33
1,3-DMU		
0.20	91.65 ± 4.29	97.46 ± 2.21
7.50	89.96 ± 3.32	91.65 ± 4.51
30.00	97.51 ± 4.31	107.66 ± 2.91
1-MU		
0.20	86.62 ± 2.19	96.31 ± 2.13
7.50	95.39 ± 3.61	91.44 ± 4.01
30.00	94.19 ± 3.44	106.51 ± 4.55
IS		
0.5	94.36 ± 2.77	–
5	97.96 ± 1.98	–
50	96.67 ± 2.17	–

Table 3
Stabilities of theophylline, 1-MX, 3-MX, 1,3-DMU, and 1-MU in rat plasma ($n = 6$).

Concentration (μg/mL)	Storage condition	Stability (%)	Stability (%)
Theophylline		0.2 μg/mL	30 μg/mL
Short term in plasma	Room temperature, for 6 h	101.91	109.87
Process (extracted sample)	4 °C, for 24 h	100.19	104.99
Freeze–thaw cycle in plasma	−70 °C, after the third cycle	92.82	97.26
Long term in plasma	−70 °C, for 20 days	93.62	101.10
Stock solution	4 °C, for 20 days	94.82	103.95
1-MX		0.2 μg/mL	30 μg/mL
Short term in plasma	Room temperature, for 6 h	96.05	95.67
Process (extracted sample)	4 °C, for 24 h	94.76	103.22
Freeze–thaw cycle in plasma	−70 °C, after the third cycle	103.29	89.27
Long term in plasma	−70 °C, for 20 days	107.61	103.63
Stock solution	4 °C, for 20 days	101.13	97.61
3-MX		0.5 μg/mL	30 μg/mL
Short term in plasma	Room temperature, for 6 h	94.75	99.15
Process (extracted sample)	4 °C, for 24 h	102.98	106.04
Freeze–thaw cycle in plasma	−70 °C, after the third cycle	88.79	89.11
Long term in plasma	−70 °C, for 20 days	107.61	103.61
Stock solution	4 °C, for 20 days	99.78	98.47
1,3-DMU		0.2 μg/mL	30 μg/mL
Short term in plasma	Room temperature, for 6 h	90.66	107.50
Process (extracted sample)	4 °C, for 24 h	108.72	99.49
Freeze–thaw cycle in plasma	−70 °C, after the third cycle	102.51	105.30
Long term in plasma	−70 °C, for 20 days	108.61	99.96
Stock solution	4 °C, for 20 days	102.16	111.71
1-MU		0.2 μg/mL	30 μg/mL
Short term in plasma	Room temperature, for 6 h	90.09	96.84
Process (extracted sample)	4 °C, for 24 h	103.19	85.19
Freeze–thaw cycle in plasma	−70 °C, after the third cycle	112.64	106.98
Long term in plasma	−70 °C, for 20 days	95.88	88.10
Stock solution	4 °C, for 20 days	87.75	92.24

(lowest quality control) and HQC (highest quality control) samples were compared before and after the stability testing described in Section 2. No stability issues were observed from any of these experiments.

4. Conclusions

A rapid, specific, and reliable LC–MS/MS-based bioanalytical method was successfully developed and validated to simultaneously quantitate levels of theophylline, 1-MX, 3-MX, 1,3-DMU, and 1-MU in rat plasma. This report represents the first time that LC–MS/MS has been applied in an assay for simultaneous quantitation of theophylline and its metabolites in rat plasma via protein precipitation. The current chromatographic conditions provided both suitably short retention times and well resolved peaks in analyses of all five analytes. The use of different collision energies can significantly reduce background noise and interference peaks without sacrificing sensitivity. Therefore, the combination of chromatographic separation and the modification of mass spectrometric parameters can efficiently shorten HPLC run times. The assay described herein also demonstrated a high degree of reproducibility and suitable precision and accuracy. The relatively short sample preparation time, together with the short LC run time, makes the present method practical for cost-effective, high-throughput sample analyses.

Acknowledgment

This research was supported by a Grant (11182KFDA603) from the Korea Food & Drug Administration in 2011.

References

[1] M.E. McManus, J.O. Miners, D. Gregor, I. Stupans, D.J. Birkett, J. Pharm. Pharmacol. 40 (1998) 388.
[2] M.W. Teunissen, I.O. Brorens, J.M. Geerlings, D.D. Breimer, Xenobiotica 15 (1985) 165.
[3] S. Kodera, M. Misawa, S. Yanaura, T. Iwata, N. Kobayashi, K. Ishimura, H. Hayakawa, Arerugi 34 (1985) 33.
[4] Y. Hamasaki, T. Ichimaru, S. Miyazaki, Jpn. J. Clin. Chem. 15 (1986) 44.
[5] J. Kizu, S. Watanabe, N. Yasuno, Y. Arakawa, S. Uzu, S. Kanda, F. Komoda, T. Iwata, H. Hayakawa, T. Hayakawa, K. Imai, Biomed. Chromatogr. 13 (1999) 15.
[6] H. Kanazawa, R. Atsumi, Y. Matsushima, J. Kizu, J. Chromatogr. A 870 (2000) 87.
[7] H. Schneider, L. Ma, H. Glatt, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 789 (2003) 227.
[8] Y. Zhang, N. Mehrotra, N.R. Budha, M.L. Christensen, B. Meibohm, Clin. Chim. Acta 398 (2008) 105.
[9] T. Arinobu, H. Hattori, T. Kumazawa, Forensic Toxicol. 27 (2009) 1.
[10] C.W. Huck, W. Guggenbichler, G.W. Bonn, Anal. Chim. Acta 538 (2005) 195.
[11] M.E. Abdel-Hamid, O.A. Phillips, J. Liq. Chromatogr. 44 (2003) 1937.
[12] Y.U. Chen, H. Junga, X. Jiang, J. Sep. Sci. 26 (2003) 1509.
[13] J. Song, K.U. Park, H.D. Park, Y. Yoon, J.Q. Kim, Clin. Chem. 50 (2004) 2176.
[14] L.J. Schaaf, B.R. Dobbs, I.R. Edwards, D.G. Perrier, Eur. J. Clin. Pharmacol. 34 (1988) 439.
[15] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882.
[16] N.A. Gomes, A. Laud, A. Pudage, S.S. Joshi, V.V. Vaidya, J.A. Tandel, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 877 (2009) 197.