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Short communication

Enatiomeric determination of tramadol and *O*-desmethyltramadol in human urine by gas chromatography–mass spectrometry

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ABSTRACT

A GC–MS assay for stereoselective determination of tramadol and its pharmacologically active phase I metabolite *O*-desmethyltramadol in human urine was developed. Nefopam was used as internal standard. The method involves a simple solid phase extraction with chiral analysis by gas chromatography–electron ionization mass spectrometry using *m*/*z* 263; 58, 249; 58, and 179; 58 for the determination of concentration of tramadol, *O*-desmethyltramadol and internal standard, respectively. Chromatography was performed on a Rt-βDEXcst column containing alkylated beta-cyclodextrins as a chiral selector. The calibration curves were linear in the concentration range 0.1–20 μg/mL ($R^2 \geq 0.998$). Intra-day accuracies ranged between 97.2–104.9%, 96.1–103.2%, and 97.3–102.8% at the lower, intermediate, and high concentration for all analytes, respectively. Inter-day accuracies ranged between 95.2–105.7%, 99.1–105.2%, and 96.5–101.2% at the lower, intermediate, and high concentration for all analytes, respectively. This method was successfully used to determine the concentration of enantiomers of T and ODT in a pharmacogenetic study.

1. Introduction

Tramadol hydrochloride (T) (1RS, 2RS)-2-[(dimethylamine) methyl-1-(methoxyphenyl)-cyclohexanol HCl] (Fig. 1) is a centrally acting analgetic drug with analgesic efficacy and potency that ranges between weak opioids and morphine. T is used as the racemate for therapy. Each enantiomer displays different binding properties for various receptors. (+)-T preferentially inhibits serotonin reuptake while (−)-T mainly inhibits noradrenalin reuptake [1,2]. (+)-T is 10-times more potent than (−)-T [3].

The metabolic fate of tramadol is unusually complex having at least 11 unconjugated metabolites and 12 conjugated compounds [4]. 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, however, formed in the liver [2,4] 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 [5]. Studies using the cloned human μ-opioid receptor have also established that (+)-ODT has approximately 200-times the affinity of the parent (±)-T [6]. Moreover, (+)-ODT had the greatest intrinsic efficacy in an in vitro screen [6].

Cytochrome P450 2D6 is a highly polymorphic gene locus with more than 70 variant alleles. Its single nucleotide polymorphisms can greatly affect the phenotype leading to complete enzyme deficiency in poor metabolizers, incomplete deficiency in intermediate metabolizers in comparison to extensive metabolizers. The pharmacokinetics of T and ODT is greatly affected by CYP2D6 deficency [7] and the metabolism catalyzed through CYP2D6 is stereoselective for (+)-ODT formation in vivo [8–10]. The genetic enzyme deficiency predispose poor metabolizers to have no or extremely low levels of (+)-ODT in blood, while ultrarapid metabolizers were shown to have unusually high (+)-ODT levels in blood [11]. Although there is no convincing evidence that the genotype-dependent pharmacokinetics differences relate to analgesic action of the drug in clinical practise, pharmacodynamic action of the drug is modified by CYP2D6 polymorphism [7,11–13]. Due to the metabolic fate of tramadol that is depedent on the activity of CYP2D6, the drug has been proposed to be used as a probe drug for easy and safe phenotyping of the liver enzyme activity in human pharmacogenetic studies [14]. For this purpose, a stereoselective determination from human urine needs to be applied.

Achiral determinations of T or ODT in biological matrices are still routinely used in bioequivalence studies. Number of achiral

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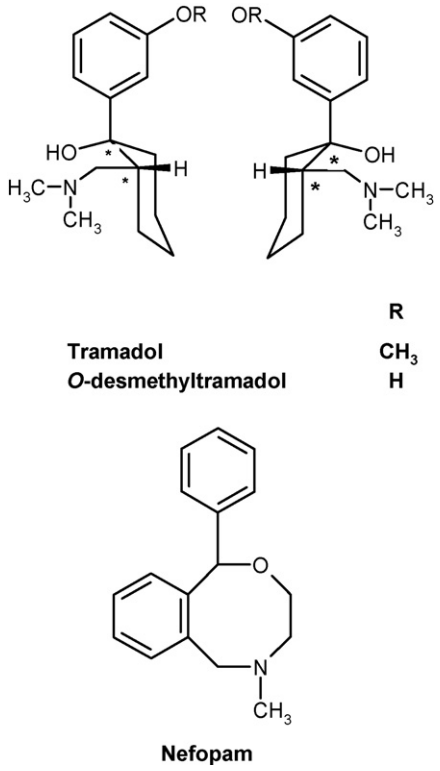

Fig. 1. Structures of tramadol, *O*-desmethyltramadol and nefopam.

methods have been developed previously using TLC [15], GC with nitrogen–phosphorous detection [16], GC with flame ionization detection [17], GC–MS [18–25], and HPLC [26–32]. Chiral analysis of T and ODT is important in clinical studies due to the different pharmacodynamic action of individual enantiomers. As the drug undergoes a stereoselective metabolism, which is dependent on the activity of liver cytochrome P450 2D6, enantiomeric determination of T and ODT in urine may be reliably used as a simple noninvasive phenotyping test for the evaluation of CYP2D6 activity in vivo. Stereoselective determinations of T and ODT have been described using CE [33–35] or HPLC [36–42], but no GC method was described so far.

The aim of this work was to develop a simple method for simultaneous enantiomeric determination of T and ODT in urine as an analytical method for CYP2D6 phenotyping in vivo. T, ODT and IS. were analysed by gas chromatography with mass spectrometric detection, which is more available than CE and can be used as alternative method to generally more costly HPLC technique. This method is easy to provide and is aplicable for routine enantiomeric determination of T and ODT in a single sample of human urine as a basis for CYP2D6 phenotype determination in vivo. Described procedure involves a simple SPE extraction for obtaining clear extract, which is prepared for injecting onto GC/MS system without derivatization step.

2. Experimental

2.1. Materials

BondElut Certify 130 SPE column and Vac Elut 20 vacuum manifold were purchased from Varian (Palo Alto, CA, USA). Automatic pipettes were obtained by Eppendorf (Hamburg, Germany). Glass vials were obtained from Jaytee Biosciences (Whitstable, UK). Heating block was purchased from Barkey (Bielefeld, Germany).

2.2. Chemicals and reagents

Standards of pure T and ODT enantiomers and racemic compounds were kindly supplied by Grünenthal (Stolberg, Germany). Internal standard nefopam hydrochloride was purchased from MP Biomedicals (Illkirch, France). Ammonium hydroxide (28%), methanol, 2-propanol and dichloromethane (gradient grade) were purchased from Merck (Darmstadt, Germany). TRIS buffer was purchased from Sigma–Aldrich (Prague, Czech Republic). Natrium acetate for the preparation of acetate buffer pH 4 was supplied by Penta Chemicals (Prague, Czech Republic). Ultra/high quality water was obtained using a Milli-Q apparatus Millipore (Bedford, MA, USA). Helium (purity 6.0) and nitrogen (purity 5.0) were purchased from Linde Gas (Prague, Czech Republic). All chemicals were obtained in p.a. grade unless specified otherwise.

2.3. Gas chromatography–mass spectrometry

A GC Fisons Instruments 8000 series chromatograph with autosampler CTC-2005, CTC Analytics (Zwingen, Switzerland) coupled to a mass spectrometer Fisons Instruments MD 800 was used. Chromatographic separation was achieved by Rt-βDEXcst capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness) from Restek (Bellefonte, PA, USA).

The oven temperature initially set at 120 °C was increased at a rate of 2.5 °C/min to 230 °C and then held for 10 min. The injector temperature was 230 °C. Pressure of carrier gas (helium) was 70 kPa. Injection was performed in splitless mode (60 s delay before opening the splitter).

The MS detector parameters were GC–MS transfer line temperature 230 °C, electron energy 70 eV. Selected ion monitoring mode for quantitative analysis from 35 to 55 min was used, *m*/*z*: 58; 263 for T, 58; 249 for ODT and 58; 179 for IS (*m*/*z* 58 was used for the quantification of both analytes and IS).

2.4. Stock and standard working solutions

Separate stock solutions of racemic T and ODT (200 μg/mL) in methanol were stored at +4 °C. Standards were prepared from stock solutions at final concentrations of 5, 25, 100, 250, 500, and 1000 μg/mL. The IS working solution was prepared at the final concentration of 1 mg/mL.

2.5. Standard samples and quality control samples

Aliquotes of blank urine obtained during a 24-h urine collection from a healthy volunteer were stored at −70 °C and used to prepare spiked urine samples. Standard working solutions of T and ODT were added to 1 mL of drug free human urine creating final concentrations from 0.1 to 20 μg/mL. Quality control samples of T and ODT at concentrations of 0.5, 5 and 10 μg/mL were prepared daily.

2.6. Sample preparation

All standard, quality control and biological samples were processed by identical method. Bond Elut Certify column was conditioned with 2 mL of methanol, followed by 2 mL of water. A mixture of 1 mL of centrifuged urine, 2 mL of TRIS buffer and 10 μL of IS working solution was applied to the column. Sample was forced through the bed at a low-flow rate by vacuum. The column was subsequently washed with 4 mL of water, 1 mL of acetate buffer pH 4 and 2 mL of methanol and dried for 5 min under 250 mmHg vacuum. Analytes were eluted by 3 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 in a heating block. The residue was redissolved in 150 μL of methanol and 2 μL was injected into the GC/MS system.

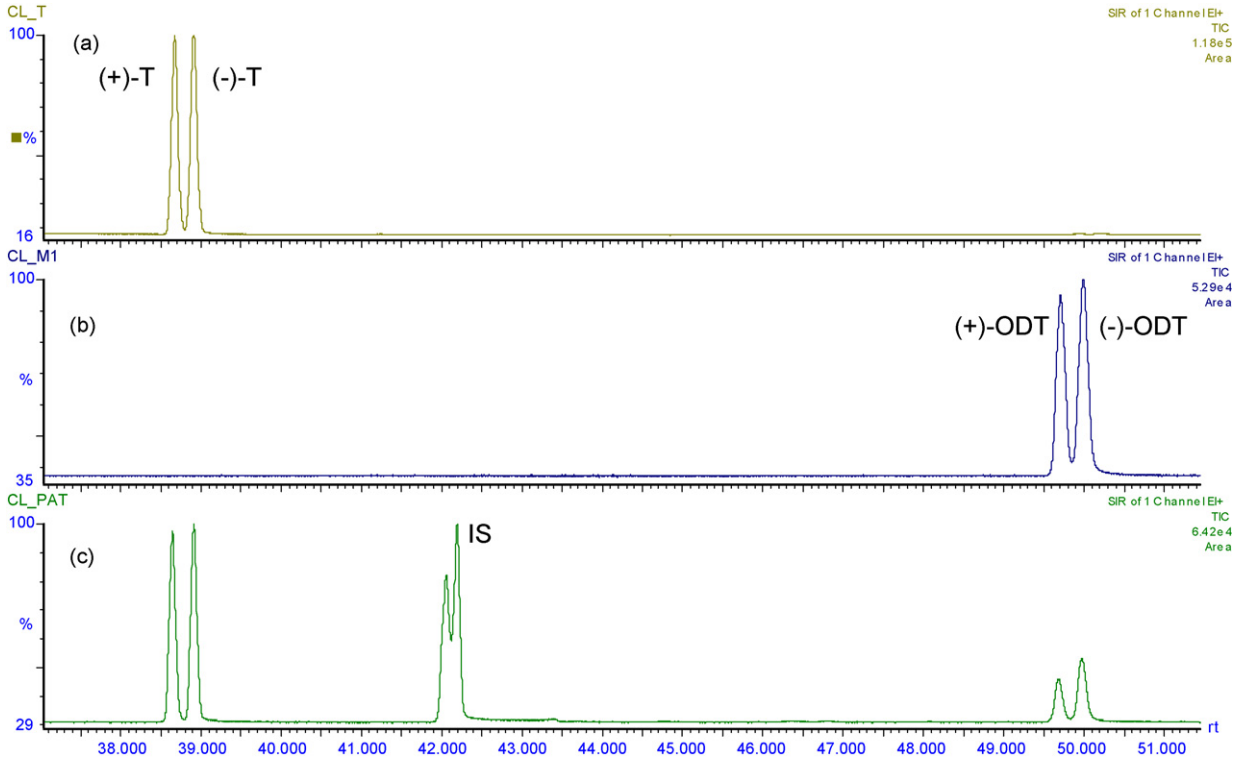

Fig. 2. SIM chromatograms obtained after SPE of a blank urine spiked with 3 μg/mL of each enantiomers of T (a), ODT (b) and a healthy volunteer urine sample (c) collected in a period of 2 h after an oral dose of tramadol drops in standardized dose of 0.7 mg/kg (1.93 μg/mL (+)-T, 1.97 μg/mL (−)-T, 0.73 μg/mL (+)-ODT and 1.08 μg/mL (−)-ODT).

2.7. Calibration

Six-point calibration curves were constructed over the whole concentration range (0.1–20 μg/mL). Normalized peak area ratio of T and ODT enantiomers/IS was measured and plotted against the theoretical concentration of the spiked standards. Least-square linear regression analysis was performed to determine correlation coefficients, slopes and intercepts. Mean accuracy was evaluated on back-calculated concentrations at each calibration level.

2.8. Precision, accuracy and recovery

Accuracy, intra- and inter-day precisions for all analytes were evaluated according to the requirements of FDA guideline on bioanalytical method validation. Intra-day variation was assessed by six replicate determinations of three concentrations over the tested range (0.5, 5 and 10 μg/mL). Intra-day accuracy was 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 standards with the same concentrations on three days. Accuracy was 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.

Recoveries of T and ODT over entire concentration range were determined by comparing peak areas obtained from processed quality control urine samples with those achieved after direct injections of methanolic standard solutions at the same concentrations.

2.9. Peak purity and selectivity

Ten different blank urine samples were analysed for peaks interfering with the detection of the analytes and the IS. The noise data from the assay of blank urine were used in the LOD and LOQ experiment.

2.10. Application of the method

To apply the newly developed method for CYP2D6 phenotyping from a single urinary sample we analysed samples from six healthy, young adult volunteers who received a single oral dose of tramadol drops in standardized 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 according to the previously published method [43]. Two subjects who carried no variant allele in their *CYP2D6* gene were classified as extensive metabolizers (EM), two intermediate metabolizers (IM) had one variant and one wild-type allele of *CYP2D6* and two poor metabolizers (PM) were homozygous carriers of variant *CYP2D6*4* allele. The study drug was administered with 150 mL of water at 8 a.m. after a 10 h overnight fast. Urine samples were collected 2 h after dosing and stored at −70 °C until analysis.

3. Results

3.1. Chromatography

Peaks of enantiomers of both analytes were well resolved. Further, no interfering peaks to T, ODT and IS were found in the urine.

Fig. 2 shows *m/z* 58 SIM chromatograms of blank urine samples spiked with 3 μg/mL of each enantiomers of T (a), blank urine sample spiked with 3 μg/mL of each enantiomers of ODT (b) and result (c) of the analysis of urine sample obtained at 2 h from a volunteer who received tramadol drops.

3.2. Extraction

Mean recovery values ± RSD for T and ODT were 86 ± 8.1% and 84 ± 9.1%, respectively. The recovery for IS was 78 ± 7.2% in concentration of 10 μg/mL.

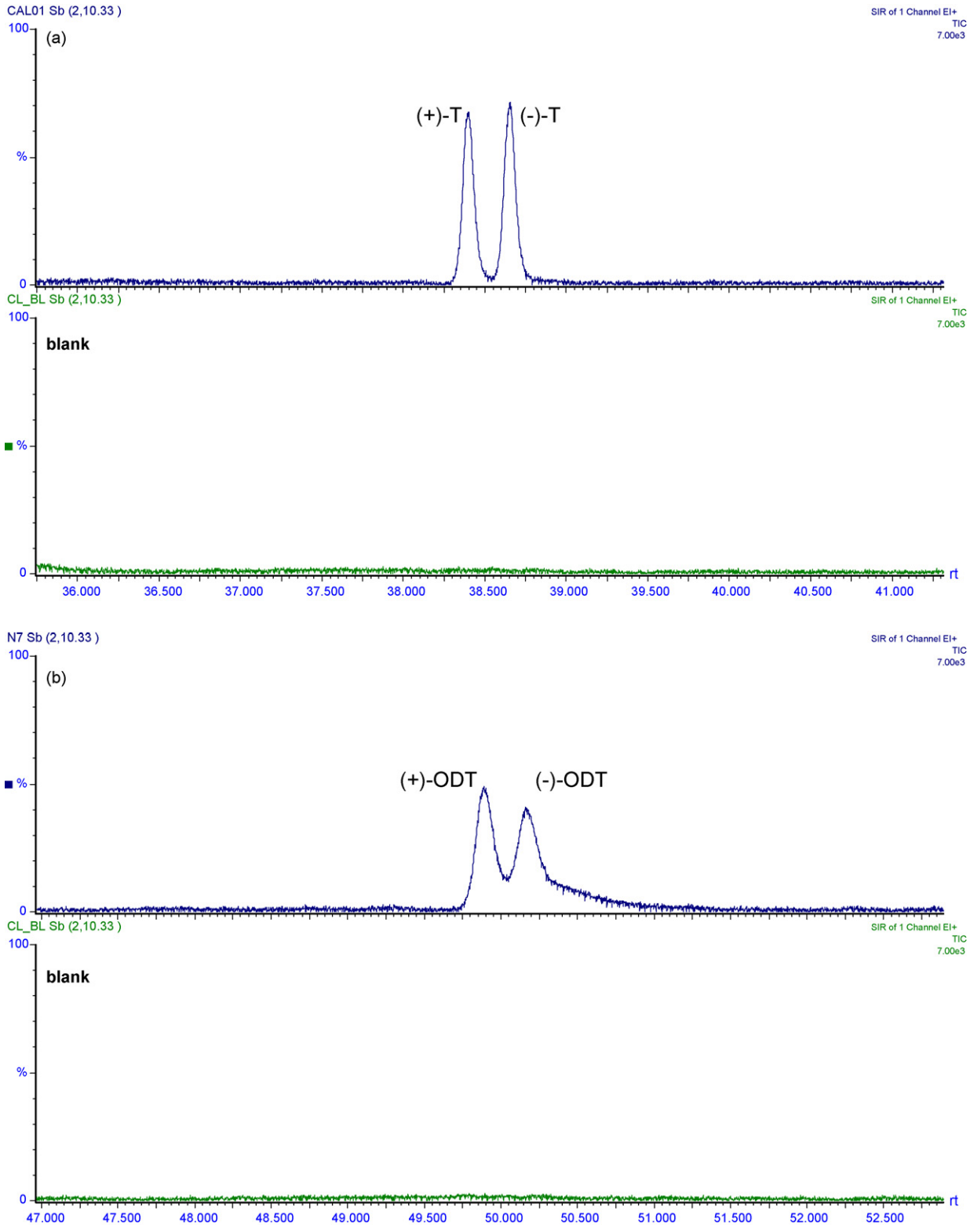

Fig. 3. SIM mass chromatograms obtained after analysis of spiked urine sample containing 0.1 μg/mL (LOQ) of each enantiomers of T (a) and ODT (b).

3.3. Limits of detection and quantification

The limit of detection (LOD, signal-to-noise greater than 3:1) for enantiomers of T and ODT were found to be 0.01 μg/mL for T and 0.03 μg/mL for ODT and the criteria for the limit of quantification (LOQ, signal-to-noise greater than 10:1) were fulfilled by the lowest point of the calibration curve (0.1 μg/mL for both enantiomers of T and ODT). Chromatograms of spiked urine sample containig 0.1 μg/mL of each enantiomers of T and ODT are presented in Fig. 3.

3.4. Calibration

Calibration curves of T and ODT were linear over the range 0.1–20 μg/mL. Mean parameters of equatations are described in

Table 1
Parameters of calibration curves.

	a	b	R^2	a	b	R^2
	(+)-T			(−)-T		
n	6	6	6	6	6	6
Mean	0.5068	−0.0126	0.9988	0.5022	−0.0144	0.9981
SD	0.0315	0.0351	0.0011	0.0292	0.0255	0.0007
RSD (%)	6.22			5.81		
	(+)-ODT			(−)-ODT		
n	6	6	6	6	6	6
Mean	0.3007	−0.0169	0.9992	0.3031	−0.0167	0.9992
SD	0.0241	0.0405	0.0005	0.0187	0.0283	0.0004
RSD (%)	8.01			6.17		

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

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

	0.5 μg/mL		5.0 μg/mL		10 μg/mL	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
Intra-day variation						
(+)-T	104.9	4.7	103.2	5.6	97.3	5.4
(−)-T	102.3	5.5	100.6	3.6	96.9	6.7
(+)-ODT	97.2	7.2	98.8	5.1	100.7	5.3
(−)-ODT	98.6	6.6	96.1	7.9	102.8	5.3
Inter-day variation						
(+)-T	105.7	5.2	105.2	2.3	96.5	2.3
(−)-T	103.2	5.3	102.6	3.8	99.8	3.1
(+)-ODT	96.4	7.5	99.1	4.2	101.2	2.3
(−)-ODT	95.2	8.0	101.2	4.6	100.5	3.7

Table 1. Correlation coefficients were higher than 0.998 and RSD values of concentrations, which were back calculated from the equation of the regresion curves, for each level of calibration curve, ranged from 98.1% to 104.3% for both enantiomers of T and ODT.

3.5. Precision and accuracy

The results obtained from the inter- and intra-day precision and accuracy studies are listed in Table 2.

Intra-day accuracies ranged between 97.2–104.9%, 96.1–103.2%, and 97.3–102.8% at the lower, intermediate, and high concentration for all analytes, respectively.

Inter-day accuracies ranged between 95.2–105.7%, 99.1–105.2%, and 96.5–101.2% at the lower, intermediate, and high concentration for all analytes, respectively.

Precision was 8.0% or less in all analyses.

3.6. CYP2D6 phenotype determination

Metabolic ratios of ODT/T concentrations for enantiomers and total analyte levels in urinary samples are shown in Table 3. The metabolic ratio based on (+)-ODT/(+)-T was clearly the most sensitive for phenotyping of CYP2D6 based on known genotype of the volunteers. The urinary levels of (+)-ODT were below the limit of detection in both poor metabolizers at 2 h post-dose and the excretion of (+)-ODT in intermediate metabolizers was lower than in the extensive metabolizers. Excretion of (−)-ODT was not specific for subjects with an active CYP2D6 enzyme. These findings are fully consistent with previously reported stereoselective production of (+)-ODT by liver CYP2D6.

Table 3
Mean (±SD) urinary metabolic ratios (enatiomer-specific and racemic) in extensive (EM), intermediate (IM) and poor (PM) metabolizers of CYP2D6.

	(+)-ODT/(+)-T	(−)-ODT/(−)-T	(±)-ODT/(±)-T
EM (n = 2)	0.63 (0.12)	0.51 (0.49)	0.57 (0.07)
IM (n = 2)	0.38 (0.01)	0.30 (0.02)	0.34 (0.02)
PM (n = 2)	0.00 (0.00)	0.51 (0.02)	0.26 (0.18)

4. Conclusions

A simple, accurate and precise method based on GC/MS has been developed for the simultaneous enantioselective determination of T and ODT in human urine. The method was verified for use in a CYP2D6 phenotyping test and may be fully recommended in clinical pharmacogenetic studies.

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