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Short communication

Enantiomeric determination of tramadol and *O*-desmethyltramadol in human plasma by fast liquid chromatographic technique coupled with mass spectrometric detection

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ABSTRACT

A rapid and sensitive method using liquid chromatography–tandem mass spectrometry (LC–MS/MS) for enantiomeric determination of tramadol and its primary phase metabolite *O*-desmethyltramadol in human plasma has been developed. Tramadol hydrochloride – ^{13}C, d_3, was used as an isotopic labeled internal standard for quantification. The method involves a simple solid phase extraction. The analytes and internal standard were separated on Lux Cellulose-2 packed with cellulose tris(3-chloro-4-methylphenylcarbamate) using isocratic elution with hexane/isopropanol/diethylamine (90:10:0.1, v/v/v) at a flow rate of 1.3 mL/min. The APCI positive ionization mass spectrometry was used with multiple reaction monitoring of the transitions at m/z 264.2 → 58.2 for tramadol, m/z 250.1 → 58.2 for *O*-desmethyltramadol and m/z 268.2 → 58.2 for internal standard. Linearity was achieved between 1–800 ng/mL and 1–400 ng/mL ($R^2 \geq 0.999$) for each enantiomer of tramadol and *O*-desmethyltramadol, respectively. Intra-day accuracies ranged among 98.2–102.8%, 97.1–109.1% and 97.4–102.9% at the lower, intermediate, and high concentration for all analytes, respectively. Inter-day accuracies ranged among 95.5–104.1%, 99.2–104.7%, and 94.2–105.6% at the lower, intermediate, and high concentration for all analytes, respectively. This assay was successfully used to determine the concentration of enantiomers of tramadol and *O*-desmethyltramadol in a pharmacogenetic study.

1. Introduction

Tramadol hydrochloride (T) (1RS, 2RS)-2-[(dimethylamine) methyl-1-(methoxyphenyl)-cyclohexanol HCl] is a centrally acting analgetic drug with analgesic activity and potency that ranges between weak opioids and morphine [1,2]. T is used in clinical practice as the racemate of the *trans*-isomer. It has been demonstrated that each enantiomer displays different binding properties for various receptors. (+)-T preferentially inhibits serotonin reuptake while (−)-T mainly inhibits noradrenalin reuptake [3,4]. (+)-T is considered 10-times more potent than (−)-T [5].

The metabolic fate of T is unusually complex having at least 11 unconjugated and 12 conjugated metabolites [6]. There are three major metabolic pathways via three distinct cytochrome P450 enzymes CYP2D6, CYP3A, and CYP2B6 forming *O*- and *N*-demethylated metabolites. Major active metabolite *O*-desmethyltramadol (ODT), which is considered to be the main agent responsible for the drug-induced opioid analgesia, is formed in the liver [4,6] predominantly via CYP2D6 enzyme. (−)-ODT also possesses potent monoamine reuptake inhibitory activity and, moreover, has been reported to potentiate the antinociceptive effects of (+)-ODT in rats [7]. Studies using the cloned human μ-opioid receptor have also established that (+)-ODT has approximately 200-times the affinity of the parent (±)-T [8]. Moreover, (+)-ODT had the greatest intrinsic efficacy in an *in vitro* screen [8].

Chiral analysis of T and ODT is therefore important in clinical studies due to the different pharmacodynamic action of individual enantiomers and stereoselective metabolism *in vivo* via polymorphic cytochrome P450 2D6.

Stereoselective determinations of T and ODT have been described using CE [9–17] as well as procedure using GC/MS [18] and mainly LC coupled with fluorescence [19–24], ultraviolet [25,26] or mass spectrometry detection [27,28].

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The objective of this study was to develop a validated normal-phase chiral LC–MS/MS method for fast enantiomeric determination of T and ODT.

2. Experimental

2.1. Chemicals and columns

Standards of T and ODT enantiomers and racemic compounds were kindly supplied by Grünenthal (Stolberg, Germany). Methanolic solution of (±)-tramadol hydrochloride – ^{13}C, d_3 was purchased from Cerilliant (Round Rock TX, USA). A Lux Cellulose-2 column (3 μm, 150 mm × 4.6 mm I.D.) packed with cellulose tris(3-chloro-4-methylphenylcarbamate) from Phenomenex (Torrance CA, USA) was used as the chiral stationary phase (CSP), preceded by a Lux Cellulose-2 security guard column (4 mm × 3 mm I.D.). BondElut Certify 50 SPE column was purchased from Varian (Palo Alto CA, USA).

2.2. LC–MS/MS conditions

Enantioseparation of T and ODT was carried out in isocratic conditions with a solution of hexane/isopropanol/diethylamine (90:10:0.1, v/v/v). The flow rate was 1.3 mL/min and the mobile phase was thermostatted at 27 ± 0.1 °C. An HPLC system (1200 RRLC Agilent, Waldbronn, Germany) was used. A mass spectrometer (3200 Q-trap MDS Sciex, Ontario, Canada) operated in APCI mode was used for analyte determination. The following MRM transitions were used for analyte quantification: m/z 264.2 → 58.2 for T, m/z 250.1 → 58.2 for ODT and m/z 268.2 → 58.2 for I.S. The dwell time for each MRM transition was set at 70 ms. MS parameters were set as follows: Nebulizer gas (GS1) and curtain gas (both N_2) were 50 and 30 psi, respectively; the heated nebulizer temperature was 225 °C and the corona discharge was 2 μA. Declustering potential (DP), entrance potential (EP), and collision energy (CE) of 30 V, 5 V, and 40 eV, respectively, were investigated for all analytes.

2.3. Sample preparation

All standard, QC and biological samples were processed by identical SPE procedure. BondElut Certify column was conditioned with 2 mL of methanol, followed by 2 mL of water. A mixture of 0.5 mL of centrifuged plasma (3000 × g for 10 min), 0.5 mL of Tris buffer and 20 μL of I.S. working solution was applied to the column. The sample was forced through the bed at a low-flow rate by vacuum. The column was subsequently washed with 2 mL of water, 1 mL of acetate buffer pH 4 and 1 mL of methanol and dried for 5 min in vacuum. Analytes were eluted by 2 mL of mixture containing isopropanol, dichloromethane and ammonium hydroxide (80:20:2, v/v/v). The extract was evaporated to dryness under a stream of nitrogen at 45 °C. The residue was redissolved in 100 μL of mobile phase and 15 μL were injected into the LC–MS/MS system.

2.4. Standard samples and quality control samples

Standard working solutions (20, 2 and 0.2 μg/mL of T or ODT racemate; 20 μg/mL of I.S.) were added to 0.5 mL of drug free human plasma creating final concentrations from 1 ng/mL to 800 ng/mL and from 1 ng/mL to 400 ng/mL of each enantiomer of T and ODT, respectively. QC samples at concentrations of 10, 200 and 600 ng/mL of each enantiomer of T and at concentrations of 10, 150 and 300 ng/mL of each enantiomer of ODT were prepared daily.

2.5. Validation

2.5.1. Selectivity and matrix effect

The method selectivity was assessed by analysing plasma extracts from six different individuals. These samples were prepared as described, but without adding analyte and I.S. Additionally, 10 samples from patients receiving structurally related drugs (codeine, morphine, fentanyl and oxycodone) and blank plasma samples spiked with *N*-desmethyltramadol were tested to exclude possible interference.

The matrix effect was calculated according to the method by Matuszewski et al. [29] as peak areas of the samples spiked after SPE procedure divided by the corresponding areas of the standard solutions dissolved directly in mobile phase. This experiment was performed for five different lots of human plasma over the whole concentration range.

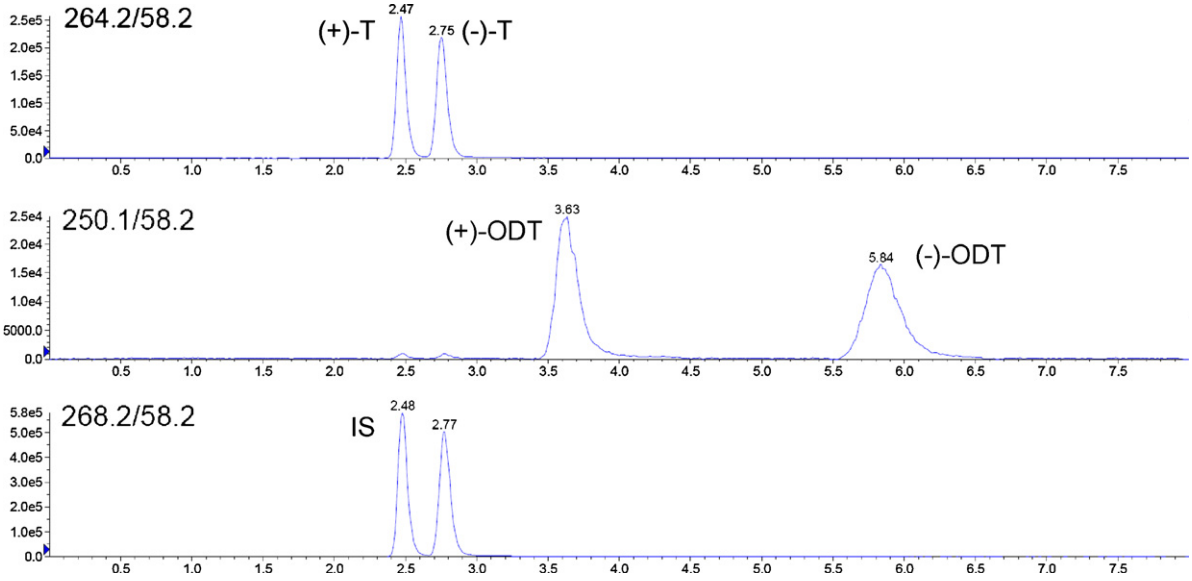

Fig. 1. MRM chromatograms obtained after SPE of plasma sample (83.5 ng/mL (+) T, 78.2 ng/mL (−)-T, 9.9 ng/mL (+)-ODT and 11.3 ng/mL (−)-ODT).

2.5.2. Calibration

Six-point calibration curves were constructed over the whole concentration range. Normalized peak-area ratio of T and ODT enantiomers/I.S. was measured and plotted against the theoretical concentration of the spiked standards. The (+)-enantiomers and (−)-enantiomers were calculated using (+)-T $^{13}C,d_3$ and (−)-T $^{13}C,d_3$, respectively. Least-square linear regression analysis was performed to determine correlation coefficients, slopes, and intercepts. Linearity was assessed by least-squares regression with a weighting index of $1/x2$.

2.5.3. Precision data and accuracy

Accuracy, intra- and inter-day precisions for all analytes were evaluated according to the requirements of FDA guideline on bioanalytical method validation [30]. Intra-day variation was assessed by six replicate determinations of three concentrations over the tested range (QC1 = 10 ng/mL of each enantiomer of T and ODT, respectively; QC2 = 200 ng/mL of each enantiomer of T and 150 ng/mL of each enantiomer of ODT; QC3: 600 ng of each enantiomer of T and 300 ng/mL of each enantiomer of ODT). Intra-day accuracies were expressed as the mean of the assays relative to the theoretical value. The intra-day precision of the method was calculated as the relative standard deviation (RSD) of the assays made for intra-day accuracy. Inter-day variation was assessed by analysing replicates of QC samples with the same concentrations on three days. Accuracies were computed as the mean of the assays relative to the nominal concentration. The inter-day precision of this method was expressed as the RSD of the assays made for inter-day accuracy.

2.5.4. Recoveries

The recoveries of all analytes over the whole concentration range were determined by comparing peak areas obtained from plasma sample and those found by direct injection of mobile phase solutions at the same concentrations.

2.6. Application of the method

To apply the method a single blood sample for CYP2D6 phenotyping was analysed from six healthy, young adult volunteers, who received a standardized oral tramadol dose of 0.7 mg/kg. The volunteers were selected according to their genotype of *CYP2D6* that was determined by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis revealing the presence of *CYP2D6*3*, **4*, **5*, **6* alleles and gene duplication/multiplication using previously published method [31]. Three subjects, who carried no active allele in their *CYP2D6* gene, were classified as poor metabolizers (PM) and three extensive metabolizers (EM) were homozygous carriers of wild-type allele. The study drug was administered with 150 mL of water at 8 a.m. after a 10-h overnight fast. Blood samples were collected 2 h after dosing and stored at −70 °C until analysis. Urinary metabolic ratios from a single spot urine collection at 2 h post-dose were obtained by recently published method for comparison [18]. The study was approved by the Ethics Committee of General Teaching Hospital in Prague.

3. Results

3.1. LC and MS/MS optimization

As was previously reported, the complete enantioeselective determination of T and ODT was obtained on different CSPs operated in reversed-phase mode [20,22,24] as well in normal-phase mode liquid chromatography [23,27,28]. Column used in our study can operate in both liquid chromatography modes. First, we tested reversed-phase to optimize the method. Different parameters such as mobile phase composition, temperature or pH were investigated.

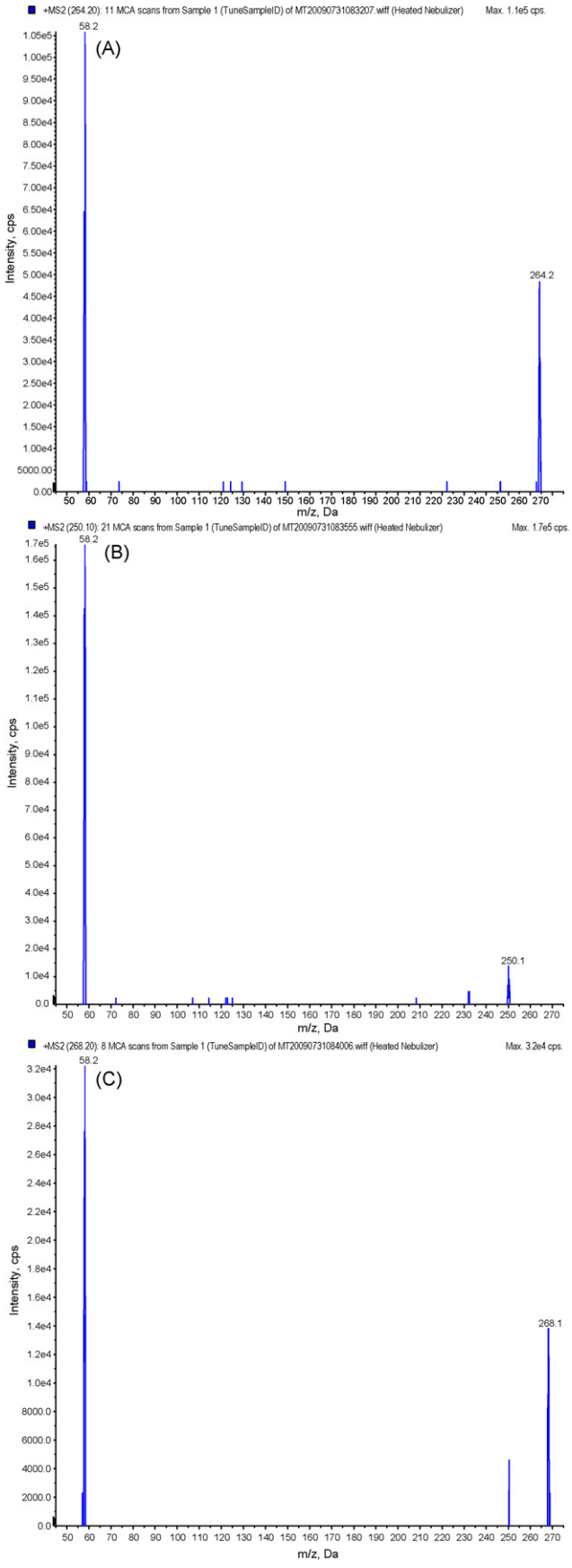

Fig. 2. Product ion spectra of $[M+H]^+$ of tramadol (A), *O*-desmethyltramadol (B) and tramadol – ^{13}C, d_3 (C).

Table 1
Parameters of calibration curves.

$n=6$	(+)-T			(−)-T		
	a	b	R^2	a	b	R^2
Mean	2.4575	0.0126	0.9990	2.4050	0.0067	0.9994
SD	0.0228	0.0074	0.0007	0.0335	0.0021	0.0003
RSD (%)	0.93			1.39		
$n=6$	(+)-ODT			(−)-ODT		
	a	b	R^2	a	b	R^2
Mean	1.6325	−0.0079	0.9995	1.6750	−0.0084	0.9997
SD	0.0773	0.0085	0.0005	0.0750	0.0072	0.0005
RSD (%)	4.73			4.48		

a: slope; b: intercept; R^2: correlation coefficient.

Sodium perchlorate, which was previously reported to improve resolution [20,22], cannot be used because of mass spectrometric detection. The absence of this compound probably resulted in very poor enantiomeric separation of all substances.

A mixture of hexane and ethanol with small amount of organic modifiers was used in all previously reported papers [23,27,28] with normal-phase mode chromatography. We used mixture of hexane and isopropanol, based on manufacturer's recommendation.

Different ratios of hexane and isopropanol, temperature and flow rate were tested for obtaining excellent enantiomeric separation. Higher content of hexane (≥95%) resulted in stronger retention of analytes and more time consuming chromatographic run. Higher content of isopropanol (≥20%) gave rise to increase in column back pressure without separation improvement. The best results were obtained for the following ratio: hexane/isopropanol/diethylamine (90/10/0.1; v/v/v). The initial flow rate was set on 0.8 mL/min resulting in time of analysis longer than 10 min. After increasing the flow rate we observed reduction of the retention times, while enantiomeric separation did not substantially change. The optimal time of analysis was achieved using flow rate of 1.3 mL/min. Tested temperature changes did not substantially affect resolution. Fig. 1 shows a chromatographic trace of real plasma sample. Enantiomers are well separated, the total chromatographic run was 8 min and the retention times of all analytes were shorter than 6 min.

Conditions of mass spectrometric detection were optimized for maximum product ion formation by direct infusion of standard solution into the MS. Product spectra of pseudomolecular ions [(M+H$^+$)] of T, ODT and I.S. are displayed in Fig. 2. It is apparent that only m/z 58, which arises from α-cleavage to the amino nitrogen, can be used as a product ion for all analytes.

3.2. Validation

3.2.1. Selectivity and matrix effect

Peaks of enantiomers of both analytes were well resolved. The order of elution of T and ODT enantiomers was determined by individual injections of mobile phase solutions for each enantiomer. No interfering peaks to T, ODT and IS were found with *N*-desmethyltramadol (MRM: m/z 250.0 → 43.8) or other potentially interfering compounds.

The average matrix effect values ranged over the whole calibration span among 91.1–98.3%, 93.1–96.2%, 91.7–98.7%, and 93.3–99.5% for (+)-T, (−)-T, (+)-ODT, and (−)-ODT, respectively. The average matrix effect for the IS was 92.2%. These results indicated that no co-eluting substances significantly influenced the ionization of analysed compounds and IS.

3.2.2. Calibration

The assay was found to be linear over the whole concentration range. The parameters of calibration lines equatations are described in Table 1. Correlation coefficients were higher than 0.999.

3.2.3. Precision and accuracy

Intra-day accuracies ranged among 98.2–102.8%, 97.1–109.1%, and 97.4–102.9% at the lower, intermediate, and high concentration for all analytes, respectively.

Inter-day accuracies ranged among 95.5–104.1%, 99.2–104.7% and 94.2–105.6% at the lower, intermediate, and high concentration for all analytes, respectively. Precision was 7.3% or less in all analyses. The results are listed in Table 2.

3.2.4. Recoveries

Mean recovery values ± RSD for T and ODT were 92 ± 6.4%, 89 ± 7.9%, respectively. The recovery for I.S. was 90 ± 6.5% in concentration of 400 ng/mL.

3.2.5. Limits of detection and quantification

The limit of detection (LOD, signal-to-noise higher than 3:1) for enantiomers of T and ODT was found to be 0.15 ng/mL for both enantiomers of T, 0.20 ng/mL for (+)-ODT and 0.30 ng/mL for (−)-ODT. The lowest limit of quantification (LLOQ) refers to the lowest concentration of each enantiomer of T and ODT in plasma, that can be analysed quantitatively by the LC–MS/MS method with precision less than or equal to 20% and accuracy within 80–120%. Criteria for LLOQ were fulfilled by the lowest point of the calibration curve (1 ng/mL for all analytes). Chromatograms of spiked plasma sample containing 1 ng/mL of each enantiomer of T and ODT and chromatograms of blank plasma samples are presented in Fig. 3.

Table 2
Inter- and intra-day variation data from the enantiomeric determination of T and ODT.

	QC 1		QC 2		QC 3	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
Intra-day variation						
(+)-T	100.2	1.5	109.1	2.8	97.8	2.5
(−)-T	102.8	5.5	103.0	7.1	98.5	2.7
(+)-ODT	98.2	3.4	97.1	3.1	102.9	5.8
(−)-ODT	101.2	3.3	97.5	3.3	97.4	2.8
Inter-day variation						
(+)-T	104.1	1.4	104.7	2.7	98.5	2.4
(−)-T	102.7	5.3	99.2	7.0	94.2	2.7
(+)-ODT	95.5	3.8	101.5	1.5	105.6	3.4
(−)-ODT	101.5	4.2	104.3	3.5	102.3	7.3

QC 1: 10 ng/mL of each enantiomer of T and ODT; QC 2: 200 ng/mL of each enantiomer of T and 150 ng/mL of each enantiomer of ODT; QC 3: 600 ng/mL of each enantiomer of T and 300 ng/mL of each enantiomer of ODT.

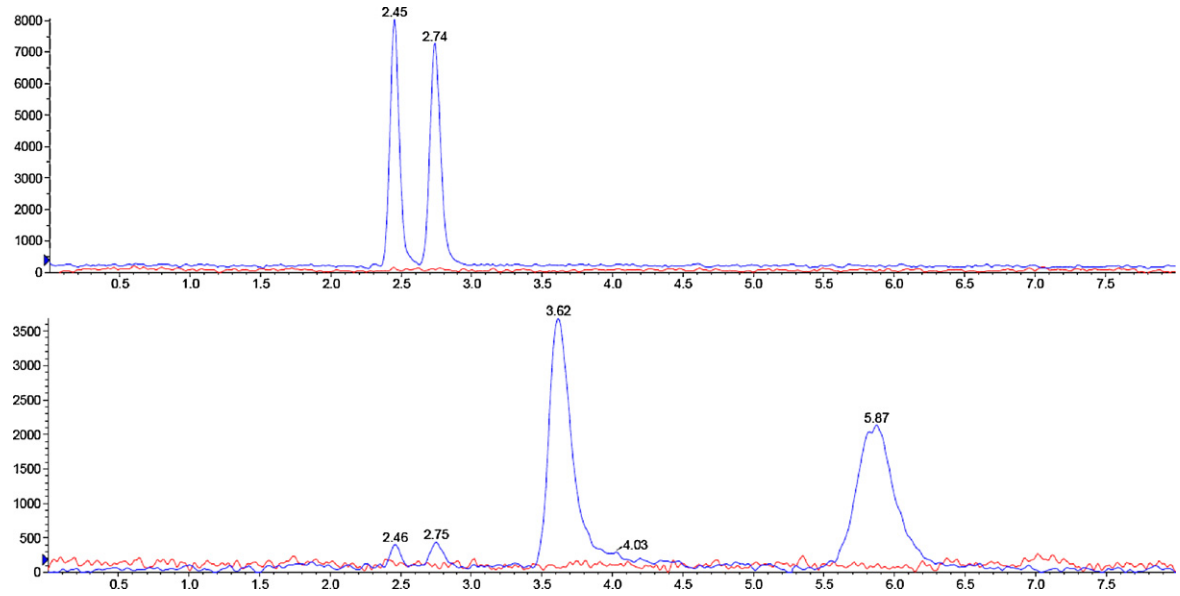

Fig. 3. MRM mass chromatograms obtained after analysis of spiked plasma sample containing 1 ng/mL (LOQ) of each enantiomer of T (264.2/58.2) and ODT (250.1/58.2). Both chromatograms contain MRM trace of blank plasma sample.

Table 3
Individual and both phenotype groups data of T and ODT plasma concentrations, metabolic ratios (MR), urinary metabolit ratios (MRu), and CYP2D6 genotypes. PM and EM group data are given as mean ± SD.

	(+)-T (ng/mL)	(−)-T (ng/mL)	(+)-ODT (ng/mL)	(−)-ODT (ng/mL)	(+)-MR	(−)-MR	(±)-MR	(±)-MRu	CYP2D6 Genotype
Subject									
EM1	21.1	25.8	13.8	20.6	1.5	1.3	1.4	0.3	*1/*1
EM2	96.4	86.1	21.4	21.8	4.5	3.9	4.2	1.1	*1/*1
EM3	54.1	47.5	10.9	9.3	5.0	5.1	5.0	1.3	*1/*1
PM1	93.1	93.8	1.2	2.8	77.6	33.5	45.6	>100	*3/*4
PM2	97.9	87.8	2.5	5.0	39.2	17.6	24.8	8.9	*4/*4
PM3	76.6	74.9	2.5	4.0	30.6	18.7	23.3	>100	*4/*4
Group									
EM	57.2 ±30.8	53,1 ±24.9	15.4 ±4.4	17.2 ±5.6	3.7 ±1.6	3.4 ±1.6	3.5 ±1.5		
PM	89.2 ±9.1	85.5 ±7.9	2.1 ±0.6	3.9 ±0.9	49.1 ±20.4	23.3 ±7.25	31.2 ±10.2		

3.3. Application of the method

Metabolic ratios (MR) of T/ODT concentrations for individual enantiomers and total analyte levels in plasma samples are shown in Table 3. The (+)-MR based on (+)-T/(+)-ODT was the most sensitive for phenotyping of CYP2D6 as evidenced from the largest difference between mean MR values in EM and PM subjects. The plasma levels of (+)-ODT were substantially lower in PMs at 2 h post-dose in comparison to EMs. These findings are fully consistent with previously reported stereoselective production of (+)-ODT by liver CYP2D6 enzyme [1,4,32]. The plasma-based MRs resulted in identical phenotyping CYP2D6 classification of the subjects as urinary based phenotyping or genotyping.

4. Discussion

In this paper, we describe chiral liquid chromatographic enantioeselective determination of T and ODT that was achieved by relatively newly developed chiral selector, which contains cellulose tris(3-chloro-4-methylphenylcarbamate). In some cases, this advanced technology provides a useful alternative to the currently most successful chiral recognition ability of commercially available cellulosis CSP chiralcel-OD [33], which was used for enantiomeric separation of T and ODT in previously published articles [20,22,25,26,28]. Chromatographic conditions and parameters of the CSP (chiral selector, particle size and column length) in combination with fast chromatographic equipment resulted in substantially shorter analysis time compared to previously published methods [20,22–28]. The previously reported retention times ranged from 5.8 min [23] to 51.1 min [20], and from 8.5 min [23] to 24.6 min [20] for (+)-T, and (+)-ODT, respectively. Both enantiomers of analytes were well separated in a total chromatographic run of 8 min in our newly developed method, while the retention time of all analytes was shorter than 6 min.

5. Conclusions

This fast method based on LC–MS/MS has been developed for the simultaneous enantioselective determination of T and ODT in human plasma. The method was verified for use by CYP2D6 phenotyping test in healthy volunteers and may be fully recommended in pharmacokinetic studies, therapeutic drug monotoring and pharmacogenetic tests.

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