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# Development and validation of a highly sensitive LC–MS/MS method for organic cation transporter (OCT) substrate tetraethylammonium (TEA) in rabbits

Jayabalan Nirmal[a], Thirumurthy Velpandian[a,*], Sundararajan Baskar Singh[b], Nihar Ranjan Biswas[c], Vasantha Thavaraj[d], Rajvardhan Azad[a], Supriyo Ghose[a]

[a] High Precision Bio-analytical Facility, Department of Ocular Pharmacology and Pharmacy, Dr. Rajendra Prasad Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110029, India
[b] Department of Biophysics, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110029, India
[c] Department of Pharmacology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110029, India
[d] Indian Council for Medical Research, Ansari Nagar, New Delhi 110029, India

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ABSTRACT

Tetraethylammonium is widely used as a probe in organic cation transporters studies. A simple, highly sensitive, and specific method using direct protein precipitation was developed using Hydrophilic Interaction Liquid Chromatography coupled with positive electrospray ionization tandem mass spectrometry for the determination of tetraethylammonium (TEA) in rabbit plasma. Isocratic separation was achieved using a ZIC-HILIC column with acetonitrile and 5 mM ammonium acetate in the ratio of 8:2 containing 0.1% formic acid. Acquisition was performed in multiple reaction monitoring mode with the transitions: $m/z$ 130 → 100 and 130 → 86 for TEA and $m/z$ 276.1 → 142.2 for internal standard (homatropine). This method was validated to determine selectivity, linearity, sensitivity, precision, accuracy, recovery and stability. A good linearity was found within a range of 1.53–784.6 ng/mL. The above method has been demonstrated for its capability to estimate the plasma levels of TEA after its topical instillation in rabbit eyes. This method provides an accurate, precise and sensitive tool for determining TEA levels for transporter studies.



## 1. Introduction

Drug transporters may play an important role in the absorption, distribution and elimination of drugs [1,2]. Organic cation transporters (OCTs) are reported to modulate the transport of drugs and xenobiotics [1–3]. OCTs are also clinically interesting since many of the currently used drugs are OCT substrates [4]. Moreover, from the recent literatures it was found that OCTs are expressed in various ocular tissues including cornea and conjunctiva [5,6]. Hence investigating the ocular disposition of a unique model substrate of OCT after topical application as an eye drop can give us an understanding towards better delivery of OCT substrates in various ocular pathological conditions.

Tetraethylammonium (TEA) is a synthetic quaternary alkylammonium compound, initially introduced as a ganglion blocking agent [7–9]. It is used in various drug transporter studies (OCT) as a model probe [10–12] and is reported to be a well transported unique substrate by all OCTs in various species studied [13–19]. So far, most of the studies used radiolabelling ($^{14}$C radiolabelled TEA) as a method for the investigation of OCT modulation by various agents [13,20,21]. The transporter studies using radiolabelled ligand require several regulatory approvals and cumbersome animal disposal methods. TEA in its halide form (either as bromide or chloride) is a highly water soluble aliphatic nitrogenous hydrocarbon. Moreover it lacks hydrophobicity and chromophore for the required retention and detection respectively, thus compromising its quantification using conventional chromatography methods.

As our laboratory has been working on the functional importance of OCT in blood ocular barriers, development of a highly sensitive method as an alternative to radiolabelled TEA for its quantification was felt as important. To achieve separation of hydrophilic TEA, Hydrophilic Interaction Liquid Chromatography (HILIC) was used [22] and to increase the sensitivity and specificity, HILIC was combined with tandem mass spectroscopy [23,24].

Hence, the aim of the present study is to develop and validate a simple, selective, rapid and reproducible liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) method for the quantitative determination of TEA in plasma samples. The developed method was further evaluated for its ability to quantify the plasma levels of TEA after its topical application into the rabbit's eye.

* Corresponding author. Tel.: +91 11 26593162; fax: +91 11 26593162/26588919.
*E-mail address:* tvelpandian@hotmail.com (T. Velpandian).

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## 2. Experimental

### 2.1. Materials and reagents

Tetraethylammonium chloride was purchased from Sigma–Aldrich (St. Louis, MO, USA). Homatropine hydrobromide was purchased from Boehringer Ingelheim, Germany. MS grade formic acid, acetonitrile and ammonium acetate was purchased from Merck, Germany. Water (18.2 MΩ) was purified using a Milli-Q purification system (Millipore Corp., Bedford, MA, USA). All other chemicals and solvent were one of the highest analytical grades available.

### 2.2. Calibration standard and quality control samples

A stock solution of TEA was prepared in water at 7.8 mg/mL. This stock solution was appropriately diluted with 50% methanol containing 0.1% formic acid to reach the required concentration. To obtain spiked working standards for calibration purpose, TEA 7.8 mg/mL stock solution was prepared by using rabbit plasma (TEA free) and serially diluted with the same plasma to reach suitable levels. A calibration curve was prepared with concentrations of 1.53, 6.12, 49.03, 196.1 and 784.6 ng/mL. Stock solution (1000 ng/mL) of internal standard (IS) was prepared by dissolving homatropine in 50% methanol. It was further diluted in the extraction solvent containing 70% acetonitrile and 0.1% formic acid to reach 500 ng/mL. Quality control (QC) samples (78.4, 157 and 627 ng/mL) were also prepared like calibration standards using rabbit plasma (TEA free).

### 2.3. LC–MS/MS

Chromatography separation was achieved using Thermo Surveyor system (Thermo Electron Corp., Waltham, MA, USA) with a quaternary pump connected to an online degasser and photodiode array detector (PDA). Chromquest software version 4.1 was used to control all parameters of HPLC. For the analytical separation of the hydrophilic compounds a Hydrophilic Interaction Liquid Chromatography column (ZIC HILIC column with the dimension of 50 mm × 4.6 mm, 3.5 μm, Merck, Darmstadt, Germany) was used.

The isocratic mobile phase consisted of acetonitrile containing 0.1% formic acid (A) and 5 mM ammonium acetate containing 0.1% formic acid (B) in the ratio of 8:2 and was pumped at the rate of 0.5 mL/min. The autosampler tray and the column were kept at ambient temperature. Twenty microlitre of sample was injected into the HPLC with a run time of 5 min.

Tandem mass spectrometric detection of analyte and internal standard (IS) was carried out using Applied Bio Systems 4000 triple quadrapole instrument (ABS Biosystems, Foster City, CA, USA) equipped with a TurboIonSpray (ESI) source that operated in the positive ion mode. Quantification was performed using multiple reaction monitoring (MRM) mode, based on molecular adduct ion and fragment ion for tetraethylammonium $m/z$ 130 → 100 (transition 1) and $m/z$ 130 → 86 (transition 2) respectively. The transition for homatropine was $m/z$ 276.1 → 142.2. Data acquisition and integration was performed by Analyst 1.4.2 software (ABS Biosystems, Foster City, CA, USA).

### 2.4. Sample preparation

Calibration standards, QC samples and rabbit plasma samples were prepared by direct protein precipitation. All plasma samples were stored at −20 °C and allowed to thaw at room temperature before processing. Briefly, 20 μL of standards or samples were mixed with 200 μL of extraction solvent (70:30 ratio of acetonitrile:water with 0.1% formic acid) containing homatropine at the concentration of 500 ng/mL as an IS. The mixture was vortexed for 1 min and centrifuged for 10 min at 7840 × $g$. The resultant supernatant was subjected for analysis.

### 2.5. Method validation

The LC–MS/MS method was validated for selectivity, linearity, sensitivity, precision, accuracy, extraction recovery, matrix effect and stability according to the currently accepted US Food and Drug Administration (FDA) Bioanalytical Method Validation Guidance [25].

#### 2.5.1. Selectivity

Selectivity was evaluated by comparing chromatograms of six blank plasma samples from six different rabbits. Blank plasma samples were evaluated using the proposed extraction procedure and chromatographic/spectroscopic conditions to make sure there were no significant interfering peaks at retention time at the concentration near the range of lowest limit of quantification (LLOQ) of the analytes.

#### 2.5.2. Calibration curve, linearity and sensitivity

Calibration curve was constructed using blank plasma (rabbit plasma without TEA and IS) at five concentrations covering the range (1.53–784.6 ng/mL) derived from the peak area ratio of TEA against IS. Concentration of TEA was calculated from these area ratios using the calibration curve. The linearity of the calibration curve was also calculated, and a correlation coefficient ($r^2$) of 0.99 or better was selected. LLOQ was defined as the lowest concentration with a coefficient of variance (%CV) < 20%.

#### 2.5.3. Precision and accuracy

Intra- and inter-day assay precisions were determined as %CV, and intra- and inter-day assay accuracies were expressed as percentage of the theoretical concentration, as accuracy (%) = (found concentration/theoretical concentration) × 100. Intra-day assays were performed using four replicates during 1 day and inter-day assays were performed on four separate days.

#### 2.5.4. Recovery and matrix effect

The concentrations of TEA used for the evaluation of recovery and matrix effect were 1.53, 49.03, 196.1 and 784.6 ng/mL ($n$ = 3) with 500 ng/mL IS for each experimented concentration. To measure the LC/MS/MS response of standard solutions (without matrix), pure solution set was prepared. Pre-extraction set was prepared by the proposed preparative procedure. Post-extraction set was prepared by dissolving analyte in the extracted blank plasma sample. The corresponding peak areas of analyte in pure solution set (A), were then compared to those of the pre-extraction set (B) or post-extraction set (C) at equivalent concentrations. The ratio of B/A is defined as the ARE (absolute recovery), and the ratio of C/A is defined as the AME (absolute matrix effect). The resulting values are expressed as percentage. To evaluate relative matrix effect (RME), blank plasma samples from six different rabbits were used to prepare spiked samples at the concentrations of 1.53, 49.03, 196.1 and 784.6 ng/mL. For each lot, samples were analysed in triplicate at each concentration. The CV of the peak area for TEA was calculated to determine inter-lot matrix variability.

#### 2.5.5. Stability

The stability of TEA was assessed by analysing QC samples at three concentrations (78.4, 157 and 627 ng/mL) exposed to different temperatures. Short-term stability was assessed by analysing QC plasma samples kept at room temperature (RT) for 6 h that exceeded the routine preparation time of samples. Long-term stability was determined by assaying QC plasma samples after storing

at −20 °C for 60 days. Freeze–thaw stability was investigated after three freeze–thaw cycles. All the samples were analysed together with freshly processed QC samples.

### 2.6. Quantitative determination of TEA in plasma

New Zealand albino rabbits of either sex weighing 1.5–2 kg were used for the study. All protocols were reviewed and approved by the Standing Institutional Animal Ethics Committee of All India Institute of Medical Sciences, New Delhi, India. Studies performed were in accordance with the Association for Research in Vision and Ophthalmology Regulations and Standards (ARVORS) guidelines. In the present study, we determined the rabbit plasma levels of TEA quantitatively after its single topical application. Twenty microlitre of TEA (0.1%, w/v pH adjusted to 7.4 in phosphate buffer) was instilled on the cornea of the right eye of the rabbit. Blood collection was performed from the marginal ear vein of rabbits at 15, 30, 60 and 120 min after the topical instillation of TEA. Blood samples were collected using standard procedure in EDTA containers and were subjected for centrifugation (2000 × *g* for 10 min) to separate the plasma. All the plasma samples were stored at −20 °C until analysis. The area under the curve of plasma concentration up to 2 h ($AUC_{0\text{-}2}$) was calculated using the standard technique.

## 3. Results and discussion

### 3.1. Optimization of MS detection and chromatographic conditions

Mass spectrometric parameters were optimized to achieve the maximum abundance of molecular adduct and fragment ions. Full scan mass spectra and fragment ion scan spectra of TEA and IS were obtained by direct infusion into the mass spectrometer at a flow rate of 5 μL/min using Harvard pump (Harvard company, Reno, Nevada, USA) connected with a Hamilton (Holliston, MA, USA) syringe (Fig. 1(A) and (B)). Two transitions were chosen for TEA, *m*/*z* 130 → 100 and *m*/*z* 130 → 86 respectively. Transition for IS was *m*/*z* 276.1 → 142.2. Ionization and fragmentation were found to be highly efficient, and as a result, a substantial detection response was obtained at the lower limit of quantification (LLOQ) 1.53 ng/mL.

The main instrument parameters of the mass spectrometer are as follows: source dependent parameters were gas 1 (30 psi); gas 2 (60 psi); curtain gas (10 psi); ion spray voltage (5500 V) and temperature (350 °C). Compound dependent parameters for analyte such as declustering potential, entrance potential, collision energy and cell exit potential were set at 57, 10, 26.5 and 8 V for transition 1 and at 57, 10, 32 and 6 V for transition 2 respectively. For IS the compound dependent parameters were 110, 5, 82 and 7 V. Collision activated dissociation and dwell time was kept at 3 (arbitrary value) and 100 ms for both analyte and IS respectively.

### 3.2. Method validation

#### 3.2.1. Selectivity

Representative MRM chromatogram of blank rabbit plasma is shown in Fig. 2(A). A spiked rabbit plasma chromatogram with TEA at concentration of 1.53 ng/mL is shown in Fig. 2(B), while an example of plasma sample chromatogram from rabbit at 30 min time point after single topical application of TEA (0.1%) is shown in Fig. 2(C). No endogenous peaks were observed at the retention times of TEA or IS. All samples were free of interference with the analyte and IS. The total LC/MS/MS analysis time was 5 min per sample.

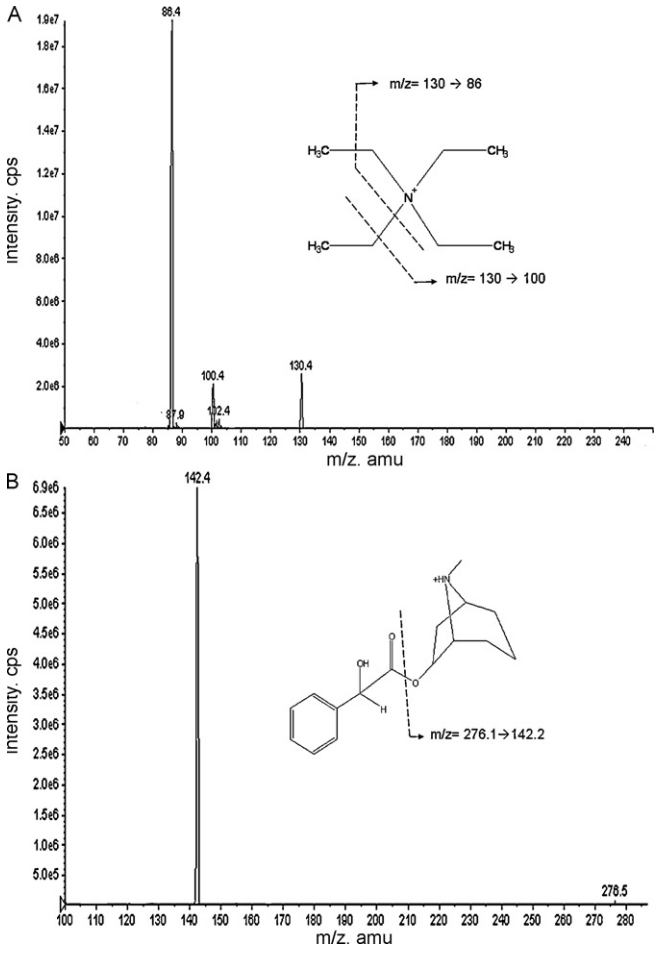


**Fig. 1.** Chemical structures and product ion spectra of TEA (A) and homatropine (B).

#### 3.2.2. Calibration curve, linearity and sensitivity

The standard curves were plotted from working standard solutions at five concentrations of TEA ranging 1.53–784.6 ng/mL. The linear regression equation of standard curve was $y = 0.438x + 19$. The standard calibration curve for TEA was linear over the range 1.53–784.6 ng/mL with $r^2 = 0.9977$. The lower limit of quantification (LLOQ) and limit of detection (LOD) were found to be 1.53 and 0.045 ng/mL respectively.

#### 3.2.3. Precision and accuracy

The intra-day precision (%CV) and accuracy of the developed method for validating TEA ranged from 5.95 to 12.48% and 99.64 to 108.56% respectively. The interday precision (%CV) and accuracy ranged from 2.21 to 12.29% and 91.81 to 102.86% respectively (Table 1). The results were within the limits of FDA criteria and also found to be reproducible.

**Table 1**
The precision and accuracy for LC/MS/MS assay of TEA in rabbit plasma ($n = 3$).

| Theoretical concentration (ng/mL) | Accuracy (%) | | Precision (%CV) | |
|---|---|---|---|---|
| | Intra-day | Inter-day | Intra-day | Inter-day |
| 1.53 | 108.19 | 97.43 | 7.60 | 12.29 |
| 6.12 | 108.56 | 91.81 | 12.48 | 11.81 |
| 49.03 | 99.64 | 102.86 | 10.42 | 9.01 |
| 196.15 | 100.10 | 100.33 | 5.95 | 5.63 |
| 784.6 | 100.07 | 99.91 | 8.17 | 2.21 |

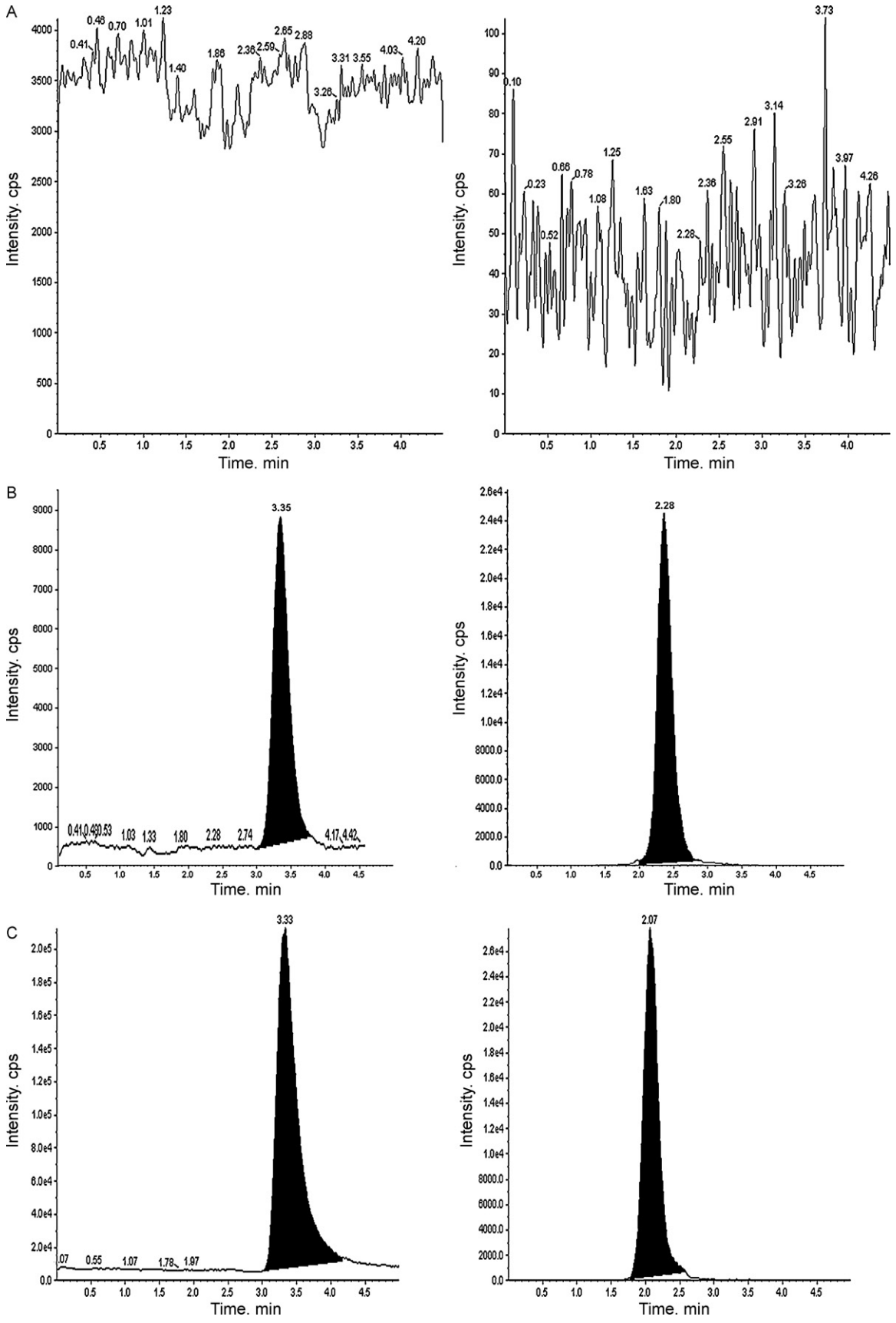


**Fig. 2.** Representative MRM chromatograms resulting from analyses of (A) blank plasma (drug and IS free), (B) 1.53 ng/mL of spiked TEA with IS (homatropine) and (C) plasma sample from a rabbit, 30 min after single topical application of TEA (0.1%).

**Table 2**
Absolute recovery (ARE), absolute matrix effect (AME) and relative matrix effect (RME) of TEA.

| Theoretical concentration (ng/mL) | ARE mean ± SD | AME mean ± SD | RME | |
|---|---|---|---|---|
| | | | Mean ± SD | %CV |
| 1.53 | 101.81 ± 3.12 | 106.8 ± 5.93 | 2.15 ± 0.38 | 14.4 |
| 49.03 | 98.61 ± 1.35 | 108.41 ± 8.16 | 48.83 ± 5.64 | 11.55 |
| 196.15 | 103.88 ± 7.47 | 107.5 ± 7.73 | 196.16 ± 4.82 | 2.46 |
| 784.6 | 101.27 ± 2.11 | 106.11 ± 6.25 | 785.29 ± 46.41 | 5.91 |

**Table 3**
Stability results, expressed as percentage of freshly prepared quality control samples ($n = 3$).

| Theoretical concentration (ng/mL) | Short term study | | Long term study | | Freeze–thaw study | |
|---|---|---|---|---|---|---|
| | Freshly prepared (mean ± SD) | After 6 h storage at RT (mean ± SD) (%)[a] | Freshly prepared (mean ± SD) | After 60 days storage at −20 °C (mean ± SD) (%)[a] | Freshly prepared (mean ± SD) | After freeze thaw cycle (mean ± SD) (%)[a] |
| 78.400 | 78.4 ± 0.86 | 78.42 ± 11.42 (100.02) | 77.76 ± 0.31 | 78.47 ± 2.33 (100.91) | 78.40 ± 3.89 | 78.39 ± 3.32 (99.90) |
| 157.00 | 157.02 ± 13.82 | 157.01 ± 7.70 (99.99) | 157.29 ± 7.59 | 156.73 ± 8.74 (99.64) | 157.0 ± 7.03 | 156.99 ± 10.99 (99.99) |
| 627.0 | 627.38 ± 51.09 | 627.25 ± 30.13 (99.97) | 627.12 ± 20.91 | 627.20 ± 52.73 (100.01) | 627.01 ± 14.49 | 626.93 ± 48.57 (99.98) |

[a] Percentage calculated from freshly prepared QC.

### 3.2.4. Recovery and matrix effect

The ARE of the proposed extraction method was about 98.61–103.88% at three different concentrations of TEA. The AME of TEA was more than 100% and RME of TEA was less than 14.4% (Table 2). The results indicate that there is no significant ion suppression or ion enhancement in the developed analytical method due to any interfering endogenous substances.

### 3.2.5. Stability

The QC samples subjected for stability studies were measured and compared with the freshly prepared QC samples. As shown in Table 3, results indicated that TEA was found stable in all tested conditions and confirmed the applicability of the method for routine analysis. Moreover, the QC samples subjected for stability studies at −80 °C for 60 days were also found to be stable (data not shown). These results indicate that there were no significant deviations when compared to freshly prepared QC samples in all the tested conditions.

### 3.3. Quantitative determination of TEA in plasma

The developed analytical method was applied to the quantification of TEA in rabbit plasma. It showed a detectable plasma concentration in the studied time points at 15, 30, 60 and 120 min. The maximum plasma concentration of 41.52 ± 13.04 ng/mL was detected at the $T_{max}$ of 30 min (Fig. 3). The area under the curve of plasma concentration up to 2 h ($AUC_{0-2}$) was found to be 21.40 ± 3.26 ng/mL*h. At 1 and 2 h the levels reached at the concentration of 1.65 ± 0.75 and 2.89 ± 1.79 ng/mL which was above the LLOQ of the present developed method.

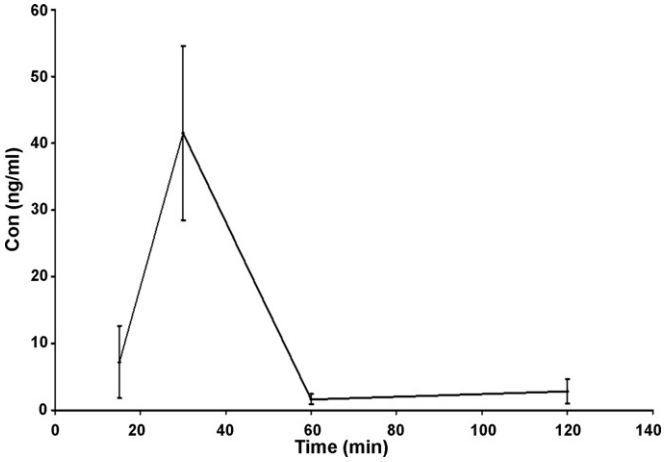


**Fig. 3.** Systemic absorption of TEA evidenced by its plasma levels after the single topical instillation of TEA at the concentration of 0.1% at the volume of 20 μL (20 μg total dose) into rabbit's eye.

## 4. Conclusion

A rapid and sensitive LC–MS/MS method has been developed for the quantitation of TEA in rabbit plasma. This method combines the separating ability of HILIC along with high resolution tandem mass spectroscopy and is capable of replacing the requirement of radiolabelling of TEA in future studies for quantitative estimations. With minor modifications, this method can also be used for *in vitro* transporter studies using cell lines. Owing to its accuracy and precision, this method is capable of quantifying TEA with simple sample preparation method and shorter run time for conducting transporter studies. This method has been successfully applied to a systemic absorption study of TEA after topical instillation into the rabbit's eye.

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## References

[1] C.J. Endres, P. Hsiao, F.S. Chung, F.S. Unadkat, Eur. J. Pharm. Sci. 27 (2006) 501.
[2] The International Transporter Consortium, Nat. Rev. Drug Dis. 9 (2010) 215.
[3] H. Koepsell, Trends Pharmacol. Sci. 25 (2004) 375.
[4] E. Mannermaa, K.S. Vellonen, A. Urtti, Adv. Drug. Deliv. Rev. 58 (2006) 1136.
[5] T. Zhang, C.D. Xiang, D. Gale, S. Carreiro, E.Y. Wu, E.Y. Zhang, Drug. Met. Disp. 36 (2008) 1300.
[6] Q. Garrett, S. Xu, P.A. Simmons, J. Vehige, J.L. Flanagan, M.D. Willcox, Invest. Ophthal. Vis. Sci. 49 (2008) 4844.
[7] M.K. Kim, C.K. Shim, Arch. Pharm. Res. 29 (2006) 605.

[8] Y.P. Tan, I. Liano, J. Physiol. 520 (1999) 65.
[9] V. Raymond, B. Lapied, Neuroscience 93 (1999) 1207.
[10] R.M. Franke, A.M. Kosloske, C.S. Lancaster, K.K. Filipski, C. Hu, O. Zolk, R.H. Mathijssen, A. Sparreboom, Clin. Cancer Res. 16 (2010) 4198.
[11] A.M. Gerlyand, D.S. Sitar, Can. J. Physiol. Pharmacol. 87 (2009) 821.
[12] I. Tamai, T. Nakanishi, D. Kobayashi, K. China, Y. Kosugi, J. Nezu, Y. Sai Y, A. Tsuji Y, Mol. Pharm. 1 (2004) 57.
[13] H. Koepsell, K. Lips, C. Volk, Pharm. Res. 24 (2007) 1227.
[14] D. Grundemann, V. Gorboulev, S. Gambaryan, M. Veyhl, H. Koepsell, Nature (Lond.) 372 (1994) 549.
[15] V. Gorboulev, J.C. Ulzheimer, A. Akhoundova, I. Ulzheimer-Teuber, U. Karbach, S. Quester, C. Baumann, F. Lang, A.E. Busch, H. Koepsell, DNA Cell Biol. 16 (1997) 871.
[16] R. Kekuda, P.D. Prasad, X. Wu, H. Wang, Y.J. Fei, F.H. Leibach, V. Ganapathy, J. Biol. Chem. 273 (1998) 15971.
[17] X. Wu, P.D. Prasad, F.H. Leibach, V. Ganapathy, Biochem. Biophys. Res. Commun. 277 (1998) 589.
[18] D.H. Sweet, J.B. Pritchard JB, Am. J. Physiol. 277 (1999) F890.
[19] H. Yabuuchi, I. Tamai, J. Nezu, K. Sakamoto, A. Oku, M. Shimane, Y. Sai, A. Tsuji, J. Pharmacol. Exp. Ther. 289 (1999) 768.
[20] H. Ueda, Y. Horibe, K.J. Kim, V.H.L. Lee, Invest. Ophthal. Vis. Sci. 41 (2000) 870.
[21] J.W. Jonker, E. Wagenaar, C.A. Mol, M. Buitelaar, H. Koepsell, J.W. Smit, A.H. Schinkel, Mol. Cell. Biol. 21 (2001) 5471.
[22] B.A. Olsen, J. Chromatogr. A 913 (2001) 113.
[23] B. Chauve, D. Guillarme, P. Cléon, J.L. Veuthey, J. Sep. Sci. 33 (2010) 752.
[24] W. Jian, R.W. Edom, Y. Xu, N. Weng, J. Sep. Sci. 33 (2010) 681.
[25] FDA Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), 2001, http://www.fda.gov/cder/guidance/index.htm.