

Development and validation of an enantioselective HPLC–UV method using Chiralpak AD-H to quantify (+)- and (−)-torcetrapib enantiomers in hamster plasma—application to a pharmacokinetic study

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Abstract

A chiral selective, accurate and reproducible high-performance liquid chromatographic (HPLC) method was developed and validated for direct separation of individual enantiomers of torcetrapib (TTB) [(+)-TTB and (−)-TTB]. TTB enantiomers and IS were extracted from a small aliquot of plasma (100 μ L) by simple liquid–liquid extraction using acetonitrile as extraction solvent. The enantiomers were resolved on Chiralpak AD-H[®] (250 mm \times 4.6 mm, 5 μ m) with the mobile phase consisting of *n*-hexane:isopropyl alcohol (IPA) in the ratio of 95:5 (v/v). The eluate was monitored using an UV detector set at 254 nm. Baseline separation of the TTB enantiomers and the internal standard (IS, DRL-17859), free from endogenous interferences was achieved. The resolution factor between the enantiomers was optimized and found to be not less than five. During method development, the IPA content in the mobile phase was optimized for separation of peaks of interest. Additionally, both flow rate and column temperature were optimized for an improved baseline separation of the enantiomers. Ratio of peak area of each enantiomer to IS was used for quantification of plasma samples. Nominal retention times of (+)-TTB, (−)-TTB and IS were 9.4, 13.8 and 17.5 min, respectively. The standard curves for TTB enantiomers were linear ($r^2 > 0.999$) in the concentration range 0.1–10 μ g/mL for each enantiomer. Absolute recovery, when compared to neat standards, was 88.7–90.0% for TTB enantiomers and 100% for IS from the hamster plasma. The lower limit of quantification (LLOQ) for each enantiomer of TTB was 0.1 μ g/mL. The inter-day precisions were in the range of 4.57–6.32 and 5.66–11.0% for (+)-TTB and (−)-TTB, respectively. The intra-day precisions were in the range of 1.60–7.36 and 2.76–13.6% for (+)-TTB and (−)-TTB, respectively. Accuracy in the measurement of quality control (QC) samples was in the range of 95.6–109% and 92.7–108% for (+)-TTB and (−)-TTB, respectively. Both enantiomers were stable in a series of stability studies, viz. bench-top (up to 12 h), auto-sampler (up to 24 h) and freeze/thaw cycles ($n = 3$). Stability of TTB enantiomers was established in hamster plasma for 15 days at −80 °C. The application of the assay to a pharmacokinetic study of (−)-TTB in hamsters is described.

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1. Introduction

Despite on-going advancement in understanding and treatment, cardiovascular diseases continue to remain the leading cause of morbidity and mortality. Although significant reductions in cardiovascular risk can be achieved by effectively lowering low-density lipoprotein cholesterol (LDL-C), treated

patients remain at substantial risk from recurring cardiovascular events. Epidemiological studies have established that higher levels of high-density lipoprotein cholesterol (HDL-C) are strongly associated with reduced cardiovascular risk, and therefore raising levels of HDL-C may be beneficial in addition to the traditionally well-accepted modality of lowering LDL-C [1]. In this regard the activity of cholesteryl ester transfer protein (CETP) appears to be inversely correlated with HDL-C levels [2]. Therefore, CETP has been projected as an attractive target for intervention to raise levels of HDL-C and potentially reduce residual cardiovascular risk [3,4].

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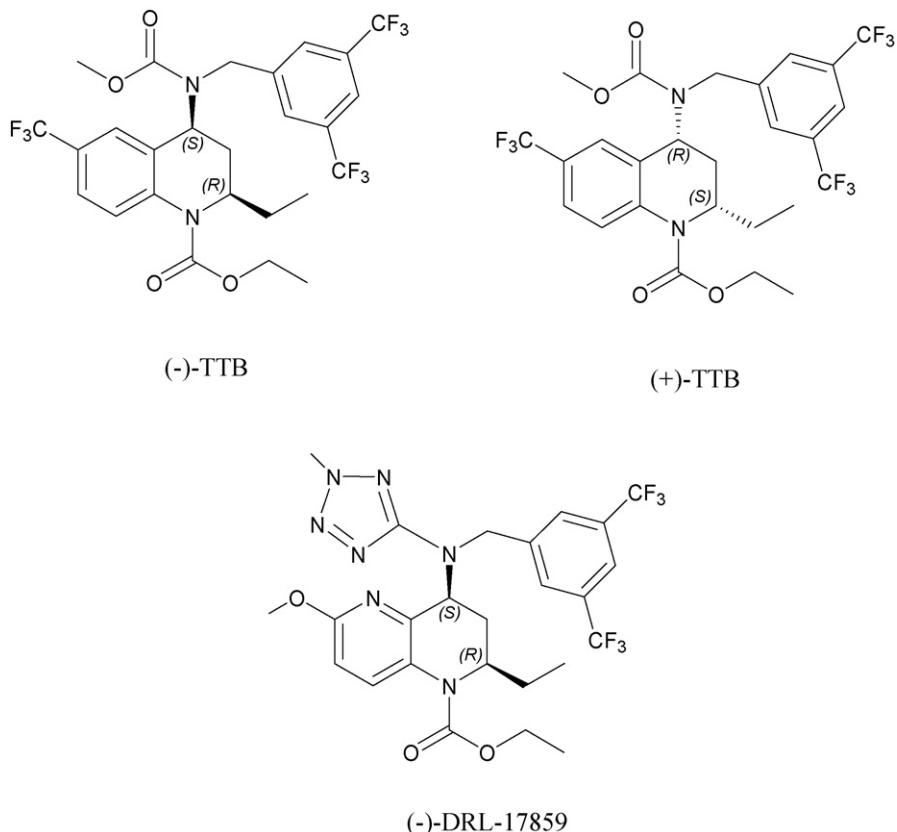


Fig. 1. Structural representation of (-)-TTB, (+)-TTB and (-)-DRL-17859.

CETP is a glycoprotein secreted mainly from the liver and circulated in plasma, bound mainly to high-density lipoprotein (HDL). CETP is endogenously expressed in hamsters, rabbits and humans [5]. Torcetrapib (TTB; CP-529,414; Fig. 1), chemically $(-)$ -(2*R*,4*S*)-4-[(3,5-bis-trifluoromethyl-benzyl)-methoxycarbonylamino]-2-ethyl-6-tri-fluoromethyl-3,4-dihydro-2*H*-quinoline-1-carboxylic acid ethyl ester is a CETP inhibitor. In clinical studies, TTB lowered CETP activity, decreased apo-B (apolipoprotein-B) and LDL-C, elevated HDL-C and apo-E with no effect on triglycerides [4]. The development of TTB was recently suspended due to higher risk of cardiovascular event in patients who received TTB along with standard of care [6].

Lee et al. have described the distribution of (–)-TTB in human plasma and different lipoprotein components of human plasma by GC–MS/MS using a structural analog of (–)-TTB (CP-456,643) as an IS [7]. In this method Lee et al. did not furnish the details of the bioanalytical method. Hitherto there is no bioanalytical method reported for the estimation of TTB enantiomers on a chiral column. In this manuscript, we are presenting the HPLC method development and validation parameters for TTB enantiomers on a Chiralpak AD-H® column in hamster plasma and application of this method to derive the pharmacokinetic parameters for (–)-TTB. In order to optimize the enantiomeric separation of TTB enantiomers, different chromatographic conditions, viz. effect of mobile-phase composition, temperature and flow rate, were also studied and are described in this paper.

2. Experimental

2.1. Chemicals and reagents

(\pm)-TTB, (-)-TTB and (-)-DRL-17859 (IS, Fig. 1) were synthesized by Discovery Chemistry Group, Dr. Reddy's Laboratories Ltd. (DRL), Hyderabad, India, using the synthetic process reported by Damon et al. [8], and were characterized using chromatographic (HPLC, LC-MS/MS) and spectral techniques (IR, UV, Mass, ^1H and ^{13}C -NMR) by the Analytical Research Group, Discovery Research, DRL, Hyderabad. Purity was found to be more than 98.7% for all three compounds. HPLC-grade *n*-hexane was purchased from S.D. Fine Chemicals, Mumbai, India. HPLC-grade acetonitrile and isopropanol (IPA) were purchased from 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

The HPLC system consisted of a Shimadzu Prominence (Koyoto, Japan) system equipped with degasser (DGU-20A5), pump (LC-20AT), column oven (CTO-20A), auto-injector with sample cooler (SIL-20AC) and ultraviolet detector (SPD-20A). A Chiralpak AD-H[®] (250 mm × 4.6 mm, 5 µm) column (Daiel Chemical Industries Ltd., Japan) coupled with guard column

(Chiralpak AD-H[®], 10 mm × 4.0 mm, 5 µm; Daicel Chemical Industries Ltd., Japan) maintained at 25 ± 1 °C in a column oven was used for the analysis. Separation characteristics such as capacity factor (*k'*), selectivity or separation factor (α) and resolution factor (*R*) were determined by using standard formulas [9]. The isocratic mobile phase system consisting of *n*-hexane:IPA in the ratio of 95:5 (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 run at a flow rate of 0.4 mL/min through the column to elute the analytes. The eluate was monitored with an UV detector set at 254 nm and the data were acquired and processed with Shimadzu VP software (Ver: 6.14SPI).

2.3. 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 and IS were stored at –10 °C, which were found to be stable for 1 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 also stored approximately at –10 °C. The working stock solution found to be stable for 15 days (data not shown). Appropriate dilutions of (±)-TTB was made in IPA to produce working stock solutions of 200, 100, 50, 20, 10, 5 and 2 µg/mL; similarly dilutions of (–)-TTB was made in IPA to produce working stock solutions of 100, 50, 25, 10, 5, 2.5 and 1 µg/mL. Working stocks were used to prepare plasma calibration standards. A working IS solution (10 µg/mL) was prepared in IPA. Calibration samples were prepared by spiking 90 µL of control pooled hamster plasma with the appropriate amount 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 (0.2, 0.6, 8.0 and 16.0 µg/mL) and 100 µL aliquots were distributed into different tubes. All the samples were stored at –80 °C.

2.4. Calibration curves

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 were 0.1, 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 µg/mL. The results were fitted to linear regression analysis with the use of 1/X weighting factor.

2.5. Extraction and recovery

Two sets of standards containing the (±)-TTB at two different concentrations (0.6 and 16.0 µg/mL) were prepared. One

set was prepared in hamster plasma and the other set was prepared in IPA. The recovery was determined by comparing peak areas of spiked plasma extracts with those of unextracted neat standards prepared in IPA. The recovery value was calculated at two different concentrations of (±)-TTB. The recovery of the IS was determined at a single concentration of 50 µg/mL.

2.6. Precision and accuracy

The intra-assay precision and accuracy were estimated by analyzing six replicates containing (±)-TTB at four different QC levels, i.e. 0.2, 0.6, 8.0 and 16.0 µg/mL, which is equivalent to 0.1, 0.3, 4.0 and 8.0 µg/mL for each enantiomer. The inter-assay precision was determined by analyzing the four levels QC samples on four different runs. The criteria for acceptability of the data included accuracy within (±)15% standard deviation (S.D.) from the nominal values and a precision of within 15% relative standard deviation except for LLOQ, where it should not exceed 20% of CV [10,11].

2.7. Sample preparation

An aliquot of 100 µL plasma sample was pipetted out into a 15-mL centrifuge tube; to this 10 µL of 10 µg/mL of IS 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 2 min, followed by centrifugation for 10 min at 1760 × g on a tabletop centrifuge (Remi Instruments, Mumbai, India). The organic layer (1.8 mL) was separated and evaporated to dryness at 40 °C using a gentle stream of nitrogen (Zymark[®] Turbovap[®], Kopkinton, MA, USA). The residue was reconstituted in 250 µL of the mobile phase and 50 µL aliquot was injected onto HPLC column.

2.8. 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 24 h (in the auto sampler at 5 °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 each enantiomers of TTB in the biomatrix during 12 h (bench-top) was determined at ambient temperature (25 ± 2 °C) at two concentrations (0.3 and 8.0 µg/mL) in quadrupletes. Freezer stability of (±)-TTB in hamster plasma was assessed by analyzing the QC samples stored at –80 °C for at least 15 days. The stability of (±)-TTB in hamster plasma following repeated freeze/thaw cycles (up to three cycles) was assessed using QC samples spiked with (±)-TTB. The samples were stored at –80 °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 stability of (±)-TTB was assessed after three freeze/thaw cycles. The samples were processed using the same procedure as described in the sample preparation section. Sam-

ples were considered stable if assay values were within the acceptable limits of accuracy (i.e., $\pm 15\%$ S.D.) and precision (i.e., $\pm 15\%$ R.S.D.) except for LLOQ, where it should not exceed 20%.

2.9. Study in hamsters

Male Golden Syrian hamsters, ~ 8 weeks of age and weighing between 90 and 120 g, were used in this study. (–)-TTB was administered *intraperitoneally* (i.p.) at a dose of 15 mg/kg in a customized solution formulation. The hamsters were anaesthetized in ether and blood samples (~ 0.25 mL) were collected from the retro-orbital plexus into microfuge tubes (containing 10 μ L of saturated EDTA) at 0.5, 1.5, 3, 5, 10, 24 and 32 h post-dosing. Plasma was harvested by centrifuging in a micro centrifuge (Biofuge, Heraeus, Germany) at 7500 $\times g$ at 4 °C for 3 min and stored at –80 °C until bioanalysis. Plasma (100 μ L) samples were spiked with IS and processed as described above. The concentrations were back-calculated using a calibration curve with (–)-TTB.

2.10. Pharmacokinetic analysis

Pharmacokinetic parameters were calculated by employing a non-compartmental analysis [12]. The peak plasma concentration (C_{\max}) and the corresponding time (T_{\max}) were directly obtained from the raw data. The area under the plasma concentration versus time curve up to the last quantifiable time point, $AUC_{(0-t)}$ was obtained by the linear and log-linear trapezoidal summation. The $AUC_{(0-t)}$ extrapolated to infinity (i.e., $AUC_{(0-\infty)}$) by adding the quotient of $C_{\text{last}}/K_{\text{el}}$, where C_{last} represents the last measurable time concentration and K_{el} represents the apparent terminal rate constant. K_{el} was calculated by the linear regression of the log-transformed concentrations of the drug in the terminal phase. The half-life ($t_{1/2}$) of the terminal elimination phase was obtained using the relationship $t_{1/2} = 0.693/K_{\text{el}}$.

2.11. Statistical analysis of data

Statistical analysis (ANOVA) was performed using Sigma Stat (Scientific software, Jandel Scientific, version 2.0, USA) and the significance level adopted for all statistical comparisons was $p < 0.05$.

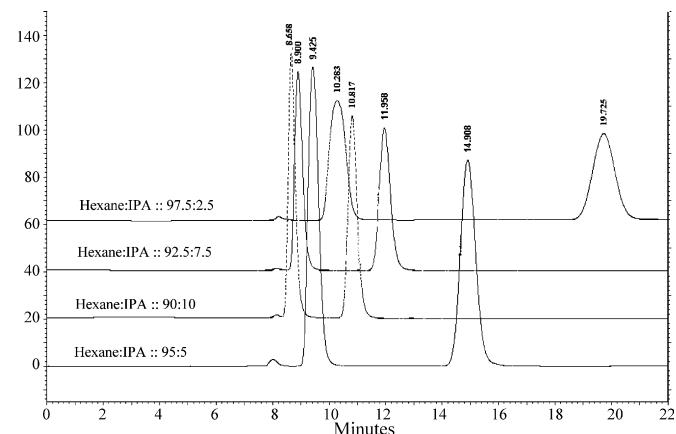


Fig. 2. Effect of IPA content upon the separation of TTB enantiomers.

3. Results and Discussion

3.1. Effect of assay conditions on the separation of (±)-TTB enantiomers

3.1.1. Effect of mobile-phase composition

Being used as the organic modifier of the mobile phase, the percentage of IPA had strong effect on the separation of the enantiomeric peaks corresponding to TTB. Fig. 2 shows the chromatograms of the (±)-TTB enantiomers employing different IPA content. The increase in percentage of IPA content in the mobile phase decreased the resolution between the two enantiomers (Table 1 and Fig. 2). In order to obtain sharp peaks without compromising on the resolution and to minimize the chromatographic retention (i.e., speed-up the run time), hexane:IPA in the ratio of 95:5 (v/v) was chosen as the mobile phase.

3.1.2. Column temperature

The examples cited in the literature demonstrate that a decrease in column temperature caused an increase in enantioselectivity [13]. In the present work, various temperature values were selected to evaluate the effects on separation. The effect of raising the column temperature in 5 °C increments was investigated on the (±)-TTB enantiomers separation. In the present context, the separation factor (α) remained constant at 1.55–1.59, resolution (R) was also found to be constant (6.44–6.28) across the tested temperature range (20–35 °C) (Table 1), indicating that temperature has no pronounced effect

Table 1
Effect of flow rate, temperature and mobile-phase composition on the separation of TTB enantiomers

Parameter	n-Hexane:IPA				Temperature (°C)				Flow rate (mL/min)				
	90:10	92.5:7.5	95:5	97.5:2.5	20	25	30	35	0.3	0.4	0.5	0.6	0.7
R	3.99	4.90	6.47	7.59	6.44	6.47	6.49	6.28	6.47	6.31	5.89	5.66	6.86
k'_1	86	88	95	102	95	94	93	93	94	74	604	513	125
k'_2	107	119	150	196	151	149	147	144	149	118	867	737	199
α	1.24	1.35	1.58	1.92	1.59	1.59	1.58	1.55	1.59	1.59	1.44	1.44	1.59

R , resolution factor; k'_1 , capacity factor for (+)-TTB; k'_2 , capacity factor for (–)-TTB; α , separation factor.

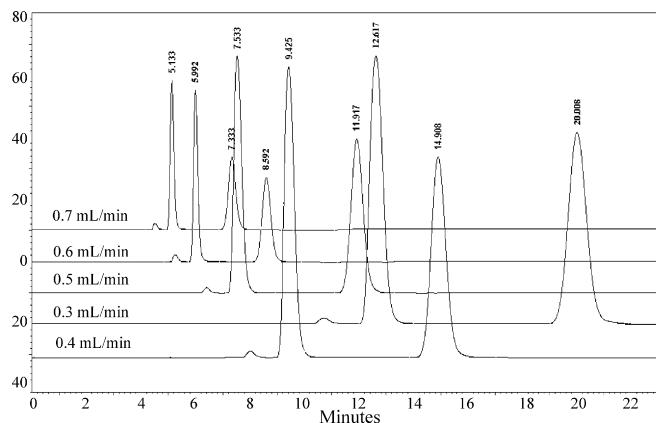


Fig. 3. Effect of flow rate upon the separation of TTB enantiomers.

on R - and α -values for the enantiomeric separation of (\pm) -TTB. During the method validation optimum column temperature for the separation of (\pm) -TTB was set at 25 °C.

3.1.3. Effect of flow rate

The influence of flow rate upon resolution was examined under the optimum mobile phase and temperature conditions, and this rate was increased from 0.3 to 0.7 mL/min (Fig. 3). It was found that the flow rate has little effect on α but significantly effects resolution (Table 1). The optimum flow rate was established at 0.4 mL/min. At this flow rate, the run time for the separation of all analytes of interest was within 23 min.

3.2. Specificity and chromatography

Under the given chromatographic conditions, specificity of this method was indicated by the absence of any endogenous interference at retention times of peaks of interest. This was

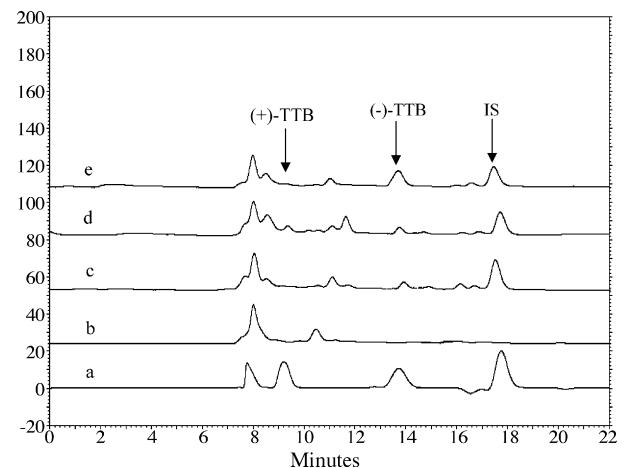


Fig. 4. HPLC chromatograms of a 50 μ L injection of (a), composite stock solution mixture consisting of (\pm) -TTB and IS (b), hamster blank plasma (c), hamster blank plasma spiked with $(-)$ -TTB at LLOQ (0.1 μ g/mL) and IS (d), hamster blank plasma spiked with (\pm) -TTB and IS (e), a 0.5 h *in vivo* plasma sample obtained from hamster dosed with $(-)$ -TTB at 15 mg/kg i.p.

confirmed by chromatograms of blank hamster plasma and plasma spiked with each enantiomers of TTB and IS. Additionally, the experiment for checking the effect of interference at the retention time(s) of individual enantiomers and an IS were performed. When single analyte was injected at the highest concentration in the chromatographic system, the chromatographic traces corresponding to the retention times of the other enantiomer and/or IS showed no interference (data not shown). Both enantiomers of TTB, i.e. $(+)$ -TTB, $(-)$ -TTB, and IS were well separated with retention time of 9.4, 13.8 and 17.5 min, respectively. Earlier, Damon et al. have also reported the elution of $(-)$ -TTB is preceded by $(+)$ -TTB [8]. Based on the retention time of pure $(-)$ -TTB at 13.8 min and the resolution of (\pm) -TTB, the peak eluted at 9.4 min was confirmed as

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

Nominal concentration (μ g/mL)	Run	$(+)$ -TTB			$(-)$ -TTB		
		Mean \pm S.D.	R.S.D.	Accuracy (%)	Mean \pm S.D.	R.S.D.	Accuracy (%)
Intra-day variation (six replicates at each concentration)							
0.1	1	0.10 \pm 0.00	4.48	101	0.11 \pm 0.01	9.89	108
	2	0.10 \pm 0.00	2.61	100	0.09 \pm 0.00	4.81	92.7
	3	0.11 \pm 0.00	6.59	109	0.10 \pm 0.01	11.4	105
0.3	1	0.30 \pm 0.01	2.72	100	0.29 \pm 0.04	13.6	96.6
	2	0.31 \pm 0.02	5.19	100	0.29 \pm 0.02	6.64	97.3
	3	0.31 \pm 0.02	4.83	105	0.30 \pm 0.02	6.51	100
4.0	1	4.07 \pm 0.10	2.53	102	3.92 \pm 0.11	2.88	98.0
	2	4.08 \pm 0.30	7.36	102	4.05 \pm 0.26	6.45	101
	3	4.29 \pm 0.17	4.00	107	4.21 \pm 0.22	5.19	105
8.0	1	7.65 \pm 0.12	1.60	95.6	7.68 \pm 0.64	8.34	96.0
	2	8.57 \pm 0.34	3.98	107	8.45 \pm 0.23	2.76	106
	3	8.51 \pm 0.39	4.56	106	8.49 \pm 0.47	5.57	106
Inter-day variation (18 replicates at each concentration)							
0.1		0.10 \pm 0.01	6.09	103	0.10 \pm 0.01	11.0	102
		0.31 \pm 0.01	4.57	102	0.29 \pm 0.03	9.03	98.1
		4.15 \pm 0.22	5.35	104	4.06 \pm 0.23	5.66	102
		8.24 \pm 0.52	6.32	103	8.21 \pm 0.59	7.20	103

R.S.D., relative standard deviation [(S.D. \times 100)/mean].

Table 3

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

Nominal concentration ($\mu\text{g/mL}$)	Stability	(+)-TTB			(-)-TTB		
		Mean \pm S.D. ^a ($n=4$, $\mu\text{g/mL}$)	Accuracy (%) ^b	Precision (% CV)	Mean \pm S.D. ^a ($n=4$, $\mu\text{g/mL}$)	Accuracy (%) ^b	Precision (% CV)
0.30	0 h (for all)	0.30 \pm 0.01	100	2.72	0.29 \pm 0.04	100	1.36
	3rd freeze/thaw	0.28 \pm 0.02	92.2	5.96	0.31 \pm 0.02	107	5.80
	12 h (bench-top)	0.27 \pm 0.01	91.0	5.30	0.27 \pm 0.02	92.3	6.93
	24 h (in-injector)	0.31 \pm 0.02	102	5.20	0.28 \pm 0.02	97.0	5.38
	15 day at -80°C	0.29 \pm 0.01	97.9	4.83	0.29 \pm 0.02	98.9	5.81
8.00	0 h (for all)	7.65 \pm 0.12	100	1.60	7.68 \pm 0.64	100	8.34
	3rd freeze/thaw	7.66 \pm 0.23	100	3.05	8.76 \pm 0.20	114	2.24
	12 h (bench-top)	7.43 \pm 0.63	97.1	8.49	7.42 \pm 0.63	96.6	8.55
	24 h (in-injector)	8.58 \pm 0.12	112	3.05	8.25 \pm 0.29	107	3.54
	15 day at -80°C	7.12 \pm 0.60	93.1	8.42	7.14 \pm 0.63	92.9	8.81

^a Back-calculated plasma concentrations.^b (Mean assayed concentration/mean assayed concentration at 0 h) \times 100.

(+)-TTB. System suitability parameters for the method were as follows: theoretical plates for (+)-TTB, (-)-TTB and IS were 7516, 7322 and 9882, respectively. Asymmetry factor for (+)-TTB, (-)-TTB was <1.24 and resolution was >6.00 between (+)-TTB and (-)-TTB; between (-)-TTB and IS was >4.00 . Fig. 4 shows a typical overlaid chromatogram for the composite stock solution comprising TTB enantiomers along with IS, control hamster plasma (free of analyte and IS), hamster plasma spiked with (-)-TTB at the 0.1 $\mu\text{g/mL}$, hamster plasma spiked with (\pm)-TTB at the 0.2 $\mu\text{g/mL}$ and an *in vivo* plasma sample obtained after i.p. administration of (-)-TTB at 15 mg/kg.

3.3. Calibration curve and reproducibility

Peak area ratio of each enantiomer of TTB to that of the IS was measured and plotted versus nominal concentration of the enantiomer to generate a calibration curve. The results were fitted to linear regression analysis with the use of $1/X$ weighting factor. A representative calibration graph of peak-area ratio (individual enantiomer to IS) versus each enantiomer concentration in the range of 0.1–10 $\mu\text{g/mL}$ was found to be linear. The average regression ($n=4$) was 0.999 for both enantiomers of TTB. The standard curve had a reliable reproducibility over the standard concentrations of the analyte across the calibration range. The lowest concentration with the R.S.D. $<20\%$ was taken as LLOQ [5] and was found to be 0.1 $\mu\text{g/mL}$ for both TTB enantiomers.

3.4. Precision and accuracy

Accuracy and precision data for intra- and inter-day plasma test samples are presented in Table 2.

3.5. Extraction recovery

The results of the comparison of neat standards versus plasma-extracted standards were estimated at 0.3 and 8.0 $\mu\text{g/mL}$

concentrations for each enantiomer of TTB. The absolute recoveries ranged from 88.7 to 90.0% for TTB enantiomers across the concentrations. The absolute recovery of IS at 50 $\mu\text{g/mL}$ was about 100%.

3.6. Stability

The predicted concentrations for each enantiomer of TTB at 0.3 and 8.0 $\mu\text{g/mL}$ samples deviated within $\pm 15\%$ of the nominal concentrations in a battery of stability tests, viz. in-injector (24 h), bench-top (12 h), repeated three freeze/thaw cycles and at -80°C for at least for 15 days (Table 3). The results were found to be within the assay variability limits during the entire process.

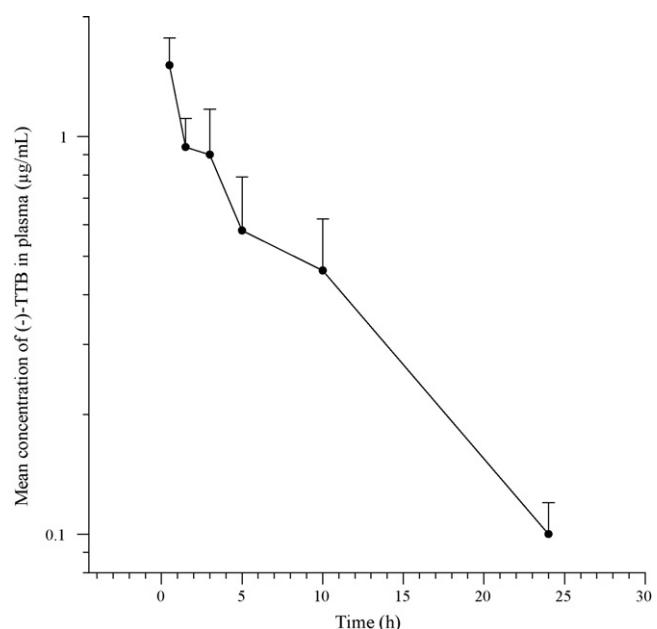


Fig. 5. Plasma concentration vs. time profiles of (-)-TTB after single-dose i.p. administration of 15 mg/kg in male hamsters. The data points are means and standard deviation bars of four observations.

3.7. Pharmacokinetics in hamsters

After a single i.p. administration of 15 mg/kg (–)-TTB to male hamsters, the plasma concentrations of (–)-TTB were determined by the described method. None of the chromatograms has shown the presence of (+)-TTB. The mean plasma concentration versus time profiles for (–)-TTB is depicted in Fig. 5. Inspection of Fig. 5 revealed that the newly developed analytical method had the required sensitivity to characterize the absorption, distribution and elimination phases of (–)-TTB following a single i.p. dose in hamsters. The pharmacokinetic parameters of (–)-TTB were calculated using a non-compartmental analysis. Maximum concentration in plasma (C_{\max} $1.52 \pm 0.24 \mu\text{g/mL}$) was achieved at $0.67 \pm 0.29 \text{ h}$ (T_{\max}). The half-life ($t_{1/2}$) of (–)-TTB was $8.21 \pm 2.38 \text{ h}$, while the $\text{AUC}_{(0-\infty)}$ was $12.23 \pm 3.85 \mu\text{g h/mL}$.

4. Conclusions

The described chiral HPLC method using Chiralpak AD-H[®] column is suitable to separate TTB enantiomers. Following the optimization of mobile-phase composition, temperature and flow rate, the method was validated for the determination of (+)-TTB and (–)-TTB in hamster plasma. The (+)- and (–)- enantiomers of TTB and IS were baseline separated with adequate specificity and selectivity. The present method is simple and applicable to routine analysis because it involved single step liquid extraction without the requirement of a chemical derivatization step. The application of the assay has been shown in a pre-clinical pharmacokinetic study involving hamsters.

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