Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 860 (2007) 227–234
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Chiral bioanalysis of torcetrapib enantiomers in hamster plasma by normal-phase liquid chromatography and detection by atmospheric pressure chemical ionization tandem mass spectrometry ☆

Ravi Kumar Trivedi [a,b], Buddhadev Layek [b], T. Santosh Kumar [b], Shivva Vittal [b], Ramesh Ganneboina [b], P.K. Dubey [a], Ramesh Mullangi [b,*], Nuggehally R. Srinivas [b]

[a] *Department of Chemistry, JNTU College of Engineering, Kukatpally, Hyderabad 500072, India*
[b] *Drug Metabolism and Pharmacokinetics, Discovery Research, Dr. Reddy's Laboratories Ltd., Miyapur, Hyderabad 500049, India*

Received 20 August 2007; accepted 25 October 2007
Available online 4 November 2007

Abstract

A highly sensitive and enantioselective assay has been developed and validated for the estimation of torcetrapib (TTB) enantiomers [(+)-TTB and (−)-TTB] in hamster plasma with chiral liquid chromatography coupled to tandem mass spectrometry with an atmospheric pressure chemical ionization interface in the negative-ion mode. The assay procedure involves liquid–liquid extraction of TTB enantiomers and IS (DRL-16126) from 100 μL hamster plasma with acetonitrile. TTB enantiomers were separated using *n*-hexane:propanol (80:20, v/v) at a flow rate of 0.7 mL/min on a Chiralpak AD column. The MS/MS ion transitions monitored were 599.2 → 340.2 for TTB and 623.2 → 298.1 for IS. Absolute recovery was found to be between 64 and 68% for TTB enantiomers and >100% for IS. The standard curves for TTB enantiomers were linear ($r^2 > 0.995$) in the concentration range 5–2500 ng/mL for each enantiomer with an LLOQ of 5 ng/mL for each enantiomer. The inter- and intra-day precisions were in the range of 10.5–12.4 and 9.15–11.5% and 3.75–12.9 and 5.16–12.5% for (+)-TTB and (−)-TTB, respectively. Accuracy in the measurement of quality control (QC) samples was in the range 91.3–105 and 88.6–111% for (+)-TTB and (−)-TTB, respectively. This novel method has been applied to the study of stereoselective oral pharmacokinetics of (−)-TTB.

Keywords: Torcetrapib; Enantioselective; Plasma; Validation; Chiralpak AD; LC–MS/MS; APCI; Pharmacokinetics

1. Introduction

Cholesteryl ester transfer protein (CETP) is a glycoprotein secreted mainly from the liver and circulated in plasma, promotes the transfer of cholesterol esters from antiatherogenic high-density lipoprotein cholesterol (HDL-C) to proatherogenic apolipoproteins very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL). Thus, the inhibition of CETP represents a novel target for the treatment of atherosclerosis by means of its beneficial effects on levels of HDL-C [1]. Torcetrapib (TTB; CP-529,414; CAS: 262352-17-0, Fig. 1),chemically (−)-(2*R*,4*S*)-4-[(3,5-bis-trifluoromethyl-benzyl)-methoxy -carbonyl-amino]-2-ethyl-6- trifluoromethyl - 3,4- dihydro- 2H-quinoline-1-carboxylic acid ethyl ester is a CETP inhibitor. In the clinic, TTB lowered CETP activity, decreased apolipoprotein-B (apo-B) and LDL; elevated HDL-C and apo-E with no effect on triglycerides [2]. While the drug showed great promise in early clinical trials, the development of TTB was recently suspended due to higher risk of cardiovascular event in patients who received TTB along with standard of care [3].

For the development of a racemic drug, it is essential to know the pharmacological effect of each enantiomer, since it is well known that the enantiomers can differ in their pharmacological, pharmacokinetic and toxicological behavior [4,5]. Pure enantiomer often exhibits higher potency, bioavailability and reduced side effects when compared to racemates. Of late, pharmaceutical companies are giving greater importance on evaluating the stereoisomeric composition of drugs

☆ DRL Publication No: 672.

* Corresponding author. Tel.: +91 40 23045439; fax: +91 40 23045438.
E-mail address: mullangiramesh@drreddys.com (R. Mullangi).

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.10.043

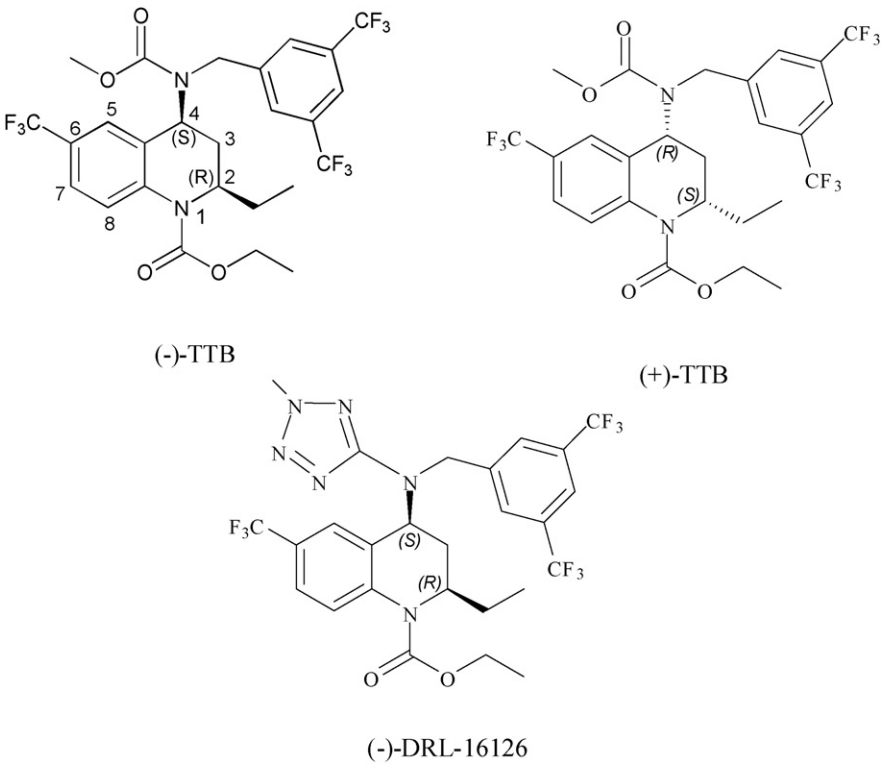

Fig. 1. Structural representation of (+)-torcetrapib, (−)-torcetrapib and (−)-DRL-16126.

having a chiral center. It is therefore important to develop enantioselective separation methods for studies on stereoselective pharmacokinetics and metabolism [6]. Typically enantiomers can be separated using different approaches, viz., formation of diastereomers prior to separation on non-chiral column or addition of chiral additives to form diastereomeric complexes followed by separation on a non-chiral column or direct separation on a chiral column [7,8]. Lee et al. [9] have described the distribution of TTB in human plasma and different lipoprotein components of human plasma by GC–MS/MS. Earlier, we have developed and validated a HPLC chiral method for the determination of TTB enantiomers [10]. Though we have achieved enantioselective separation of TTB enantiomers, the limitations of this method, viz., higher LLOQ (0.1 μg/mL) and long run time, prompted us to develop a chiral bioanalytical method on LC–MS/MS. Hyphenation of LC with mass spectrometry has advantages in providing shorter run time, higher specificity and sensitivity along with much lower LLOQ; hence, it is gaining importance in qualitative and quantitative measurement of compounds. Chiral LC–MS/MS is gaining popularity in bioanalysis, trace analysis and metabolites identification. To the best of our knowledge, no quantitative LC/APCI-MS/MS method suitable for the routine stereoselective analysis of TTB enantiomers has been reported yet. In this manuscript, we are presenting the LC/APCI-MS/MS method for determination of TTB enantiomers without post-cloumn reagent addition on a Chiralpak AD column in hamster plasma and application of this method to derive the pharmacokinetic parameters for (−)-TTB in hamsters.

2. Experimental

2.1. Chemicals and reagents

(±)-TTB, (−)-TTB and (−)-DRL-16126 (IS, Fig. 1) were synthesized using a reported synthetic route [11]. All the compounds were characterized using chromatographic (HPLC, LC–MS/MS) and spectral techniques (IR, UV, Mass, ^{1}H and ^{13}C NMR) by the Analytical Research Group, Discovery Research, DRL, Hyderabad. Purity was found to be more than 98.7% for all the compounds. HPLC grade *n*-hexane was purchased from S.D. Fine Chemicals, Mumbai, India. HPLC grade acetonitrile and isopropanol (IPA) were purchased from Rankem, Ranbaxy Fine Chemicals Limited, New Delhi, India. All other reagents purchased from Qualigens (Mumbai, India) were of analytical reagent grade. Control hamster plasma was obtained from Department of Pre-clinical and Safety Evaluation, Discovery Research, DRL, Hyderabad.

2.2. HPLC operating conditions

An Agilent (Agilent Technologies, Waldbronn, Germany) 1100 series LC system equipped with degasser (G1379A), quaternary pump (G1311A) along with auto-sampler (G1367A) was used to inject 40 μL aliquots of the processed samples on a Chiralpak AD (250 mm × 4.6 mm, 10 μm) column (Daicel Chemical Industries Ltd., Japan) coupled with guard column (Chiralpak AD, 10 mm × 4.6 mm, 10 μm, Daicel Chemical Industries Ltd., Japan), which was kept at ambient temperature.

The isocratic mobile phase, a mixture of *n*-hexane and IPA (80:20, v/v) was filtered through a 0.45-μm hydrophilic PVDF filter (Cat No.: HVLP 04700, Millipore, USA) and then degassed ultrasonically for 15 min was delivered at a flow rate of 0.7 mL/min into the mass spectrometer atmospheric pressure chemical ionization chamber.

2.3. Mass spectrometry operating conditions

Quantitation was achieved by MS/MS detection in negative-ion mode for analyte and IS using a MDS Sciex (Foster City, CA, USA) API 4000 Q-Trap mass spectrometer, equipped with a Turboionspray™ interface at 250 °C. The needle current was set at −3 V. The common parameters, viz., nebulizer gas, curtain gas, auxiliary gas and collision gas were set at 45, 10, 50 and at medium, respectively. Where as the compounds parameters, viz., declustering potential (DP), collision energy (CE), collision exit potential (CXP) and entrance potential (EP) for both TTB enantiomers and IS were −80, −28, −7, −10 and −60, −22, −7, −10, respectively. We have used nitrogen as auxiliary and nebulizing gas type in our MS detector gas in order to lower the risk of explosion. Detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring the transition of the *m/z* 599.2 precursor ion to the *m/z* 340.2 product ion for TTB enantiomers and *m/z* 623.2 precursor ion to the *m/z* 298.1 product ion for IS. Quadrupoles Q1 and Q3 were set on low resolution. The analytical data were processed by Analyst software (Version 1.4.1).

2.4. Standard solutions

Primary stock solutions of (±)- and (−)-TTB for preparation of standard and quality control (QC) samples were prepared from separate weighing. The primary stock solutions were prepared in IPA (1000 μg/mL). The IS stock solution of 1000 μg/mL was prepared in IPA. The stock solutions of (±)-TTB, (−)-TTB and IS were stored at 4 °C, which were found to be stable for one month (data not shown) and successively diluted with IPA to prepare working solutions to prepare calibration curve (CC). Another set of working stock solutions of (±)- and (−)-TTB were made in IPA (from primary stock) for preparation of QC samples. Working stock solutions were stored approximately at 4 °C for a week (data not shown). Appropriate dilutions of (±)-TTB stock solution was made in IPA to produce working stock solutions of 50, 20, 10, 5, 2, 1, 0.5, 0.2 and 0.1 μg/mL, similarly dilutions of (−)-TTB was made in IPA to produce working stock solutions of 25, 10, 5, 2.5, 1, 0.5, 0.25, 0.1 and 0.05 μg/mL. Working stocks were used to prepare plasma calibration standards. A working IS solution (5 μg/mL) was prepared in IPA. Calibration samples were prepared by spiking 90 μL of control pooled hamster plasma with the appropriate working solution of the analyte (10 μL) and IS (10 μL) on the day of analysis. Samples for the determination of precision and accuracy were prepared by spiking control hamster plasma in bulk with (±)-TTB at appropriate concentrations (10, 30, 1500 and 4000 ng/mL equivalent to 5, 15, 750 and 2000 ng/mL of each enantiomer) and 100 μL aliquots were distributed into different tubes. All the samples were stored at −80 ± 10 °C.

2.5. Recovery

Two sets of standards containing the (±)-TTB and IS at two different concentrations (30 and 4000 ng/mL) equivalent to 15 and 2000 ng/mL of each enantiomer were prepared. One set was prepared in hamster plasma and the other set was prepared in methanol. The recovery was determined by comparing peak areas of spiked plasma extracts with those of unextracted neat standards prepared in methanol [12]. The recovery value was calculated at two different concentrations of (±)-TTB. The recovery of the IS was determined at a single concentration of 500 ng/mL.

2.6. Sample preparation

To an aliquot of 100 μL plasma sample, IS solution (10 μL) equivalent to 500 ng was added and mixed for 15 s on a cyclomixer (Remi Instruments, Mumbai, India). After the addition of 2 mL of acetonitrile, the mixture was vortexed for 3 min; followed by centrifugation for 4 min at 3200 rpm on Multifuge 3_{SR} (Heraus, Germany). The organic layer (1.8 mL) was separated and evaporated to dryness at 50 °C using a gentle stream of nitrogen (Turbovap®, Zymark® Kopkinton, MA, USA). The residue was reconstituted in 250 μL of the mobile phase and 40 μL was injected onto LC–MS/MS system.

2.7. Validation procedures

A full validation according to the FDA guidelines [13] was performed for the assay in hamster plasma.

2.7.1. Specificity and selectivity

The specificity of the method was evaluated by analyzing hamster plasma samples from at least six different sources to investigate the potential interferences at the LC peak region for analyte and IS.

2.7.2. Matrix effect

The effect of hamster plasma constituents over the ionization of TTB enantiomers and IS was determined by comparing the responses of the post-extracted plasma standard QC samples ($n = 4$) with the response of analytes from neat standard samples (10 μL of required working stock sample spiked into 90 μL of methanol instead of blank plasma) at equivalent concentrations [12,14]. Matrix effect for (±)-TTB was determined at low and high concentrations, viz., 30.0 and 4000 ng/mL (equivalent to 15 and 2000 ng/mL of each enantiomer), whereas the matrix effect over the IS was determined at a single concentration of 500 ng/mL.

2.7.3. Calibration curve

Calibration curves were acquired by plotting the peak-area ratio of each enantiomer of TTB: IS against the nominal concentration of calibration standards. The concentrations used for

each enantiomer were 5, 10, 25, 50, 100, 250, 500, 1000 and 2500 ng/mL. The results were fitted to linear regression analysis with the use of $1/X^2$ weighting factor. The calibration curve had to have a correlation coefficient (r) of 0.995 or better for both enantiomers. The acceptance criteria for each back-calculated standard concentration were ±15% deviation from the nominal value except at LLOQ, which was set at ±20% [13].

2.7.4. Precision and accuracy

The intra-assay precision and accuracy were estimated by analyzing six replicates containing (±)-TTB at four different QC levels, i.e., 10, 30, 1500 and 4000 ng/mL, which is equivalent to 5, 15, 750 and 2000 ng/mL for each enantiomer. The inter-assay precision was determined by analyzing the four levels QC samples on three different runs. The criteria for acceptability of the data included accuracy within ±15% deviation (S.D.) from the nominal values and a precision of within ±15% relative standard deviation (R.S.D) except for LLOQ, where it should not exceed ±20% of S.D. [13].

2.7.5. Stability experiments

The stability of (±)-TTB and IS in the injection solvent was determined periodically by injecting replicate preparations of processed samples for up to 8 h (in the auto-sampler at 4 °C) after the initial injection. The peak areas of the analyte and IS obtained at initial cycle were used as the reference to determine the stability at subsequent points. Stability of (±)-TTB in the biomatrix during 6 h (bench-top) was determined at ambient temperature (25 ± 2 °C) at two concentrations (30 and 4000 ng/mL equivalent to 15 and 2000 ng/mL of each enantiomer of TTB) in quadruplicates. Freezer stability of (±)-TTB in hamster plasma was assessed by analyzing the LQC and HQC samples stored at −80 ± 10 °C for at least 15 days. The stability of (±)-TTB in hamster plasma following three freeze–thaw cycles was assessed using QC samples spiked with (±)-TTB. The samples were stored at −80 ± 10 °C between freeze/thaw cycles. The samples were thawed by allowing them to stand (unassisted) at room temperature for approximately 2 h. The samples were then returned to the freezer. The samples were processed using the same procedure as described in the sample preparation section. Samples were considered stable if assay values were within the acceptable limits of accuracy (i.e., ±15% S.D.) and precision (i.e., ±15% R.S.D.).

2.8. Pharmacokinetic study

Male Golden Syrian hamsters, ∼8 weeks of age and weighing between 90 and 120 g were used in this study. (−)-TTB was administered orally at a dose of 30 mg/kg in a 0.25% sodium CMC suspension. The hamsters were anaesthetized in ether and blood samples (∼0.25 mL) were collected from the retro-orbital plexus into microfuge tubes (containing 10 μL of saturated EDTA) at 0.5, 1, 2, 3, 5, 8, 10 and 24 h post-dosing. Plasma was harvested by centrifuging the blood using Biofuge (Hereaus, Germany) at 7500 × g for 3 min and stored at −80 ± 10 °C until bioanalysis.

An aliquot of 100 μL of thawed plasma samples were spiked with IS and processed as mentioned in Sample preparation section. Along with study samples, QC samples at low, medium and high concentration were assayed in duplicate and were distributed among unknown samples in the analytical run. The criteria for acceptance of the analytical runs encompassed the following: (i) not more than 33% of the QC samples were greater than ±15% of the nominal concentration (ii) not less than 50% at each QC concentration level must meet the acceptance criteria. Plasma concentration–time data of (−)-TTB was analyzed by non-compartmental method using WinNonlin Version 5.1 (Pharsight Corporation, Mountain View, CA).

3. Results

3.1. Liquid chromatography

The chromatographic conditions, in particular the mobile phase composition and column selection were optimized through several trials to achieve good resolution and symmetric peak shapes for analytes and IS. To begin with the optimization of the chiral resolution of TTB enantiomers, we have used previously developed LC conditions [10] on different polysaccharide chiral stationary phase (CSP) normal columns, viz., Chiralpak AD (Chiral Technologies, 4.6 mm × 250 mm, 10 μm), Chiralpak AD-H (Chiral Technologies, 4.6 mm × 250 mm, 5 μm), Chiralcel OJ (Chiral Technologies, 4.6 mm × 250 mm, 5 μm) and Chiralcel OD (Chiral Technologies, 4.6 mm × 250 mm, 5 μm). Among the selected CSPs the required resolution and symmetric peak shapes were achieved on Chiralpak AD column. The resolution of TTB enantiomers was not good on Chiralcel OD and Chiralcel OJ columns; where as on Chiralpak AD-H, though the resolution was good, the run time was found to be longer. In order to achieve the reduced run time, the mobile phase composition was optimized to *n*-hexane: IPA (80:20, v/v) at a flow rate of 0.7 mL/min; this enabled elution of the analytes and IS within 9 min. The retention times of (+)-TTB, (−)-TTB and IS were approximately 4.67, 5.18 and 8.36 min, respectively.

3.2. Mass spectrometry

In order to optimize APCI conditions for TTB and IS, quadrupole full scans were carried out in negative ion detection mode. During a direct infusion experiment, the mass spectra for TTB (both enantiomers) and IS revealed peaks at m/z 599.2 and 623.2, respectively as deprotonated molecular ions, $[M-H]^-$. The product ion mass spectrum for TTB shows the formation of characteristic product ions at m/z 500, 370, 340, 299 and 227 (Fig. 3). Following detailed optimization of mass spectrometry conditions (provided in Section 2.3) m/z 599.2 precursor ions to the m/z 340.2 was used for the quantitation of TTB. For IS m/z 623.2 precursor ions to the m/z 298.1 was used for quantification purpose.

The proposed fragmentation pattern for TTB is depicted in Fig. 2. The sequential loss of 100 Da (due to ring opening between C4–C5, loss of methoxy group from C4 carbamate and ethoxycarbonyl from 1st position carbamate), 159 Da (due to

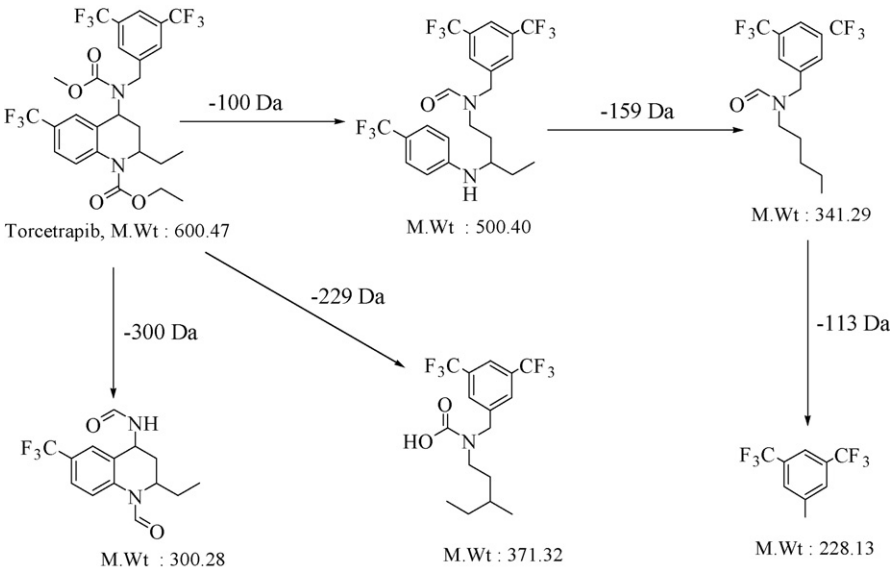

Fig. 2. Proposed fragmentation pattern for torcetrapib (TTB).

loss of trifluoromethyl aniline at C2) and 113 Da (due to cleavage at benzylic position) from *m/z* 599 generated the product ions at *m/z* 500, 340 and 227, respectively. The product ion at *m/z* 370 was generated directly from *m/z* 599 due to loss of 229 Da (due to cleavage between C4–C5 and at C2 position). Similarly, the product ion at *m/z* 299 was generated directly from *m/z* 599 due to loss of 300 Da (loss of methoxy group and bistrifluoromethylbenzyl from C4 carbamate and ethoxy group from 1st position).

3.3. Recovery

A simple liquid–liquid extraction with acetonitrile proved to be robust and provided cleanest samples. The results of the comparison of neat standards versus plasma-extracted standards were estimated for each enantiomer of TTB at 15 and 2000 ng/mL and the absolute mean recovery at LQC and HQC was found to be 68.92 ± 9.46 and 64.36 ± 6.28%, respectively. The absolute recovery of IS at 500 ng/mL was 106.97 ± 12.6%.

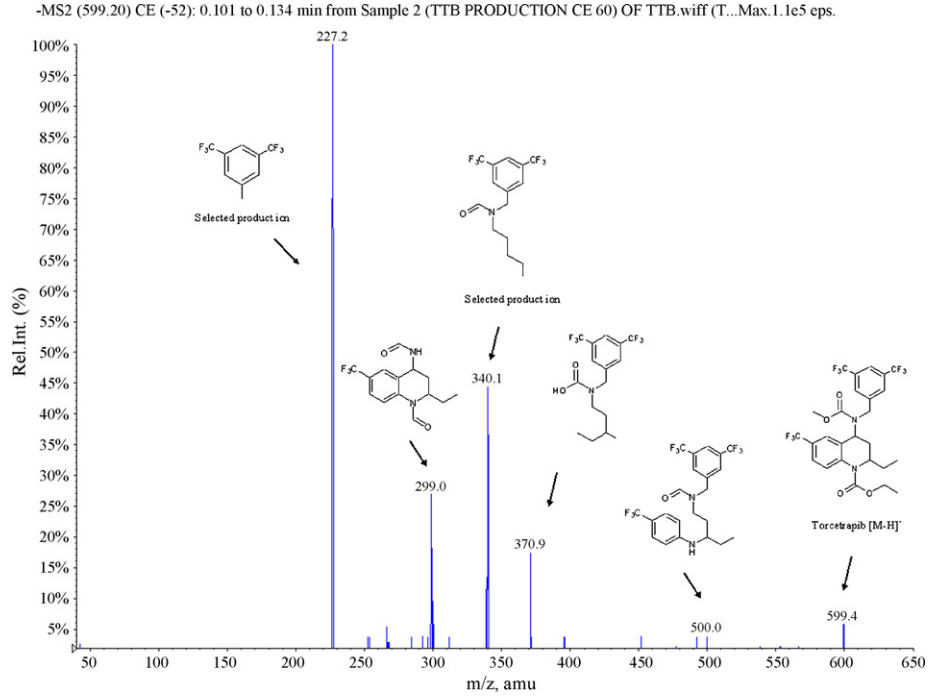

Fig. 3. MS/MS spectra of TTB showing prominent precursor to product ion transitions.

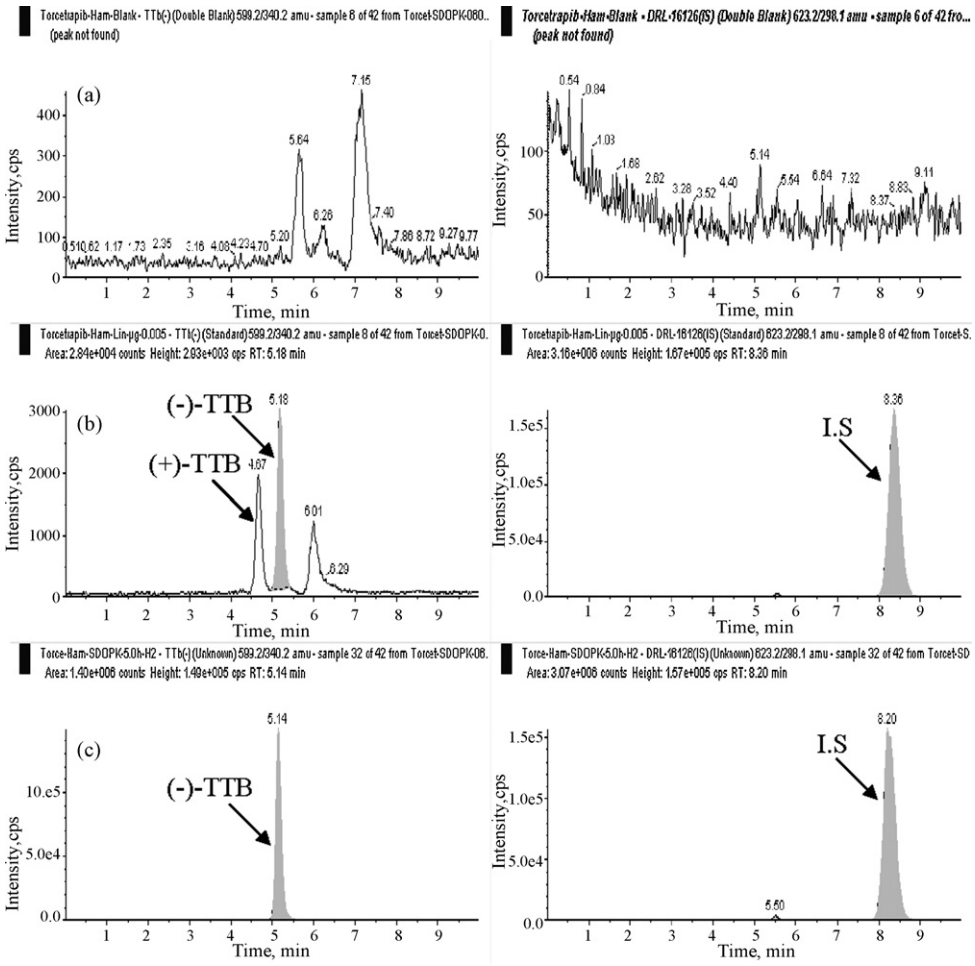

Fig. 4. Typical MRM chromatograms of TTB (left panel) and IS (right panel) in (a) hamster blank plasma (b) hamster plasma spiked with (±)-TTB at LLOQ (5.00 ng/mL) and IS (c) a 5.0 h *in vivo* plasma sample showing (−)-TTB peak obtained following oral dose of (−)-TTB to hamster along with IS.

3.4. Validation procedures

3.4.1. Matrix effect, specificity and selectivity

Average matrix effect values obtained were 3.01 and 10.9 for (+)-TTB and 5.65 and 13.3% for (−)-TTB in hamster plasma at QC low (15 ng/mL) and QC high (2000 ng/mL) concentrations, respectively. No significant peak-area differences were observed. Matrix effect on IS was found to be 6.80% at tested concentration of 500 ng/mL.

Fig. 4 shows a typical overlaid chromatogram for the control hamster plasma (free of analyte and IS), hamster plasma spiked with (±)-TTB at LLOQ and IS and an *in vivo* plasma sample obtained at 5.0 h after oral administration of (−)-TTB. No interfering peaks from endogenous compounds are observed at the retention times of analyte and IS in both the matrices. The retention time of (+)-TTB, (−)-TTB and IS were 4.67, 5.18 and 8.36 min, respectively. The total chromatographic run time was 9.0 min.

3.4.2. Calibration curve

The plasma calibration curve for each enantiomer was constructed using nine calibration standards (viz., 5.00–2500 ng/mL). The calibration standard curve had a reliable reproducibility over the standard concentrations across the calibration range. Calibration curve was prepared by determining the best fit of peak-area ratios (peak-area analyte/peak-area IS) versus concentration, and fitted to the $y = mx + c$ using weighing factor ($1/X^2$). The average regression ($n = 3$) was found to be $\geq$0.995. The lowest concentration with the R.S.D. < 20% was taken as LLOQ and was found to be 5.00 ng/mL. The % accuracy observed for the mean of back-calculated concentrations for three calibration curves for (+)-TTB and (−)-TTB was within 90.9–109 and 91.3–107, respectively; while the % deviation values ranged from −9.97 to 8.26 and −9.49 to 6.32, respectively (Table 1).

3.4.3. Accuracy and precision

Accuracy and precision data for intra- and inter-day plasma samples are presented in Table 2. The assay values on both the occasions (intra- and inter-day) and in hamster plasma was found to be with in the accepted variable limits.

3.4.4. Stability

The predicted concentrations for each enantiomer of TTB at 15 and 2000 ng/mL samples deviated within ±10–15% of the nominal concentrations in a battery of stability tests, viz.,

Table 1
Precision and accuracy data of back-calculated concentrations of calibration samples for (+)-TTB/(−)-TTB in hamster plasma

Nominal concentration (ng/mL)	(+)-TTB			(−)-TTB		
	Mean ± S.D. (n = 3)	Deviation (%)	Accuracy (%)	Mean ± S.D. (n = 3)	Deviation (%)	Accuracy (%)
5	5.45 ± 0.36	8.26	109	5.02 ± 0.41	0.33	100
10	9.98 ± 0.88	−0.17	99.8	10.5 ± 1.08	4.76	105
25	26.4 ± 1.72	5.12	105	24.8 ± 3.04	−0.81	99.2
50	47.2 ± 5.06	−6.01	94.3	45.7 ± 6.81	−9.49	91.3
100	106 ± 7.47	5.21	106	99.5 ± 14.1	−0.50	99.5
250	263 ± 19.8	4.88	105	258 ± 32.5	3.16	103
500	472 ± 46.6	−6.05	94.3	496 ± 64.7	−0.84	99.2
1000	1070 ± 77.8	6.54	107	1068 ± 38.9	6.32	107
2500	2273 ± 70.1	−9.97	90.9	2348 ± 237	−6.46	93.9

Accuracy (%) = (C_{mean}/C_{exp}) × 100; deviation (%) = ($C_{mean} - C_{exp}$)/C_{mean} × 100.

Table 2
Intra- and inter-day precision of determination of (+)-TTB/(−)-TTB in hamster plasma

Nominal concentration (ng/mL)	Run	(+)-TTB			(−)-TTB		
		Mean ± S.D.	R.S.D.	Accuracy (%)	Mean ± S.D.	R.S.D.	Accuracy (%)
Intra-day variation (six replicates at each concentration)							
5	1	4.58 ± 0.55	12.0	91.7	5.17 ± 0.53	10.3	103
	2	5.27 ± 0.20	3.75	105	5.56 ± 0.52	9.31	111
	3	5.24 ± 0.62	11.8	105	5.35 ± 0.57	10.7	107
15	1	14.1 ± 1.73	12.3	93.8	14.6 ± 1.40	9.60	97.2
	2	14.3 ± 1.23	8.62	95.3	14.0 ± 1.74	12.5	93.4
	3	15.8 ± 1.29	8.17	105	15.2 ± 1.40	9.17	102
750	1	685 ± 86.4	12.6	91.3	664 ± 36.3	5.46	88.6
	2	711 ± 55.9	7.86	94.8	762 ± 39.3	5.16	102
	3	771 ± 79.5	10.3	103	749 ± 73.1	9.76	100
2000	1	2066 ± 244	11.8	103	2066 ± 244	11.8	103
	2	1862 ± 228	12.2	93.1	1803 ± 93.6	5.19	90.2
	3	1961 ± 252	12.9	98.0	2053 ± 230	11.2	103
Inter-day variation (Eighteen replicates at each concentration)							
5		5.03 ± 0.56	11.2	101	5.38 ± 0.52	9.79	107
15		14.7 ± 1.55	10.5	98.0	14.6 ± 1.52	10.4	97.4
750		722 ± 79.6	11.0	96.3	725 ± 66.3	9.15	96.7
2000		1963 ± 243	12.4	98.1	1974 ± 226	11.5	98.7

R.S.D: relative standard deviation (S.D. × 100/mean).

Table 3
Stability data of (+)-TTB/(−)-TTB quality controls in hamster plasma

Nominal concentration (ng/mL)	Stability	(+)-TTB			(−)-TTB		
		Mean ± S.D.[a] n = 6 (ng/mL)	Accuracy (%)[b]	Precision (% CV)	Mean ± S.D.[a] n = 6 (ng/mL)	Accuracy (%)[b]	Precision (% CV)
15	0 h (for all)	15.8 ± 1.29	105	8.17	15.2 ± 1.40	102	9.17
	3rd freeze–thaw	15.0 ± 1.07	95.3	7.12	14.8 ± 1.20	97.4	8.09
	6 h (bench-top)	14.2 ± 1.46	89.9	10.3	14.0 ± 1.14	92.2	8.16
	8 h (in-injector)	15.4 ± 0.89	97.9	5.78	14.8 ± 1.72	97.1	11.6
	15 day at −80 °C	14.9 ± 1.89	94.6	12.7	14.7 ± 1.68	96.7	11.4
2000	0 h (for all)	1961 ± 252	98.0	12.9	2053 ± 229	103	11.2
	3rd freeze–thaw	1923 ± 251	98.1	13.1	1995 ± 213	97.2	10.7
	6 h (bench-top)	1788 ± 33.6	91.2	1.88	1995 ± 101	97.2	5.05
	8 h (in-injector)	1887 ± 109	96.2	5.76	2193 ± 179	107	8.16
	15 day at −80 °C	1913 ± 231	97.6	12.1	1814 ± 169	88.4	9.34

[a] Back-calculated plasma concentrations.
[b] (Mean assayed concentration/mean assayed concentration at 0 h) × 100.

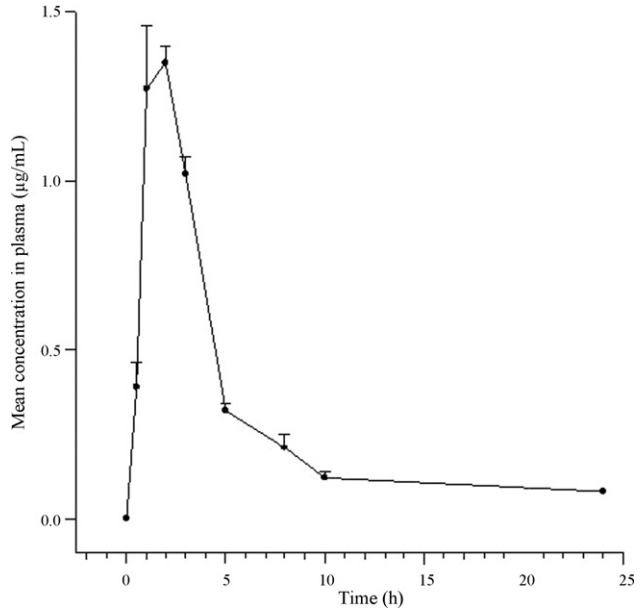

Fig. 5. Mean plasma concentration–time profile of (−)-TTB in hamster plasma following oral dosing.

in-injector (8 h), bench-top (6 h), repeated three freeze/thaw cycles and at −80 ± 10 °C for at least for 15 days (Table 3). The results were found to be within the assay variability limits during the entire process.

3.5. Pharmacokinetic study

The sensitivity and specificity of the assay were found to be sufficient for accurately characterizing the pharmacokinetics of (−)-TTB in hamsters following oral dosing. Profiles of the mean plasma concentration versus time were shown in Fig. 5. Maximum concentration in plasma (C_{max} 1.38 ± 0.05 μg/mL) was achieved at 1.33 ± 0.58 h (t_{max}). The half-life ($t_{1/2}$) of (−)-TTB was 11.06 ± 1.40 h, while the $AUC_{(0-\infty)}$ was 8.11 ± 0.21 μg h/mL.

4. Discussion and conclusions

To the best of our knowledge, no published LC/APCI-MS/MS methods are available for the determination of (±)-TTB in biological matrix. Validated methods are essential for the determination of plasma concentrations in pre-clinical species for pharmacokinetics, toxicokinetic studies and in *in vitro* plasma protein binding studies. The developed method utilizes small volume of plasma (100 μL) and it involves liquid/liquid extraction of plasma with acetonitrile (i.e., protein precipitation) without post-column reagent addition or chemical derivatization step. The applicability of this method in pre-clinical pharmacokinetic studies has been demonstrated in hamsters.

In conclusion, we have developed and validated a highly sensitive, specific, reproducible and high-throughput enantioselective LC/APCI-MS/MS assay to quantify (±)-TTB using structurally close IS from small volume of hamster plasma for the first time. The (+)- and (−)-enantiomers of TTB and IS were baseline separated with adequate specificity and selectivity. From the results of all the validation parameters and applicability of the assay, we can conclude that the present method can be useful for pre-clinical pharmacokinetic studies.

Acknowledgement

This work is part of one of the authors (R.K.T.) doctoral thesis with Jawaharlal Nehru Technological University, Kukatpally, Hyderabad, India.

References

[1] P.J. Barter, K.A. Rye, Curr. Opin. Lipidol. 12 (2001) 377.
[2] R.W. Clark, T.A. Sutfin, R.B. Ruggeri, A.T. Willauer, E.D. Sugarman, G. Magnus-Aryitey, P.G. Cosgrove, T.M. Sand, R.T. Wester, J.A. Williams, M.E. Perlman, M.J. Bamberger, Arterioscler. Thromb. Vasc. Biol. 24 (2004) 490.
[3] G.B. Jensen, J. Hampton, Br. Med. J. 334 (2007) 326.
[4] K. Williams, E. Lee, Drugs 30 (1985) 333.
[5] K.D. Tripathi, Ind. J. Pharmacol. 25 (1993) 73.
[6] N.R. Srinivas, Ind. J. Pharm. Sci. 63 (2001) 265.
[7] A.J. Hutt, M.R. Hadley, S.C. Tan, Eur. J. Drug Metab. Pharmacokinet. 3 (1994) 241.
[8] J. Haginaka, J. Pharm. Biomed. Anal. 27 (2002) 357.
[9] S.D. Lee, K.M. Wasan, A. Calcagni, M. Avery, F. McCush, C. Chen, Pharm. Res. 23 (2006) 1025.
[10] R.K. Trivedi, P.K. Dubey, R. Mullangi, N.R. Srinivas, J. Chromatogr. B 857 (2007) 224.
[11] M. Guino, P.H. Phua, J.C. Caille, K.K. Hii, J. Org. Chem. 72 (2007) 6290.
[12] R. Dams, M.A. Huestis, W.E. Lambert, C.M. Murphy, J. Am. Soc. Mass Spectrom. 14 (2003) 1290.
[13] 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 Center for Veterinary Medicine (CV), May 2001. http://www/fda.gov/cder/guidance/index.htm.
[14] Ph. Hubert, P. Chiap, J. Crommen, B. Boulanger, E. Chapuzet, N. Mercier, S. Bervoas-Martin, P. Chevalier, D. Grandjean, P. Lagorce, M. Lallier, M.C. Laparra, M. Laurentie, J.C. Nivet, Anal. Chim. Acta 391 (1999) 135.