Journal of Pharmaceutical and Biomedical Analysis 55 (2011) 282–287

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Precolumn derivatization followed by liquid chromatographic separation and determination of tramiprosate in rat plasma by fluorescence detector: Application to pharmacokinetics

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ARTICLE INFO

Article history:
Received 21 August 2010
Received in revised form
28 December 2010
Accepted 17 January 2011
Available online 22 January 2011

Keywords:
Alzheimer disease
Tramiprosate
Precolumn derivatization
Design of experiments
Pharmacokinetics

ABSTRACT

Alzheimer disease (AD) is characterized pathologically by extracellular amyloid deposits composed of amyloid β (Aβ) protein. A simple and rapid method using HPLC with fluorescence detector was developed and validated for determination of tramiprosate in rat plasma. Pre-column derivatization of the deproteinized rat plasma was carried out using o-phthaldialdehyde (OPA) as a fluorescent reagent in presence of 3-mercaptopropionic acid. The liquid chromatographic separation was achieved on a Kromasil C_{18} column using methanol:acetonitrile: 20 mM phosphate buffer pH 7.5 (8.0:17.5:74.5 v/v/v) as a mobile phase in an isocratic elution mode. The eluents were monitored by a fluorescence detector set at 330 and 450 nm of excitation and emission wavelength respectively. Vigabatrin was used as an internal standard. The method was linear within the range 30.0–1000.0 ng/mL. Design of experiments (DOE) was used to evaluate the robustness of the method. The developed method was applied to study the pharmacokinetics of tramiprosate in rats.

1. Introduction

Alzheimer disease (AD) is one of the most common forms of dementia and leading cause of disability in population aged above 65 years [1]. All over the world, more than 24 million people have dementia and it is expected to be 80 million by 2040 [2]. The imbalance between Aβ production and clearance leads to accumulation of the peptides in AD patients. Glycosaminoglycans (GAGs) bind to the soluble Aβ peptides and promote the formation of neurotoxic insoluble fibrils which are the component of plaques [3–9]. Drugs that protect against Aβ-induced neurotoxicity may alter the changes in hippocampus volume and show therapeutic value in the treatment of AD. A major strand of current research is targeted at modifying the deposition of Aβ protein. This could be achieved by reducing the generation and aggregation of Aβ or by promoting clearance of amyloid deposits. Studies on genetic of mutations that cause early AD prove that the prevention of Aβ aggregation may eventually prevent or delay AD [10]. Five cholinesterase inhibitors viz., donepezil, rivastigmine, galantamine and tacrine, and the N-methyl-D-aspartate (NMDA)-receptor modulator memantine are currently approved for treatment of AD. However these drugs are modestly efficacious [11] and unable to prevent or reverse the disease progression. The most advanced disease-modifying drug (DMD) candidate in development is tramiprosate (TMPS) (3-aminopropane-1-sulphonic acid) (Fig. 1). It mimics the anionic portion properties of GAGs, binds to the soluble Aβ peptides; and maintains the peptides in their soluble form thus promoting their clearance from brain. Yet TMPS is not approved by FDA, however, it is often commercialized as Alzhemed™.

To effectively optimize the treatment outcome and reduce the incidence of side effects, drug doses and scheduling should be personalized. A reliable therapeutic drug monitoring (TDM) regimen should be established [12–15]. The first step in this chain of decisions is always the development and implementation of reliable analytical methods, which should be suitable for the repeated determination of drug plasma levels over long periods of time. Analytical methods employed for the quantitative determination of drugs and their metabolites in biological samples are the key determinants of pharmacokinetic study.

Recently we have developed a liquid chromatographic method using evaporative light scattering detector (ELSD) for analysis of TMPS [16], so it seems to be essential to develop a sensitive analytical method for determining drug concentrations in blood plasma. For the pharmacokinetic study, a matrix based standard curve must cover the entire range of concentrations in the unknown biological samples. Instead, the standard curve

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0731-7085/$ – see front matter © 2011 Published by Elsevier B.V.
doi:10.1016/j.jpba.2011.01.013

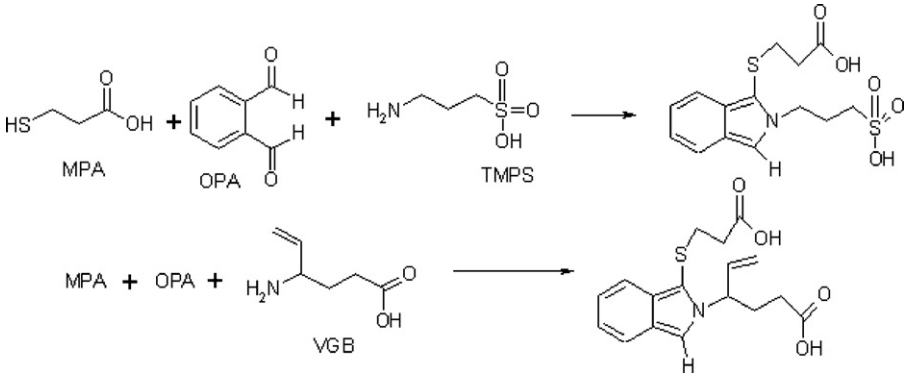

Fig. 1. Reaction of tramiproste (TMPS) and vigabatrin (VGB) with OPA (o-phthalaldehyde) and MPA (3-mercaptopropanoic acid).

should be re-determined or samples are re-assayed after dilution with the matrix [17]. Therefore, it is a challenge to develop a sensitive and high-throughput assay with wide linear range for quantitation of TMPS in biological samples in the pharmacokinetic study. The most commonly used techniques for determining drug concentrations in blood plasma include high-performance liquid chromatography (HPLC) with mass spectrometry (MS) [18], HPLC with ultraviolet (UV) detection [19], and microparticle enzyme immunoassay [20]. Although these techniques meet many or all of the generally accepted criteria for validation, the HPLC/UV assay is cumbersome due to the presence of interfering peaks in the chromatograms, lack of chromophore in TMPS, requiring tedious extraction procedures and long running time to resolve the peaks [21]. The advantages of quantitative LC/MS assay over other techniques such as immunoassay or HPLC/UV include enhanced selectivity, lower detection limit and higher throughput [22]. However, clinical laboratories have been slow to incorporate LC/MS because of the concerns of over expensive instruments, technical difficulty, limited capacity and long turnaround time. The fluorescence detector (FL) is considered to be an attractive alternative to conventional detectors such as UV–vis due to its high sensitivity. In order to overcome the limitations of LC/MS and LC/UV, a simple and rapid precolumn derivatization method with o-phthaldialdehyde (OPA) in presence of 3-mercaptopropionic acid (MPA) [23–25] followed by HPLC-FL detection was attempted for determination of blood concentrations of TMPS.

The present work, describes a simple, rapid and highly sensitive method for analysis of TMPS in rat plasma. De-proteinated plasma was derivatized with OPA in presence of MPA under alkaline conditions. The iso-indoles thus formed were separated by reversed phase HPLC under isocratic conditions and monitored by fluorescence detection. The method was validated according to the guidelines of the International Conference on Harmonization (ICH) [26]. Robustness of the method was studied by means of experimental design combined with statistical evaluation of the data (ANOVA). The developed method was applied to study pharmacokinetic (PK) of TMPS in Wistar rats and the pharmacokinetic parameters were obtained.

2. Experimental

2.1. Chemicals and solutions

TMPS (97% purity), vigabatrin (VGB), o-phthaldialdehyde (OPA), 3-mercaptopropionic acid (MPA) and mercaptoethanol were obtained from Sigma–Aldrich (USA). Boric acid, sodium hydroxide, disodium hydrogen orthophosphate dihydrate, methanol, acetonitrile were purchased from Merck, Mumbai, India. Taurine, pioglitazone, simvastatin, metformin, atorvastatin, acetaminophen, caffeine, ibuprofen, pheniramine, dextromethorphan, nicotine, pseudoephedrine, diphenhydramine, acetylsalicylic acid and phenylephrine were obtained from local industries in Hyderabad, India. Glass-distilled and de-ionized water (Nanopure, Barnsted, USA) was used. The 0.1 M borate buffer (pH 10.0) was prepared by dissolving 0.6200 g of boric acid in approximately 80 mL of water. After adjusting pH to 10.0 using 1.0 M NaOH the volume was adjusted to 100.0 mL with water. Stock solution of OPA was prepared by dissolving 100.0 mg OPA into 3.0 mL of methanol. The working solution for derivatization was prepared by adding 0.3 mL of OPA stock solution and 20 μL MPA to 4.0 mL 0.1 M borate buffer (pH 10). The storage life of the stock solution protected from light was at least 4 weeks at 4 °C. The working solution was stable for at least 4 weeks at 4 °C. Stock solution of TMPS was prepared by dissolving 10 mg of TMPS in 70 mL of water in 100 mL standard flask, mixture was sonicated for 10 min and the solution was made up to mark with water. Stock solution was stored at 4 °C. Calibration plasma was prepared in five concentrations by spiking blank rat plasma with TMPS.

2.2. Instrumentation and chromatographic conditions

The HPLC system consisting of two LC-20AT pumps, an RF-10AXL fluorescence detector, a SIL-20AC autosampler, a DGU-20A3 degasser and CBM-20A communications bus module (all from Shimadzu, Kyoto, Japan), was used. Reversed phase Kromasil C18 (Hichrome) (250 × 4.6 mm i.d.; particle size 5 μm) column was used for analysis. The chromatographic and the integrated data were recorded using HP-Vectra (Hewlett Packard, Waldron, Germany) computer system using LC-Solution data acquiring software (Shimadzu, Kyoto, Japan). The mobile phase consisting of a mixture of methanol:acetonitrile: 20 mM phosphate buffer pH 7.5 (8.0:17.5: 74.5 v/v/v) was delivered in an isocratic mode at a flow-rate of 1.0 mL/min. The eluents were monitored by a fluorescence detector set at 330 and 450 nm of excitation and emission wavelength respectively. VGB was used as an internal standard.

2.3. Sample preparation

200 μL of rat plasma sample of TMPS was spiked with 100 μL of internal standard (VGB), proteineous material was removed by precipitation with 500 μL of acetonitrile. The mixture was vortexed for 2 min and then centrifuged for 10 min at 3000 × g. After centrifugation, 100 μL of the supernatant was transferred to an autosampler vial (1500 μL). To this vial containing 100 μL of the supernatant, 800 μL of borate buffer and 100 μL of derivatizing reagent were

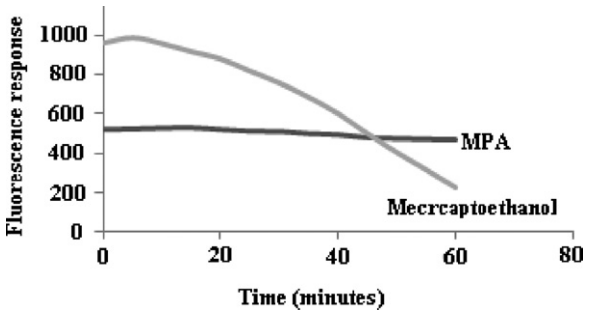

Fig. 2. Comparison of stability of isoindole derivative of TMPS with MPA and mercaptoethanol.

added. After mixing and sonicating for one min 20 μL of the reaction mixture was injected on to the HPLC-FL system

2.4. Animals

Wistar rats, six (180–200 g) housed under standard conditions and had ad libitum access to water and standard laboratory diet throughout the experiments were used in the present study. After a single dose by oral administration of 20 mg/Kg of TMPS to healthy wistar rats ($n = 6$), blood samples (1.0 mL) were collected for the determination of analyte concentrations. Serial blood samples were collected into the processed test tubes at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 h post dose. Specimens were thawed and allowed to reach room temperature, derivatized with OPA and MPA, then analyzed by HPLC. The concentrations of TMPS were determined from the calibration curve on the same day.

3. Results and discussion

3.1. Method development

For the LC method development, the methods reported by Mercolinia et al. [15] and Vermeij et al. [25] were selected as starting point. Both methods were described for determination of topiramate, pregabalin, gabapentin and VGB in human plasma. So, the selectivity of the method had to be improved to make it suitable for pharmacokinetic study of TMPS in rat plasma. The Mercolinia et al. method uses dansyl chloride as a derivatizing reagent, which needs 50 °C temperature and 10 min to complete whereas Vermeij et al. derivatization method need just 1 min to complete at room temperature, so this method was used for starting method development. In our study, a Kromasil C_{18} (Hichrome) (250 × 4.6 mm i.d.; particle size 5 μm) column was used because it gave good separation. After varying methanol, acetonitrile and buffer concentration at various level, the mobile phase consisting of a mixture of methanol:acetonitrile: 20 mM phosphate buffer pH 7.5 (8.0:17.5: 74.5 v/v/v) was selected and delivered in an isocratic mode at a flow-rate of 1.0 mL/min at room temperature.

3.2. Derivatization

OPA reacts with primary amino groups in presence of a thiol (Fig. 1) under alkaline conditions at room temperature [27]. The fluorescence response of the formed iso-indole derivatives generally decreases after 10–20 min. The use of MPA compared to mercaptoethanol prolongs the life-time of such derivatives [28]. Fig. 2 shows the comparative stability of fluorescence response of the formed iso-indole derivatives of TMPS with MPA and mercaptoethanol.

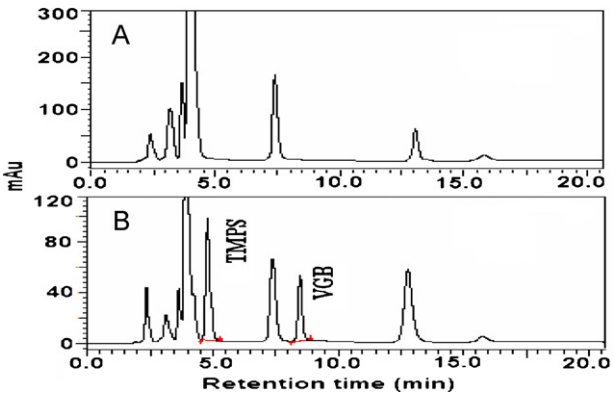

Fig. 3. HPLC-FL chromatograms of (A) blank and (B) plasma obtained from rat after 5 h of oral dose of TMPS (20 mg/kg) spiked with internal standard VGB.

3.3. Method validation

3.3.1. Selectivity

It could be seen from Fig. 3 that no endogenous components extracted from blank plasma eluted at the retention times of the peaks of either TMPS or VGB. The developed method was found to be selective for determination of TMPS in rat plasma without interference from the endogenous constituents of plasma. The structurally correlated compounds taurine and some commonly co-administered drugs such as pioglitazone, simvastatin, metformin, atorvastatin, acetaminophen, caffeine, ibuprofen, pheniramine, dextromethorphan, nicotine, pseudoephedrine, diphenhydramine, acetylsalicylic acid and phenylephrine were tested for exogenous selectivity. Taurine did not interfered with TMPS peak, and other drugs have no primary amine to iso-indole derivative and none had interference with TMPS. So, the developed method may be considered as selective for exogenous as well as endogenous substances.

3.3.2. Calibration curve and linearity

The standard calibration curve was constructed using blank plasma samples spiked with TMPS at five different concentrations from 30, 50, 100, 500, and 1000 ng/mL. The data were subjected to the statistical analysis using a linear-regression model. The calibration curves were obtained by weighted linear regression (weighing factor $1/x^2$) using the Microsoft Excel 2000 software. The suitability of the calibration model was confirmed by back calculating the concentrations of the calibration standards. The developed method was linear over the tested concentrations with correlation coefficient $r^2 = 0.999$, the calibration curve was described by equation $y = 29.2970x + 5.6573$.

3.3.3. Accuracy and precision

Three concentrations viz., 30, 500, 1000 ng/mL, in five replicates were used to validate the accuracy and precision of the developed method. The results showed that the intra- and inter-day accuracy (%bias) for the method ranged between −1.13% and −7.20%, respectively (Table 1). The %CV of intra- and inter-day precision was <8.44%. The developed method was thus found to meet generally accepted requirements of accuracy and precision over the studied concentration ranges.

3.3.4. Limit of detection (LOD) and quantitation (LOQ)

After sample clean-up, the extracts from spiked plasma were injected into the chromatographic systems. The analysis was carried out at decreasing concentrations to determine the minimal concentration with a signal-to-noise ratio of 3:1. The LOD and LOQ for TMPS were 9.0 and 30.0 ng/mL respectively.

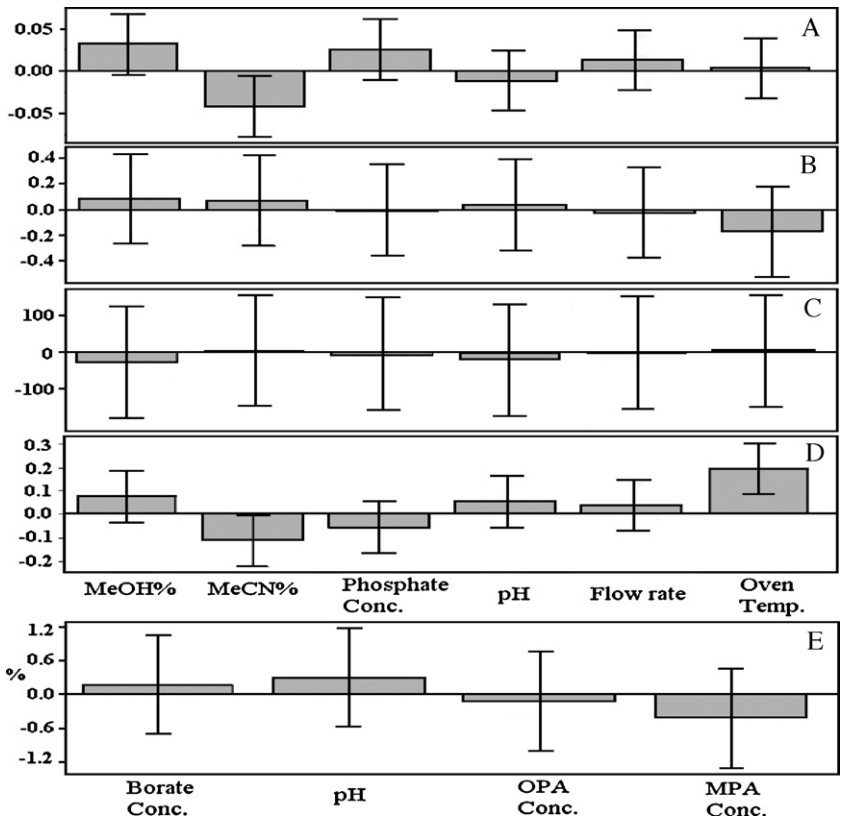

Fig. 4. Scaled and centered coefficient plots of (A) capacity factor; k', (B) resolution; Rs, (C) number of theoretical plates; N, (D) tailing factor; T_f and (E) coefficient of variation; CV.

3.3.5. *Matrix effects and recovery*

As matrix-effects in bioanalysis can be of major influence, identification of the matrix-effect was performed by preparing six blank samples. After sample preparation the aliquot was injected, while infusing a standard solution of TMPS and VGB. As shown (see Fig. 2A and B) no matrix-effect was observed. Quantification of the matrix-effect was performed by preparing six post-extraction spiked calibration lines from six different batches of plasma and comparing the slopes of these calibration lines with slopes of neat calibration lines. Slope of the neat calibration curve was 0.002045. The extraction recoveries of TMPS from biological matrix of rats were determined by using an internal standard method. The results showed that the overall recovery of TMPS was 96.53% (Table 1).

Table 1
Intra- and inter-day assay performance data of TMPS.

Nominal conc. (ng/mL)		30.00	500.00	1000.0
Intra-day				
Run 1	Mean conc. (ng/mL)	28.05	484.12	971.40
	SD	1.34	12.55	18.26
	Precision (%CV)	4.78	2.59	1.88
	Accuracy (% bias)	−6.50	−3.18	−2.16
Run 2	Mean conc. (ng/mL)	27.84	484.50	976.40
	SD	1.48	15.33	20.03
	Precision (%CV)	5.32	3.16	2.05
	Accuracy (% bias)	−7.20	−3.20	−2.36
Run 3	Mean conc. (ng/mL)	29.47	486.25	988.66
	SD	1.58	21.08	9.41
	Precision (%CV)	5.36	4.34	0.95
	Accuracy (% bias)	−1.77	−2.83	−1.13
Inter-day				
	Mean conc. (ng/mL)	28.09	489.59	978.40
	SD	2.37	10.36	17.47
	Precision (%CV)	8.44	2.12	1.78
	Accuracy (% bias)	−6.37	−2.08	−2.16
Overall recovery = 96.53%, $n = 5$				

3.3.6. *Stability*

TMPS in plasma was subjected to three freeze/thaw (−10 to −30 °C to room temperature) cycles. The obtained bias of TMPS was −6.40% of the theoretical value and coefficient of variation (CV) was 5.13%. No significant degradation of the TMPS was observed even after 48 h storage period in the autosampler tray with the bias of −6.80% and CV 7.19%. In addition, the long-term stability of TMPS in quality control samples after 30 days of storage at −10 to −30 °C and room temperature stability for 48 h was also evaluated with the bias of −9.20% and −5.70%, CV 5.84% and 4.14% respectively.

3.3.7. *Robustness*

A "Plackett–Burman" design was used to test the robustness of chromatographic separation and sample preparation. Experimental design is a useful tool in this kind of studies as it facilitates the investigation of several parameters at the same time while reducing the number of experiments [22].

(i) *Chromatographic separation*

Six factors which are likely to be significant in practical use of the method: methanol and acetonitrile content of the mobile phase, pH and molarity of the phosphate buffer, flow-rate and column oven temperature were investigated using three variables with upper and lower limits as shown in Table 2a. The experiments were run randomly with plasma sample spiked with 100 ng/mL TMPS. The selected responses were capacity factor (k'), resolution (Rs), number of theoretical plates (N) and tailing factor (T_f). The design matrix with the factor settings is shown in Table 2b. Plotting the scaled and centered coefficient plots (Fig. 4) for k', Rs, N and T_f revealed

Table 2
Robustness of chromatographic separation.

(a) Selected parameters and their variations	–	0	+
MeOH in mobile phase (vol.%)	7	8	9
MeCN in mobile phase (vol.%)	16.5	17.5	18.5
Molarity of phosphate buffer (mM)	15	20	25
Final pH of mobile phase	7.4	7.5	7.6
Flow rate (mL/min)	0.9	1.0	1.1
Oven temperature (°C)	20	25	30

(b) Plackett–Burman experimental design

Exp. no.	MeOH	MeCN	Phosphate	pH	Flow rate	Temp.	k'	Rs	N	T_f
3	7	16.5	15	7.6	1.1	30	0.831	2.123	2107	1.312
2	9	16.5	15	7.4	0.9	30	0.852	2.152	2257	1.368
4	7	18.5	15	7.4	1.1	20	0.723	2.783	2309	0.679
11	9	18.5	15	7.6	0.9	20	0.779	2.812	2058	0.775
10	7	16.5	25	7.6	0.9	20	0.807	2.758	2253	0.826
6	9	16.5	25	7.4	1.1	20	0.863	2.543	2078	0.853
5	7	18.5	25	7.4	0.9	30	0.795	2.223	2153	0.798
1	9	18.5	25	7.6	1.1	30	0.823	2.678	2219	1.213
9	8	17.5	20	7.5	1.0	25	0.853	2.949	2294	0.898
8	8	17.5	20	7.5	1.0	25	0.841	3.000	2378	0.876
7	8	17.5	20	7.5	1.0	25	0.859	2.962	2265	0.875

k': capacity factor; Rs: resolution; N = no. of theoretical plates; T_f: tailing factor.

that different combinations of significant parameters will not drastically affect responses, so that the developed method for analysis was considered to be robust.

(ii) *Sample preparation*

Three variables with upper and lower limits as shown in Table 3a were investigated. The design matrix with the factor settings is shown in Table 3b. The experiments were run randomly with plasma sample spiked with 100 ng/mL. The selected response was coefficient of variation (CV). The results are summarized as scaled and centered coefficient plots in Fig. 4. It revealed that different combinations of significant parameters will not drastically affect the response, so that the developed method for sample preparation was considered to be robust.

3.4. Application to a pharmacokinetic study

The application of the developed HPLC-FL method was demonstrated by analysis of plasma samples collected from healthy wistar rats received single oral dose of 20 mg/Kg of TMPS. The chromatograms of the corresponding blank plasma show that no interferences occur at the retention times of TMPS and VGB.

Table 3
Robustness of sample preparation.

Selected parameters and their variations	–	0	+
Molarity of borate buffer (mM)	75	100	125
pH of borate buffer	9.5	10	10.5
OPA stock volume added to working solution (mL)	0.25	0.30	0.35
MPA volume added to OPA working solution (μL)	15	20	25

(b) Experimental design

Exp. no.	Molarity of borate	pH of borate	OPA	MPA	Responses (CV)
6	75	9.5	0.25	15	2.48
5	125	9.5	0.25	25	2.05
3	75	10.5	0.25	25	1.27
7	125	10.5	0.25	15	3.83
8	75	6.5	0.35	25	1.79
1	125	9.5	0.35	15	1.63
2	75	10.5	0.35	15	2.88
9	125	10.5	0.35	25	2.32
10	100	10.0	0.30	20	0.98
11	100	10.0	0.30	20	0.99
4	100	10.0	0.30	20	1.01

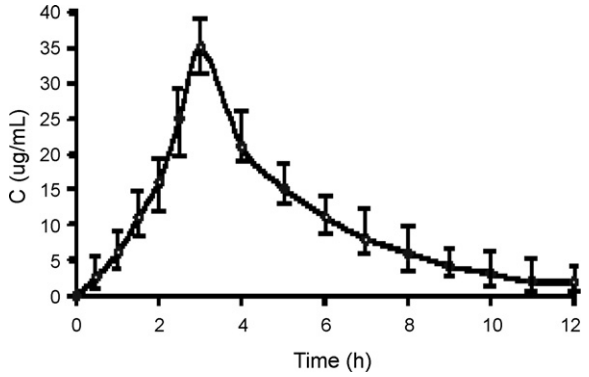

Fig. 5. Rat plasma concentration–time profile of TMPS after an oral administration of 20 mg/kg to 6 rats. Each point represents a mean ± S.D. (n = 6).

Plasma concentration–time profile of TMPS; is plotted and shown in Fig. 5. The obtained pharmacokinetic parameters of TMPS were t_{max} 3.0 h, maximum plasma concentration (C_{max}) 35 μg/mL, area under plasma concentration–time curve (AUC) 660.53 μg/mL/h, half life $t_{1/2}$ 3.13 h. Fig. 6 shows the cumulative excretion of TMPS for the same subject at regular time intervals after the dose. Statistical analysis was performed using Microsoft Excel 2000 while pharmacokinetic software, 'Ramkin', based on noncompartment model was used to calculate the [AUC] from the plasma drug concentration vs. time profiles.

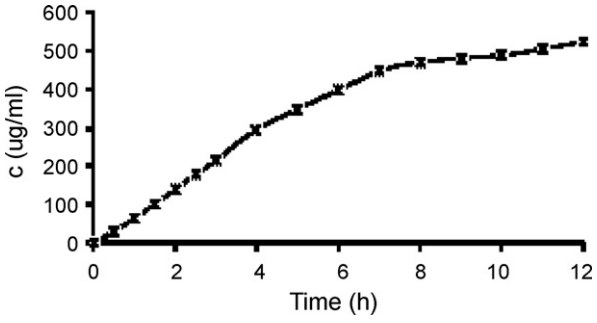

Fig. 6. Cumulative excretion of TMPS in rat urine after a single 20 mg/kg dose.

4. Conclusions

A simple, rapid and robust precolumn derivatization RP-HPLC-FL method for determination of TMPS in rat plasma was developed. Experimental design was found to be very helpful tool in testing robustness of the analytical methods during the pre-validation phase. The preceding exploration of its limits was very useful in identifying potential problematic factors and how to control them. The experimental results with respect to linearity, accuracy, precision, specificity and sensitivity demonstrate the reliability of the procedure for its intended application. The method was successfully applied to the pharmacokinetic study in rats.

Acknowledgements

The authors wish to thank Dr. J.S. Yadav, Director, IICT; Dr. M. Vairamani, Head, Analytical Chemistry Division for encouragement and permission to communicate the manuscript for publication. The authors also thank Mr. Sachin B. Agwane, Scientist, Pharmacology Division, IICT for his assistance in collection of samples of rat plasma. One of the authors Mr. Pawan K. Maurya thanks University Grants Commission (UGC), New Delhi, India, for a Research Fellowship.

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