Determination of theophylline in rabbit plasma by triple quadrupole LC/MS

Abstract

Aim and Background: A simple, sensitive and rapid method was developed for quantitation of theophylline in rabbit plasma utilizing Triple Quadrupole LC/MS. **Materials and Methods:** An aliquot of 0.1 mL of plasma sample was extracted with ethyl acetate using Heidolph Vortex. The chromatographic separation was performed by using HyPURITY ADVANCE™ C18 Column (3 × 50 mm) with a mobile phase of 80% methanol and 20% 2 mM ammonium acetate buffer followed by MS/MS detection. The analyte was quantitated in positive ionization mode. Multiple reaction monitoring (MRM) using the transition m/z 181.1→124.2 and m/z 180.2→110.1 was performed to quantify theophylline with internal standard (IS, Phenacetin), respectively. The method had a total chromatographic runtime of 3 min and linear calibration curves over the concentration range of 50.418–5062.063 ng/mL. The lower limit of quantification (LLOQ) was 50.418 ng/mL Sodium heparin (3.50%) used as an anticoagulant to prepare rabbit plasma and samples were maintained at 10°C in the auto sampler during the assay period. Inter and intraday batch precision and accuracy of the method were determined by using six quality control samples. **Results:** Average accuracies for the assay were 89 to 106%, inter and intra-day coefficients variation (CV) of <9% and the recovery is 39.30% for theophylline and 57.00% for Phenacetin. **Conclusion:** Currently we are extensively using this method in our laboratory for quantitative analysis of theophylline in rabbit plasma samples and proved to be simple, accurate and precise.

Key words: Phenacetin, rabbit plasma, sodium heparin, theophylline, triple quadrupole LCMS

**A. R. Suresh Babu,
B. Thippeswamy,
A. B. Vinod,
E. G. Ramakishore,
S. Anand,
Duraisamy Senthil**

Drug Discovery Research, Bioanalytical Division, G7 Synergon Private Limited, No. 537, 9th Cross, 5th Main, Tata Nagar, Sahakaranagar Post, Bangalore, India

Address for correspondence:
Dr. A. R. Suresh Babu, Drug Discovery Research, Bioanalytical Division, G7 Synergon Private Limited, No. 537, 9th Cross, 5th Main, Tata Nagar, Sahakaranagar Post, Bangalore - 560 092, India. E-mail: sureshbabu.ar@g7synergon.in

INTRODUCTION

Theophylline is a bronchodilator used in the treatment of acute, chronic asthma and patients with a chronic obstructive pulmonary disease. It has been shown to be extensively metabolized *in vivo* and eliminated almost exclusively by cytochrome P-450-mediated hepatic oxidation, predominantly by 8-hydroxylation to 1, 3-dimethyluric acid. The latter pathway accounts for almost half of the total theophylline clearance.[1] In addition, theophylline is converted from *N*-demethylated to 1-methylxanthine and 3-methylxanthine. The former is further oxidized by xanthine oxidase to 1-methyluric acid, which is the only theophylline 1-demethylation product seen in human plasma and urine. Asthmatic patients receive a variety of theophylline dosage forms (oral, intravenous, rectal) in different dose schedules. Serum theophylline shows considerable individual variations in patients, presumably due to wide variations in the extent of metabolism. It seems that theophylline levels, required for optimal bronchodilator effect, range between 8 and 20 μg/mL.[2]

In recent years, a number of laboratories have reported for determination of theophylline in biological fluids by many methods such as UV, HPLC, HPTLC, GC,GC-MS, fluorescence immunoassay, fluorescence polarized immunoassay, radio immunoassay, capillary electrophoresis and reflectance photometry assay.[3] Most of these methods are insensitive to concentrations obtained after single dose administration of this drug or otherwise time consuming and/or expensive. So we therefore developed a simple, rapid, sensitive, specific, robust and novel

assay method that makes it an attractive procedure in high throughput for quantification of this drug in rabbit plasma. The results indicate the suitability of this method in bioavailability studies.

EXPERIMENTAL PROCEDURE

Reagents
The pure substances of theophylline with Phenacetin (internal standard; IS) were obtained from Cipla and Sigma Aldrich India, respectively. Chemical structures are presented in Figure 1. Stock solutions of theophylline (1 mg/mL) and IS (1 mg/mL) were separately prepared in 5 mL volumetric flasks with methanol. HPLC grade methanol from JT Baker, *t*-butyl methyl ether and ammonium acetate were from Merck (Worli, Mumbai, India), HPLC type 1 water from Milli Q System (Millipore, Bedford.MA USA) was used and blank rabbit plasma from healthy rabbits was obtained from Bioneeds, Bangalore India.

Apparatus/instrumentation
Chromatographic analysis was performed using Agilent 1200 Series LC System (Agilent technologies, INC; VBA) equipped with G1312B Binary pump, G1315C DAD detector, G1316B thermostatic column compartment, G1329B auto sampler equipped with a G1330B thermostat. Mass spectrometric detection was performed in Triple Quadrupole LC/MS 6410 instrument with Agilent technologies, USA, using MRM. A turbo electro-spray interface in positive ionization mode was used. Mass data acquisitions with integration were controlled by Agilent MassHunter ChemStation (B.01.03) software.

LC-MS/MS conditions
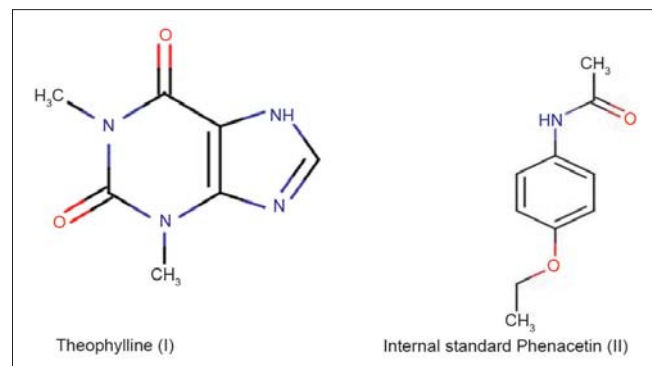Chromatography was performed on HyPURITY ADVANCE C18 Column (3 × 50 mm), maintained at 40°C. The mobile phase composition was 2 mM ammonium acetate buffer: methanol 20:80 v/v (Binary Flow), which was pumped at flow rate of 0.2 mL/min without splitter. The auto sampler temperature was set at 10°C. The main working parameters of the mass spectrometer are summarized in Table 1.

Preparation of standard and quality control samples
Stock solutions of theophylline at a concentration of 1 mg/mL were prepared by dissolving the accurately weighed reference substance in methanol. The stock solution was then serially diluted with methanol water (50:50 v/v) to give working solutions at the following concentrations: 50.418, 100.836, 252.091, 504.181, 1214.859, 2429.790, 4049.650 and 5062.063 ng/mL. The other stock solution was independently diluted in a similar way to achieve quality control (QC) solution at concentrations of 0.5128 (LLOQ), 2.5128 (low), 24.4176 (medium) and 40.3597 ng/mL (high). Internal standard working solution (0.5 μg/mL) was prepared by diluting the 1 mg/mL stock solution of Phenacetin with methanol water (50:50 v/v). All the solutions were kept at 4–8°C and were brought to room temperature before use.

Both the calibration standard and quality control samples used for validation and pharmacokinetic study were prepared by spiking 100 μL sodium heparin plasma with 50 μL internal standard correspondingly.

Extraction procedure for plasma samples
A 0.1 mL of plasma sample was mixed with 50 μL of internal work solution. The mixture was vortexed for 30 s, and 0.5 mL aliquot of extraction solvent (ethyl acetate) was added. The analyte and IS were extracted from plasma by vortexing for 10 min using Heidolph Vibramax 110 (Germany). Then sample was centrifuged using micro centrifuge (5415R Eppendorf) for 5 min at 10000 rpm. Following

Figure 1: Chemical structures of Theophylline (I) and internal standard Phenacetin (II)

Table 1: Tandem mass spectrometer main working parameters	
Source temperature (°C)	350
Dwell time per transition (milliseconds)	200
Gas flow/Drying gas (L/min)	6
Nebulizer gas (psi)	25
Fragmentor (V)	200
Collision energy (V)	10
Ion spray voltage (V)	4000
Mode of analysis	Positive
Ion transition for theophylline m/z	181.1→124.2
Ion transition for phenacetin m/z	180.2→110.1

centrifugation, the supernatant was transferred to clean glass test tubes and then evaporated to dryness using TurboVap LV Evaporator (Zymark, Hopkinton, MA, USA) at 40°C under a stream of nitrogen. The residues were reconstituted with 100 μL of mobile phase and aliquots of 5 μL were injected into the chromatographic system.

Bioanalytical method validation
Selectivity and lower limit of quantification
To investigate the method selectivity, rabbit plasma blank samples from six different lots were pretreated and analyzed at LLOQ.

Calibration curve: A calibration curve was constructed from a blank sample (blank plasma processed without an IS), a zero sample (plasma processed with IS) and eight non-zero samples covering the total range (50.418–5062.063 ng/mL), including LLOQ. Eight samples of each concentration were measured and the curves were fitted by a linear weighted ($1/x^2$) least squares regression method through the measurement of peak-area ratio of analyte to IS. The calibration curve had to have a correlation coefficient (r^2) of 0.999 or better. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except LLOQ, which was set at 20%.

Recovery
Recovery of theophylline was evaluated by comparing the mean peak areas of control samples ($n = 6$) extracted low (256.385 ng/mL), medium (2441.761 ng/mL) and high (4035.969 ng/mL) concentration levels compared with reference solutions (unprocessed). Recovery of IS was evaluated by comparing between Blank + IS samples ($n = 6$) against reference solutions (unprocessed) of the same concentration.

Precision and accuracy
To evaluate the precision and accuracy of theophylline quantification method, QC samples at four concentration levels (51.277, 256.385, 2441.761 and 4035.969 ng/mL) were analyzed in six replicates. The whole experiment was reproduced for accuracy checking in three consecutive days (data not shown). The assay precision was calculated by using relative standard deviation (RSD) method, and accuracy was expressed as relative error (RE %), i.e. (measured concentration - nominal concentration)/(nominal concentration) × 100.

Stability
Stability of stock solutions and working solutions of theophylline and IS, which were stored at 4–8°C for 15 days and at room temperature (25°C) for 5 h, were tested by comparing the instrument response with that of freshly prepared solutions. The analyte were considered stable when the intensities ranged between 85 and 115% of the initial solutions. The stability of theophylline in rabbit plasma was evaluated by analyzing replicates ($n = 6$) of plasma samples that were exposed to different conditions (time and temperature) at two concentrations (256.385 and 4035.969 ng/mL). These results were compared with results obtained from freshly prepared plasma samples. The analyte was considered to be stable in biological matrix and an acceptance criterion is ±15%. The long term stability was determined after exposure of the spiked samples at -20°C for 10 days. The freeze-thaw stability was evaluated after complete three freeze thaw cycles (-20°C to -25°C) on consecutive days.

Matrix effects
The matrix effects (MEs) were determined based on Matuszewski *et al.*,[4] whether the potential ion suppression or enhancement owing to the co-eluting matrix components existed in the present experiment. The corresponding peak areas of theophylline from spike-after-extraction samples at one concentration level (256.385 ng/mL) were compared in respective unprocessed or aqueous standard. MEs of IS were investigated at concentration level (0.5 μg/mL) in similar way.

RESULTS AND DISCUSSION

LC-MS/MS optimization
Electrospray MS–MS was used to analyze the compound theophylline. Positive ionization was selected to quantify the analyte because positive ion mass spectrometry gave a protonated molecular ion without adduct formation over negative ionization. The combination of chromatographic separation by HPLC and successive mass filtrations by monitoring the transition of protonated ion to product ion provided excellent specificity for theophylline and internal standard. The positive ion electro-spray mass spectrum of analyte and IS gave a protonated molecular ions at m/z 124.2, m/z 110.1 and product ions at m/z 181.1 m/z 180.2, respectively.

Thus, MRM technique was chosen for the assay development. The MRM state file parameters were optimized to maximize the response for analyte. The approach applied to development of this method was based on literature survey done on theophylline,

which form adducts for the quantization by LC–MS/MS.[5-9] Therefore, sensitivity, robustness and ruggedness of the method are questionable. There is a need of rugged method in high-throughput bioanalysis. This method is robust, simple and rapid, which makes it an attractive procedure in high-throughput bioanalysis.

Selection of mobile phase and internal standard

Different mobile phases were evaluated to improve HPLC separation and enhance sensitivity in MS. A binary system using a mobile phase of 80% methanol and 20% 2 mM of ammonium acetate buffer was optional for analyte with respect to peak shape and mass spectral response. Under this condition, the retention times of both analyte and IS were approximately 1.730 and 1.852 min, respectively. The total run time for each sample was 3 min. The use of an internal standard was required in the LC–MS/MS assay for two reasons: To compensate for loses during extraction and to compensate for the variable detection sensitivity of MS. We chose Phenacetin as an internal standard based on its similarity with theophylline properties such as pKa, solubility along with chromatographic and extraction properties. It was expected to give similar recovery on liquid–liquid extraction and MS/MS response in the positive ion mode.

Calibration curves

Calibration curve was linear over the concentration range of 50.418–5062.063 ng/mL for the analyte. The eight-point calibration curve gave acceptable results for analyte and was used for all calculations. The mean correlation coefficient of weighted ($1/x^2$) calibration curve generated during the validation was

0.999 for analyte [Figure 2]. Table 2 summarizes the calibration curve results for the analyte. The precision and accuracy for analyte covering the concentration of 50.418–5062.063 ng/mL ranged from 1.17 to 9.49 and 90.42 to 105.85%, respectively. The calibration curve obtained as described above was suitable for generation of acceptable data for concentrations of analyte in the samples during validations for theophylline in rabbit plasma.

Specificity

The method specificity was examined by analyzing blank plasma extract [Figure 3] and spiked only with internal standard [Figure 4]. As shown in Figure 3, no significant interference in blank plasma traces was seen from endogenous substances in drug-free rabbit plasma at the retention time of analyte. Figure 4 shows the absence of interference from the internal standard to MRM channels of the analyte. Figure 5 depicts a representative ion chromatogram for the LLOQ (50.418 ng/mL) of the calibration curve. Excellent sensitivity was observed for 5 µL injection volume corresponding to 50.418 ng/mL on-column.m

Table 2: Precision and accuracy data of back-calculated concentrations of calibration samples for theophylline in rabbit plasma

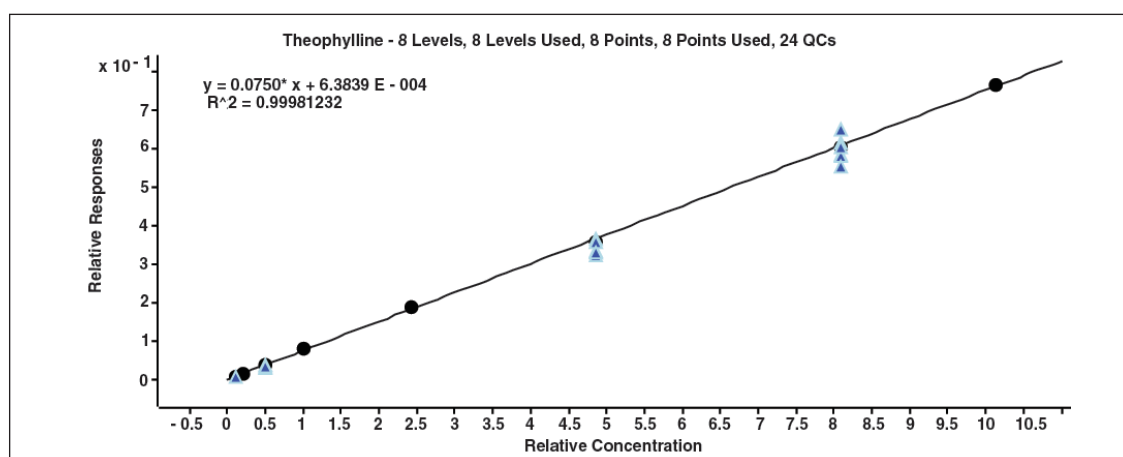| Nominal concentration (ng/ml)	n	Precision (%)	Accuracy (%)
50.418	5	9.49	90.42
100.836	5	1.65	102.98
252.091	5	3.79	100.70
504.181	5	1.17	105.85
1214.859	5	2.24	103.43
2429.790	5	4.44	101.33
4049.650	5	8.16	93.98
5062.063	5	7.07	95.87

Figure 2: Calibration curve for extracted theophylline from rabbit plasma
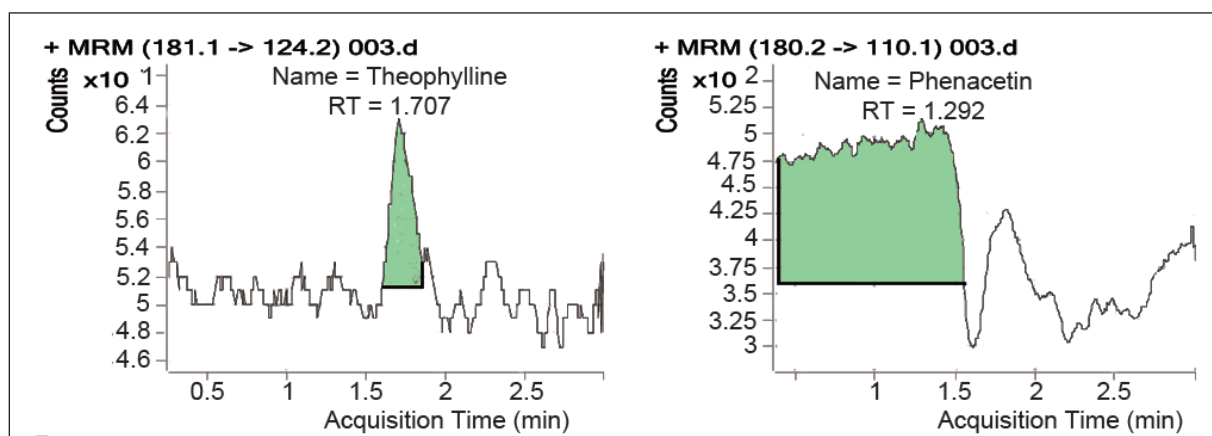
Figure 3: LC-MRM ion-chromatograms resulting from the analysis of blank (drug and internal standard free) rabbit plasma for theophylline (I) and IS (II)
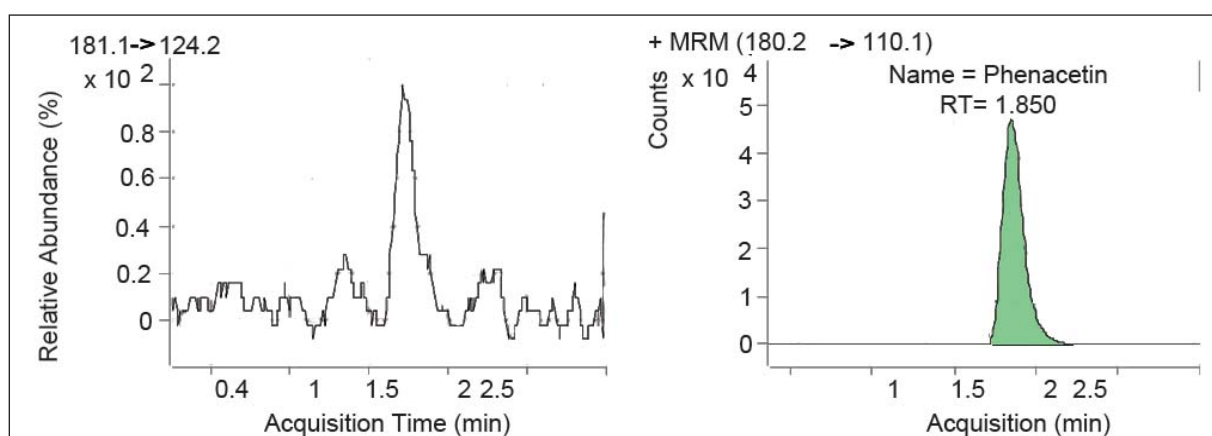

Figure 4: LC-MRM ion-chromatograms resulting from the analysis of blank (drug-free spiked with IS) rabbit plasma for theophylline (I) and IS (II)
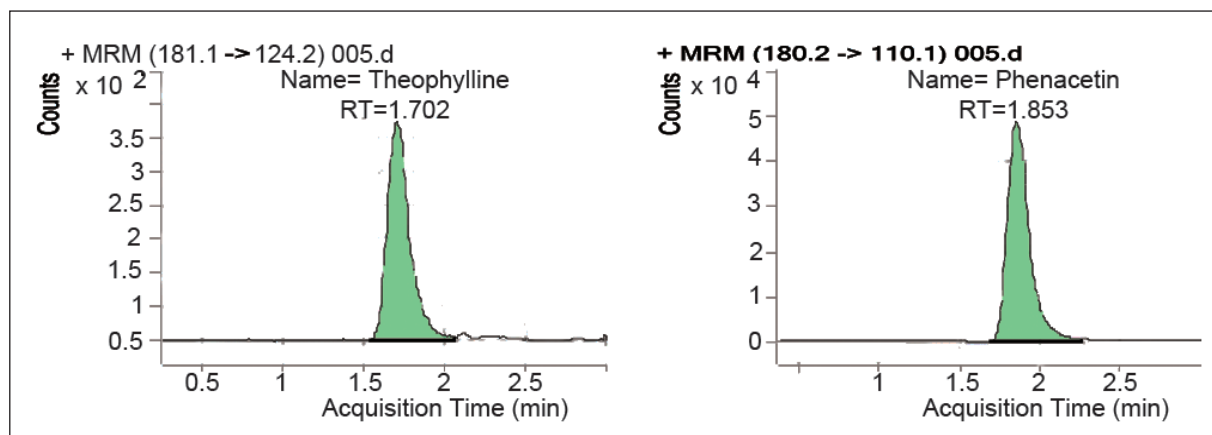

Figure 5: Representative LC–MRM ion-chromatograms resulting from the analysis of 50.418 ng/ml (LLOQ) of *theophylline* (I) spiked with the IS (II) 0.5 µg/mL per sample)

Recovery

The extraction recovery of theophylline was 39.30% on average and the recovery of IS was 57% at concentration used in the assay (0.5 µg/mL). Recovery of analyte and IS were consistent, precise and reproducible.

Lowest concentration

The LLOQ of theophylline in rabbit plasma assay was 50.418 ng/mL. Although peaks were detected at the concentration of 25 ng/mL with a signal-to-noise ratio above 5, the precision and accuracy did not meet the acceptance criteria (<±20%). The between-batch

Table 3: Precision and accuracy data from between-batch experiment for theophylline in Rabbit plasma

Nominal concentration (ng/ml)	*n*	Precision (%)	Accuracy (%)
LLOQ (51.277)	24	5.56	89.04
Low concentration (256.385)	24	5.29	91.07
Medium concentration (2441.761)	24	7.02	96.50
High concentration (4035.969)	24	2.18	99.17

Table 4: Stability of rabbit plasma samples of theophylline

Spiked concentration (ng/ml)	*n*	Precision (%)	Accuracy (%)
Freeze thaw stability			
Low concentration (256.385)	6	4.58	98.70
High concentration (4035.969)	6	3.22	95.29
Long-term frozen storage stability			
Low concentration (256.385)	6	3.99	103.21
High concentration (4035.969)	6	2.55	97.63

precision at the LLOQ expressed as RSD was 2.40%. The between-batch accuracy expressed as relative error (RE) was 107.98% [Table 3]. The within batch precision was 9.5% and the accuracy was 106.2% for theophylline.

Precision and accuracy

All the values are summarized in Table 3. The middle and upper quantitation levels of theophylline ranged from 256.385 to 4035.969 ng/mL in rabbit plasma. For the between-batch experiment, the precision ranged from 2.18 to 7.02%. For within-batch experiment, the precision and accuracy for analyte met the acceptance criteria (<±15%) and precision was below 5.56% at all concentrations tested.

Freeze-thaw stability

The freeze-thaw stability of the analyte was determined by measuring the assay precision and accuracy for samples, which underwent three freeze-thaw cycles. The stability data were used to support repeat analysis. The frozen plasma samples containing analyte was thawed at room temperature for 2–3 h, refrozen for 12–24 h, repeated this cycle three times and then analyzed. The results showed that the analyte was stable in rabbit plasma through three freeze-thaw cycles. The precision ranged from 3.22 to 4.58% and the accuracy ranged from 95.29 to 98.70% [Table 4]. The results demonstrated that rabbit plasma samples could be thawed and refrozen without compromising the integrity of the samples.

Long-term storage stability

The sample long-term storage stability at −20°C was evaluated to establish acceptable storage conditions for pharmacokinetic samples. Aliquots of rabbit plasma samples spiked with analyte at concentrations of 256.385 to 4035.969 ng/mL were analyzed on day 1. Then samples from the same pools were analyzed against calibration curves from freshly prepared standards after storage at −20°C for 10 days. The precision and accuracy for the analyte on day 10 ranged from 2.55 to 3.989 and 97.63 to 103.21%, respectively.

CONCLUSIONS

In summary, theophylline is quantified in heparinized rabbit plasma by Triple Quadrupole LC/MS positive ionization mode without adduct formation using MRM. The method described is simple, rapid, sensitive, specific and fully validated as per FDA guidelines. The cost effectiveness, simplicity and speed of liquid–liquid extraction and sample total runtime of 3 min per sample make it an attractive procedure. The validated method allows quantization of theophylline in 50.418–5062.063 ng/mL concentration range.

ACKNOWLEDGMENTS

REFERENCES

1. Kizu J, Watanabe S, Yasuno N, Arakawa Y, Uzu S, Kanda S, *et al*. Development and clinical application of high performance liquid chromatography for simultaneous determination of plasma levels of theophylline and its metabolites without interference from caffeine. Biomed Chromatogr 1999;13:15-23.

2. Jenne JW, Wyze E, Rood BS, Macdonald FM. Pharmacokinetics of theophylline. Pharmacokinetics of theophylline: Application to adjustment of the clinical dose of aminophylline. Clin Pharma Ther 1972;13:349-60.

3. Mirfazaelian M, Goudarzi M, Tabatabaiefar M, Mahmoudian A. Quantitative thin layer chromatography method for determination of theophylline in plasma. J Pharma Pharmaceut Sci 2002;5:131-4.

4. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Monolithic silica based capillary reversed-phase liquid chromatography/ electrospray mass spectrometry for plant metabolomics. Anal Chem 2003;75:3019-30.

5. Lensmeyer GL, Poquette MA. Clinically important drug interactions in epilepsy: Interactions between antiepileptic drugs and other drugs. Ther Drug Monit 2001;23:473-81.

6. Streit F, Christians U, Schiebel HM, Napoli KL, Ernst L, Linck A, *et al*. Validation of an assay for routine monitoring of sirolimus using HPLC with mass spectrometric detection. Clin Chem 1996;42:1417-25.

7. Kirchner GI, Vidal C, Jacobsen W, Franzke A, Hallensleben K.

The quantification of sirolimus by high-performance liquid chromatography-tandem mass spectrometry and microparticle enzyme immunoassay in renal transplant recipients. J Chromatogr B 1999;721:285-94.

8. Taylor PJ, Johnson AG. Matrix effects: The Achilles heel of quantitative high-performance liquid chromatography-electrospray-tandem mass spectrometry. J Chromatogr B 1998;718:251.

9. Salm P, Taylor PJ, Lynch SV, Pillans PI. Quantification and stability of everolimus (SDZ RAD) in human blood by high-performance liquid chromatography-electrospray tandem mass spectrometry. J Chromatogr B 2002;772:283.

How to cite this article: Suresh Babu AR, Thippeswamy B, Vinod AB, Ramakishore EG, Anand S, Senthil D. Determination of theophylline in rabbit plasma by triple quadrupole LC/MS. Pharm Methods 2011;2:211-7.
Source of Support: Nil, **Conflict of Interest:** None declared.

New features on the journal's website

Optimized content for mobile and hand-held devices
HTML pages have been optimized of mobile and other hand-held devices (such as iPad, Kindle, iPod) for faster browsing speed.
Click on **[Mobile Full text]** from Table of Contents page.
This is simple HTML version for faster download on mobiles (if viewed on desktop, it will be automatically redirected to full HTML version)

E-Pub for hand-held devices
EPUB is an open e-book standard recommended by The International Digital Publishing Forum which is designed for reflowable content i.e. the text display can be optimized for a particular display device.
Click on **[EPub]** from Table of Contents page.
There are various e-Pub readers such as for Windows: Digital Editions, OS X: Calibre/Bookworm, iPhone/iPod Touch/iPad: Stanza, and Linux: Calibre/Bookworm.

E-Book for desktop
One can also see the entire issue as printed here in a 'flip book' version on desktops.
Links are available from Current Issue as well as Archives pages.
Click on View as eBook