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Research Article

Capillary electrophoretic chiral determination of mirtazapine and its main metabolites in human urine after enzymatic hydrolysis

Capillary electrophoresis and liquid-phase microextraction using porous polypropylene hollow fibers were employed for the enantioselective analyses of mirtazapine and its metabolites demethylmirtazapine and 8-hydroxymirtazapine in human urine. Before the extraction, urine samples (1.0 mL) were submitted to enzymatic hydrolysis at 37°C for 16 h. Then, the enzyme was precipitated with trichloroacetic acid, the pH was adjusted to 8 with 0.5 mol/L phosphate buffer solution (pH 11) and 15% sodium chloride was further added. The analytes were transferred from the aqueous donor phase, through *n*-hexyl ether (organic solvent immobilized in the fiber), into 0.01 moL/L acetic acid solution (acceptor phase). The electrophoretic analyses were carried out in 50 mmol/L phosphate buffer solution (pH 2.5) containing 0.55% w/v carboxymethyl-β-cyclodextrin. The method was linear over the concentration range of 62.5–2500 ng/mL for each mirtazapine and 8-hydroxymirtazapine enantiomer and 62.5–1250 ng/mL for each demethylmirtazapine enantiomer. The quantification limit was 62.5 ng/mL for all the enantiomers. Within-day and between-day assay precision and accuracy were lower than 15% for all the enantiomers. Finally, the method proved to be suitable for pharmacokinetic studies.

Keywords:
Capillary electrophoresis / Enantioselective analysis / Liquid-phase microextraction / Metabolites / Mirtazapine
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1 Introduction

Mirtazapine (MRT) is available as a racemic mixture (*rac*-) and it is widely used for the treatment of depressed patients [1, 2]. More recently, studies have even suggested that MRT could be used in social anxiety [3], posttraumatic stress disorders [4], Parkinson's disease [5] and also for the treatment of progressive multifocal leukoencephalopathy caused by immunodeficiency virus infection [6]. MRT has a dual-action profile, combining the enhancement of the noradrenergic neurotransmitter system with specific actions on particular serotonergic receptor subtypes. This pharmacological profile tends to be stereoselective [7].

In vitro metabolism studies have shown that MRT is extensively biotransformed in the liver preferentially *via* cytochrome P450 (CYP) iso-enzymes, including hydroxylation, demethylation and N-oxidation reactions, followed by conjugation, as well as direct N-glucuronidation. However, this metabolic pathway appears to be enantioselective with the (+)-(*S*)-enantiomer undergoing hydroxylation (approximately 40%) *via* CYP2D6, whereas the (−)-(*R*)-enantiomer is preferentially biotransformed by demethylation (approximately 25%) *via* CYP3A4 [8]. The major metabolite formed by hydroxylation, 8-hydroxymirtazapine (8-OHM), as well as the active metabolite formed by demethylation, demethylmirtazapine (DMR), are conjugated with glucuronic acid or sulfuric acid [9]. In addition, differences in the disposition of (+)-(*S*)-MRT in extensive metabolizers and poor metabolizers have also been demonstrated; on the other hand, these differences were not observed for the other enantiomer [10]. Although higher concentrations of the (−)-(*R*)-MRT in human plasma have been reported [9, 11, 12] after oral administration of *rac*-MRT, *R/S* enantiomer ratios <1 have also been observed for some patient samples [11–15].

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Abbreviations: **CM-β-CD,** carboxymethyl-β-CD; **CYP,** cytochrome P450; **DMR,** demethylmirtazapine; **HP-β-CD,** hydroxypropyl-β-CD; **LID,** lidocaine; **LPME,** liquid phase microextraction; **MRT,** mirtazapine; **8-OHM,** 8-hydroxymirtazapine; **rac,** racemic; **S-β-CD,** sulfated-β-CD

The stereoselective pharmacokinetic properties of MRT and its metabolites have been traditionally evaluated by using chromatographic methods and liquid–liquid extraction [11, 13] or solid-phase extraction [12, 16, 17] for sample preparation. However, these techniques require high amounts of organic solvents in the extraction step or as mobile phase. Fanali *et al.* [18] reduced the use of organic solvent by using nano-liquid chromatography for the enantioselective analysis of MRT, DMR and 8-OHM, but solid-phase extraction was still used for sample preparation.

In contrast, enantioseparations by capillary electromigration methods require no or minimum amounts of organic solvents. Furthermore, high efficiency and resolution, as well as, high selectivity and fast analysis have been observed using capillary electrophoresis (CE) or capillary electrochromatography systems [19–25]. Hence, Mandrioli *et al.* [26] and Aturki *et al.* [27] employed CE and capillary electrochromatography, respectively, for the analysis of MRT and its metabolites, but solid-phase extraction was still used for sample preparation.

An alternative approach to overcome the drawbacks of using organic solvents in sample preparation and in the analysis is the combination of CE system with microextraction techniques. Microextraction techniques such as liquid-phase microextraction (LPME) have been recently developed and have shown to be excellent alternatives for sample preparation [28]. This technique is based on the passive diffusion of analytes with suitable partition coefficient from an aqueous sample, through the organic phase immobilized in the pores of the hollow fiber, and further into the acceptor solution placed inside the lumen of the hollow fiber, which is collected and injected in the analytical system [29]. Substantial reductions in the use of toxic and expensive organic solvents, as well as, high analyte preconcentration and efficient sample clean up ability have been observed using both two- and three-phase LPME system [28]. In the two-phase system, the acceptor solution is the same organic solvent immobilized in the pores of the hollow fiber, whereas in the three-phase system, the acceptor solution is an aqueous solution. Thus, after extraction, the aqueous acceptor solution from the three-phase system is directly compatible with reversed phase high-performance liquid chromatography or CE systems.

Recently, our group has used this technique for sample preparation in several methods for the enantioselective analysis of drugs and metabolites in biological samples, including MRT [14, 15, 30, 31].

To the best of our knowledge, just a few methods exist for the chiral analysis of drugs using LPME coupled to capillary electrophoresis [30, 32, 33], and none for the chiral analysis of MRT and its metabolites. On this basis, the aim of the present study was to develop and validate a three-phase LPME procedure and a CE chiral method for the simultaneous analysis of the enantiomers of MRT, 8-OHM and DMR in human urine.

2 Materials and methods

2.1 Chemicals and reagents

rac-MRT (98%), *rac*-8-OHM and *rac*-DMR (Fig. 1) were kindly supplied by Analytical Control Labs, N.V. Organon (Oss, Netherlands). Lidocaine (LID), used as internal standard, was purchased from Sigma-Aldrich (St. Louis, MO, USA). The solvents (chromatographic grade) and chemical reagents (analytical grade) were purchased from Merck (Darmstadt, Germany), Mallinckrodt (Paris, KT, USA) and J. T. Baker (Phillipsburg, NJ, USA). Carboxymethyl-β-cyclodextrin (CM-β-CD, degree of substitution, DS∼3) used as chiral selector was purchased from Fluka (Buchs, Switzerland). All solutions used as CE running buffers and in CE rinse cycle procedure were filtered through Millex-HA 0.45 mm disk filters from Millipore® (Millipore Corporation, Bedford, MA, USA) and degassed by ultrasound for 5 min. All aqueous solutions were prepared with water purified with a MILLI-Q-PLUS® system. The β-glucuronidase enzyme (Type HP-2, from *Helix pomatia*, 122 700 unit/mL^{-1} β-glucuronidase; 1150 unit/mL sulfatase) used in the enzymatic hydrolysis procedure was supplied by Sigma-Aldrich.

The stock (1 mg/mL) and working (5, 10, 25, 50, 100 and 200 μg/mL) solutions of *rac*-MRT and its metabolites were prepared by appropriate dilutions in methanol. The internal standard solution (LID) was prepared in methanol at the concentration of 40 μg/mL. The solutions were stored frozen at −20°C and protected from light.

2.2 Apparatus and CE conditions

The enantioseparations were performed on an Agilent Technologies CE system (Waldbronn, Germany) model

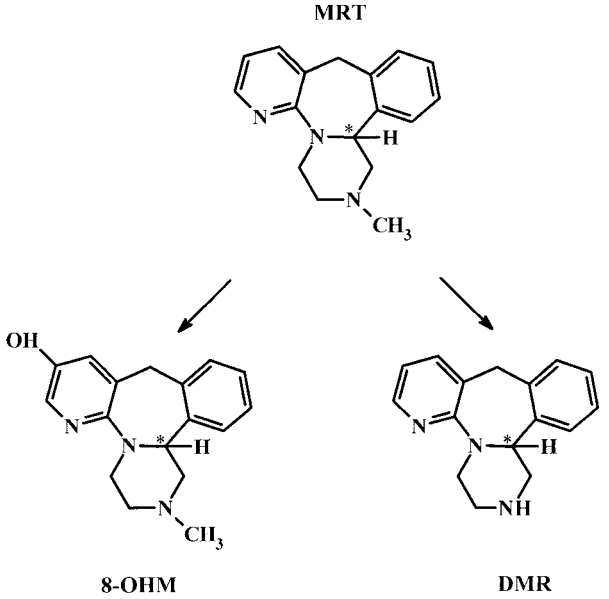

Figure 1. Chemical structures of MRT, DMR and 8-OHM; (*) denotes the chiral center.

G1600A consisting of an analyzer, an automatic sampler, a diode array detector (operating at 205 nm) and an Agilent ChemStation software for data acquisition. An uncoated fused silica capillary (Agilent Technologies) of 50 μm i.d., 80 cm in total length, and 72 cm in effective length was used. Before first use, the capillary was conditioned by rinsing with 1.0 mol/L sodium hydroxide for 1 min at 30°C, followed by 0.1 mol/L sodium hydroxide for 10 min at 20°C and water for 10 min at 20°C. At the beginning of each working day, the capillary was rinsed with 0.1 mol/L sodium hydroxide for 10 min, followed by water for 10 min. Between consecutive analyses, the capillary was rinsed with 0.1 mol/L sodium hydroxide for 2 min, water for 2 min and running buffer for 5 min. After daily use, the capillary was washed with 0.1 mol/L sodium hydroxide for 10 min, followed by water for 10 min. When not in use, the capillary was stored in water. The electrophoretic separations were carried out in 50 mmol/L phosphate buffer (pH 2.5) containing 0.55% w/v CM-β-CD in the normal mode (+25 kV). The samples were hydrodynamically injected at a pressure of 50 mbar for 20 s in the capillary thermostated at 25°C.

2.3 Enzymatic hydrolysis procedure

The hydrolysis reactions were performed using 100 μL of β-glucuronidase enzyme (*H. pomatia*, Type HP-2) and 0.5 mL of 0.5 mol/L phosphate buffer solution (pH 5) added to 1.0 mL urine samples. The samples were kept at 37°C under constant shaking for 16 h. The hydrolyzed samples were added with 100 μL of 40% tricholoacetic acid solution, vortex-mixed (1 min), left to stand (20 min) and then centrifuged (5 min at 1800 *g*) [34]. The upper phases were transferred to clean tubes and submitted to LPME.

2.4 Liquid-phase microextraction procedure

The samples were treated using a modified version of a published method developed by our group recently [15] and illustrated in Fig. 2. Briefly, a 25 μL syringe was connected to the top end of a piece of hollow fiber (8.0 cm) and the bottom end was closed. Urine samples previously submitted to enzymatic hydrolysis were added with 2 mL of 0.5 mol/L phosphate buffer solution (pH 11) and 15% sodium chloride, resulting in a donor phase pH of 8.0. *n*-Hexyl ether was immobilized in the pores of the fiber and 0.01 mol/L acetic acid solution was used as acceptor solution. After extraction for 45 min at 35 ± 2°C under ultrasonification at 25 kHz, the acceptor solution was transferred to the injection vial and directly analyzed.

2.5 Method Validation

Calibration curves were assessed by analyzing 1.0 mL drug-free urine samples spiked with 62.5, 125, 312.5, 625, 1250 and 2500 ng/mL of each MRT and 8-OHM enantiomer and 62.5, 125, 312.5, 625 and 1250 ng/mL of each DMR enantiomer, in duplicate for each concentration. LID was used as internal standard (1000 ng/mL). Plots of drug/internal standard peak area ratios *versus* analyte concentrations were constructed and the relationship was determined by linear regression lines. The quantification limit (LOQ) of the method was assayed by analyzing 1.0 mL urine samples spiked with 62.5 ng/mL ($n = 5$ of each compound enantiomer) against calibration curves analyzed in the same conditions. The selectivity of the method was assured by analyzing 25 μL standard solutions of several drugs (1 mg/mL), using the same electrophoretic conditions as described for MRT and its metabolites. In cases in which the drug migration time (t_m) was similar to the enantiomers of MRT, DMR and 8-OHM, the experiment was repeated after submitting the drug to the extraction procedure. The efficiency of the extraction procedure was evaluated by analyzing the urine samples spiked with 100, 875, 1750 ng/mL of MRT and 8-OHM enantiomers and 100, 500 and 1000 ng/mL of DMR enantiomers ($n = 3$ of each compound enantiomer) against calibration curves obtained by the analysis of analytes not submitted to extraction. The precision (expressed as coefficient of variation CV) and accuracy (expressed as relative error E) were obtained by within-day ($n = 5$ of each compound enantiomer) and between-day (three consecutives days) assays using urine samples spiked with 100, 875, 1750 ng/mL of MRT and 8-OHM enantiomers and 100, 500, 1000 ng/mL of DMR enantiomers.

Aliquots of the quality control samples ($n = 3$) at the concentration of 100 and 1750 ng/mL of MRT and 8-OHM enantiomers, as well as 100 and 1000 of DMR enantiomers were used to determine the freeze–thaw cycle and short-term room temperature (23 ± 2°C, for 12 h) stability tests. The peak areas obtained from both stability tests were compared with the peak areas obtained with freshly prepared samples. Student's t-test was applied, with the significance level at $p \leq 0.05$.

2.6 Method application

To assess the applicability of the method, the cumulative urinary excretion rate of the MRT, DMR and 8-OHM enantiomers was determined in the urine samples collected from an healthy volunteer after a single oral administration of 45 mg of *rac*-MRT (Remeron®, N.V. Organon). Urine samples were taken prior to dosage and at 0–4, 4–8, 8–12, 12–16, 16–24, 24–48, 48–54 and 54–72 h intervals. The urine volumes were recorded and 10 mL aliquots were stored frozen (−20°C) until analysis ($n = 2$). The subject had signed written and informed consent prior to the investigation, and the study was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences of Ribeirão Preto—University of São Paulo (CEP-FCFRP/USP, process number 03/2003).

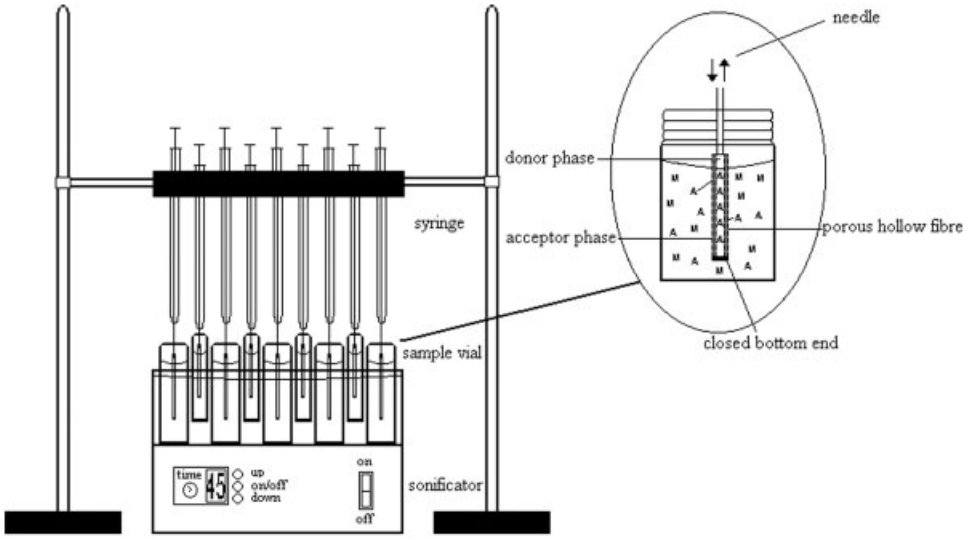

Figure 2. Schematic representation of the multiple LPME device using hollow fiber with sealed end. (A) target analytes and (M) matrix.

3. Result and discussion

3.1 Electrophoresis conditions

The enantiomeric separation of MRT and its main metabolites was optimized by evaluating several parameters, including chiral selectors (type and concentration), running buffer (type, pH and electrolyte concentration), applied voltage (kV) and capillary temperature (°C). In these experiments, the standard solutions were prepared in 5 mmol/L phosphate buffer solution, pH 5 (1:10 dilution of the running buffer) to produce a stacking effect and to improve peak efficiency. The enantioselective resolution of MRT, 8-OHM and DMR was evaluated by employing cyclodextrins (CDs) as chiral selectors: hydroxypropyl-β-cyclodextrin (HP-β-CD), sulfated-β-cyclodextrin (S-β-CD) and carboxymethyl-β-cyclodextrin (CM-β-CD) added to 50 mmol/L phosphate buffer solution (pH 2.5).

3.1.1 Influence of CD type

At this pH value, the analytes were protonated and the ionization of the acid silanols was small, Hence the electrosmotic flow was minimal. The uncharged HP-β-CD and CM-β-CD (not charged at pH 2.5) do not migrate under these conditions and the electrophoretic mobility was the only force responsible for the migration rate of the analyte enantiomers from the anode toward the detector. The mobility was reduced for the enantiomer with greater interaction with the CD [35]. Under these conditions, simultaneous baseline chiral resolutions of MRT, DMR and 8-OHM were achieved using CM-β-CD and a partial separation was observed using HP-β-CD. On the other hand, anionic S-β-CD (negatively charged over the whole pH range) possesses a self-electrophoretic mobility and the analyte–chiral selector complex migrated only under reverse polarity, and, in addition, high levels of current ($\approx$100 μA) were produced, resulting in poor resolution due to an increase in Joule heating within the capillary.

3.1.2 Influence of CD concentration

The CD concentration affects largely the enantiomer separation as well as the levels of current (for charged CD), EOF and peak tailing [36]. Unfortunately, changes in the concentration of HP-β-CD (20–40 mM) and S-β-CD (0.2–1%) do not improve the chiral resolution of MRT and its metabolites. Therefore, the simultaneous baseline chiral resolution of MRT, DMR and 8-OHM was only possible by using CM-β-CD. At low CM-β-CD concentration (0.1%) there was not enough chiral selector available to form complexes and, therefore, no enantiomer separation was possible for 8-OHM. A suitable chiral resolution was observed in the range of 0.24–0.55% and in this case, the resolution did not change significantly with the CD concentration, but the reproducibility was better at high concentrations. However, at concentrations higher than 0.55%, the analysis time increased significantly for MRT, DMR and 8-OHM, and the reproducibility decreased again (Fig. 3). Thus, 0.55% of CM-β-CD was selected for further experiments.

3.1.3 Influence of buffer composition

In order to investigate the influence of the buffer composition in the enantioseparation of MRT, DMR and 8-OHM, several experiments were performed at optimum pH and buffer concentration using Tris, acetate, borate and

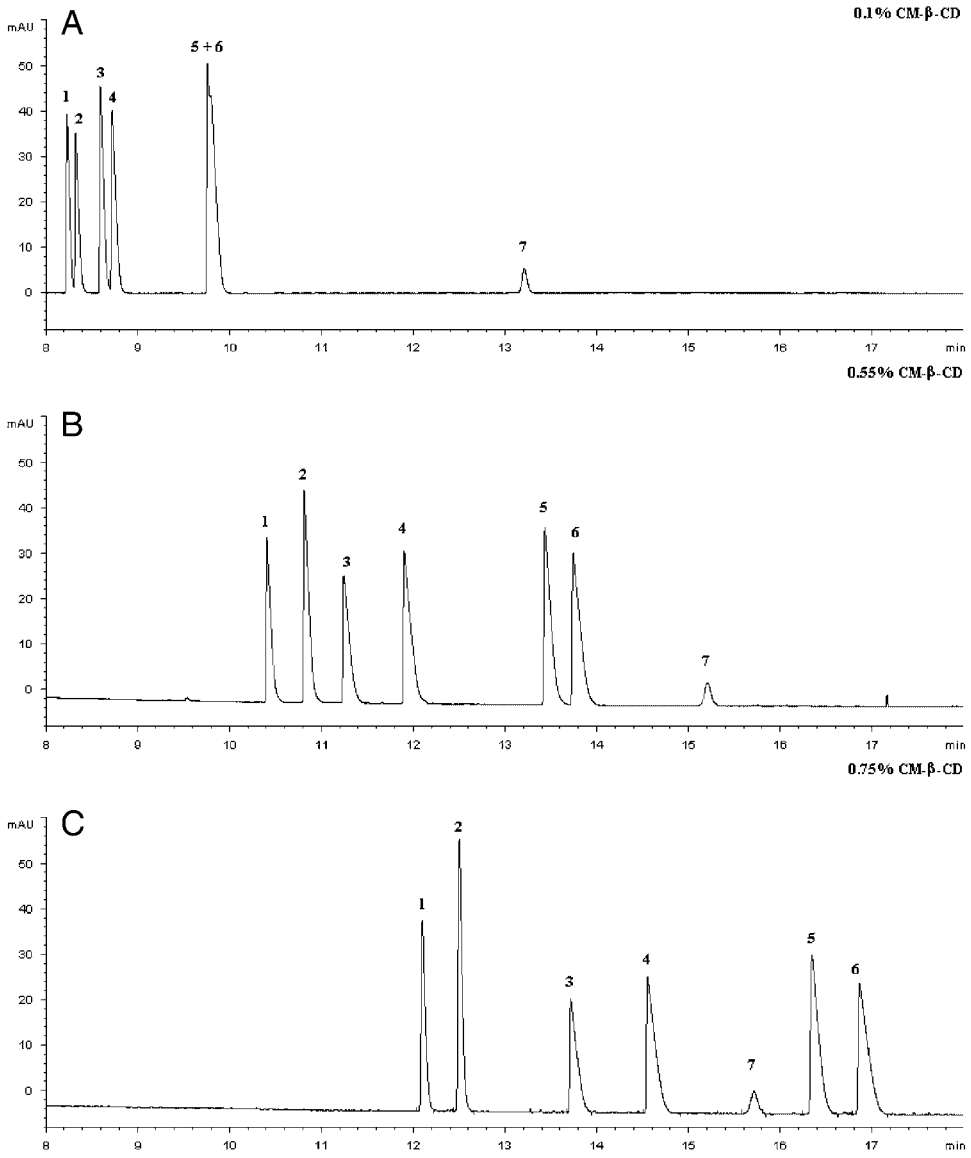

Figure 3. Effect of CM-β-CD concentration on chiral resolution of MRT, DMR, 8-OHM and LID standard solutions. Electrophoretic conditions: uncoated fused silica capillary, 80 cm (72 cm effective length) × 50 μm i.d.; running buffer, 50 mmol/L phosphate buffer solution (pH adjusted to 2.5 with phosphoric acid) containing CM-β-CD (w/v); injection (50 mbar, 20 s) at the anodic end of the capillary; applied voltage, +25 kV; detection wavelength, 205 nm; capillary temperature, 25°C. (A) 0.1% CM-β-CD, (B) 0.55% CM-β-CD and (C) 0.75% CM-β-CD. (1) (−)-(*R*)-DMR, (2) (−)-(*R*)-MRT, (3) (+)-(*S*)-DMR, (4) (+)-(*S*)-MRT, (5) (−)-(*R*)-8-OHM, (6) (+)-(*S*)-8-OHM and (7) LID.

phosphate buffer solutions. Baseline resolution showing low conductibility (current ≈ 50 μA) and high reproducibility was achieved for MRT, DMR and 8-OHM using phosphate buffer solutions. Only minor improvements in the resolution of MRT, DMR, 8-OHM enantiomers and internal standard were obtained when the buffer concentration was increased from 50 to 100 mmol/L, but increases in the analysis time were observed. Consequently, 50 mmol/L, were selected for further experiments. The influence of buffer pH was evaluated at values of 2.0, 2.5, 3.5, 5.0, 7.0 and 9.0. No peak separation was observed using buffer solutions below pH 2.5, whereas at pH values higher than this value resulted in lower resolution.

3.1.4 Effect of temperature and applied voltage

The operating temperature and voltage were optimized in combination with the choice of electrolyte concentration and capillary dimensions in order to produce suitable resolution, analysis time and acceptable level of current.

Optimum conditions were obtained using 50 mmol/L phosphate buffer solution (pH 2.5) containing 0.55% w/v of CM-β-CD under a constant voltage of +25 kV at 25°C. Under these conditions, suitable chiral resolution was observed for all target analytes in 18 min with acceptable levels of current (Fig. 4). The samples were injected for 20 s at 50 mbar. In

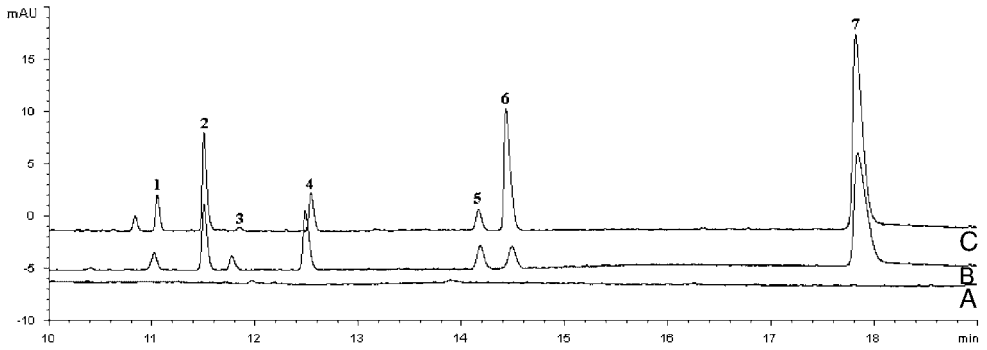

Figure 4. Representative CE electropherograms from (A) blank urine; (B) urine spiked with 1000 ng/mL internal standard and 312.5 ng/mL MRT and its metabolites enantiomers; (C) urine sample collected between 8 and 12 h after single oral dosing with 45 mg *rac*-MRT from a healthy volunteer. Electrophoretic conditions: uncoated fused silica capillary, 80 cm (72 cm effective length) × 50 μm i.d.; running buffer, 50 mmol/L phosphate buffer solution (pH adjusted to 2.5 with phosphoric acid) containing 0.55% w/v carboxymethyl-β-cyclodextrin (CM-β-CD); injection (50 mbar, 20 s) at the anodic end of the capillary; applied voltage, +25 kV; detection wavelength, 205 nm; capillary temperature, 25°C. All samples were pre-treated by LPME. (1) (−)-(*R*)-DMR (400.1 ng/mL), (2) (−)-(*R*)-MRT (539.7 ng/mL), (3) (+)-(*S*)-DMR (66.2 ng/mL), (4) (+)-(*S*)-MRT (226.4 ng/mL), (5) (−)-(*R*)-8-OHM (169.8 ng/mL), (6) (+)-(*S*)-8-OHM (919.3 ng/mL) and (7) LID.

Table 1. Mean recoveries and linearities of the analytical method

Analytes	Mean recovery		Linearity		
	%	CV (%)	Calibration curve equation	r^2	CV (%)
(+)-(*S*)-MRT	75.9	8.9	0.0006*x*–0.0284	0.9976	13.7
(−)-(*R*)-MRT	80.0	4.6	0.0006*x*–0.0330	0.9969	15.4
(+)-(*S*)-DMR	23.4	5.7	0.0004*x*–0.0712	0.9893	13.5
(−)-(*R*)-DMR	23.9	7.7	0.0004*x*–0.0919	0.9928	14.1
(+)-(*S*)-8-OHM	23.6	10.6	0.0005*x*–0.0238	0.9974	13.9
(−)-(*R*)-8-OHM	23.8	10.0	0.0005*x*–0.0271	0.9978	14.4

r^2, determination coefficient; CV, coefficient of variation for recovery and slope of the calibration curves.
The linear range was 62.5–2500 ng/mL for each MRT and 8-OHM enantiomer and 62.5–1250 ng/mL for each DMR enantiomer.

order to guarantee the reproducibility of the migration times and of the selectivity, the buffer reservoir was changed after nine consecutive analyses. The migration order was defined by analyzing the pure enantiomers obtained by High-Performance Liquid Chromatography semipreparative analysis [11]. Under the adopted experimental conditions, the (−)-(*R*)-isomer migrated first and the (+)-(*S*)-isomer was the second migrating peak both for the drug and for the metabolites.

3.2 Optimization of the hydrolysis and LPME procedures

The three-phase LPME procedure for the extraction of MRT, 8-OHM and DMR from human urine was based on a method developed recently by our group for the analysis of these drugs in plasma samples [15]. Preliminary experiments were performed using 1.0 mL human urine samples spiked with MRT and its metabolites (1250 ng/mL of each enantiomer), alkalinized with 2 mL of 0.5 mol/L (pH 11) phosphate buffer solution and supplemented with 10% sodium chloride. Other experimental conditions were similar to the previous method reported.

After establishing these initial conditions, the extraction method was adapted to analyze the enantiomers of MRT, 8-OHM and DMR after hydrolysis. Hence, a hydrolysis step (acid, basic and enzymatic) was evaluated before the extraction procedure. The acid and basic hydrolysis proved to be inappropriate for the simultaneous determination of MRT enantiomers and its metabolites in human urine due to the presence of interfering peaks when drug-free urine sample was analyzed (data not shown). On the other hand, the addition of 100 μL β-glucuronidase enzyme solution and 0.5 mL of 0.5 mol/L phosphate buffer solution (pH 5) to 1.0 mL human urine, before LPME extraction procedure and CE analysis, proved to be suitable for the analysis of MRT enantiomers and its metabolites. The high selectivity of the CE system and the excellent clean-up properties of the three-phase LPME technique, as well as the specific enzymatic hydrolysis resulted in electropherograms, which were completely free from endogenous interfering peaks (Fig. 4).

Table 2. Within-day precision and accuracy for the analysis of MRT, DMR and 8-OHM enantiomers in human urine

Drug/metabolite	Quantification limit			100 ng/mL			875/500 ng/mL			1750/1000 ng/mL		
	Precision		Accuracy	Precision		Accuracy	Precision		Accuracy	Precision		Accuracy
	CV[a)]	Conc[b)]	*E*[c)]	CV[a)]	Conc[b)]	*E*[c)]	CV[a)]	Conc[b)]	*E*[c)]	CV[a)]	Conc[b)]	*E*[c)]
(+)-(S)-MRT	9.7	58.3	−6.8	12.3	102.9	2.9	9.4	780.2	−10.8	4.7	1524.4	−12.9
(−)-(R)-MRT	13.6	58.3	−6.8	14.2	110.3	10.3	11.0	792.1	−9.5	6.6	1534.5	−12.3
(+)-(S)-DMR	6.9	58.0	−7.2	2.6	93.1	−6.8	5.1	584.6	9.1	7.8	972.4	−2.7
(−)-(R)-DMR	5.0	59.2	−5.2	8.2	93.4	−6.6	7.9	611.0	14.2	11.5	977.4	−2.3
(+)-(S)-8-OHM	10.7	58.5	−6.4	7.9	93.8	−6.2	11.3	805.3	−8.0	8.6	1554.1	−11.2
(−)-(R)-8-OHM	11.0	56.5	−9.5	8.5	94.2	−5.8	9.8	768.0	−15.0	4.0	1486.4	−15.0

a) CV, coefficient of variation (expressed as percentage).
b) Conc, concentration analyzed (expressed as ng/mL).
c) *E*, relative error (expressed as deviation from the theoretical values).
Number of analysis, $n = 5$.

Table 3. Between-day precision and accuracy for the analysis of MRT, DMR and 8-OHM enantiomers in human urine

Drug/metabolite	100 ng/mL			875/500 ng/mL			1750/1000 ng/mL		
	Precision		Accuracy	Precision		Accuracy	Precision		Accuracy
	CV[a)]	Conc[b)]	*E*[c)]	CV[a)]	Conc[b)]	*E*[c)]	CV[a)]	Conc[b)]	*E*[c)]
(+)-(*S*)-MRT	9.3	99.6	−0.3	9.4	796.4	−7.4	8.7	1583.9	−9.5
(−)-(*R*)-MRT	9.4	103.4	3.8	5.7	794.5	−7.2	9.0	1621.7	−7.3
(+)-(*S*)-DMR	7.5	94.3	−5.7	13.1	551.0	2.9	10.4	1041.1	4.1
(−)-(*R*)-DMR	6.9	95.9	−4.1	13.0	570.5	6.6	11.3	1061.7	6.2
(+)-(*S*)-8-OHM	4.7	96.1	−3.9	10.6	819.0	−5.6	9.9	1649.9	−5.7
(−)-(*R*)-8-OHM	7.8	93.3	−6.7	10.4	779.2	−10.1	6.8	1573.5	−10.1

a) CV, coefficient of variation (expressed as percentage).
b) Conc, concentration analyzed (expressed as ng/mL).
c) *E*, relative error (expressed as deviation from the theoretical values).
Number of days, $n = 3$.

Table 4. Evaluation of the interference of some drugs with the chiral analysis of MRT and its metabolites

Drug	t_m[a)]	Drug	t_m[a)]	Drug	t_m[a)]
MRT	8.164/9.495	Disopyramide	11.579	Mexiletine	10.496
DMR	7.869/8.933	Etosuximide	ND	Mianserin	ND
8-OHM	10.758/11.131	Phenylbutazone	ND	Phenobarbital	ND
Albendazole	ND	Phenoprofen	ND	Procainamide	8.375
Amiodarone	ND	Phenproporex	11.260	Propiophenone	ND
Amitriptyline	ND	Haloperidol	ND	Propoxyphene	ND
Atropine	17.684	Imipramine	ND	Propranolol	15.327/15.695
Carbamazepine	ND	Lercanidipine	ND	Thioridazine	ND
Chlorpromazine	ND	Levomepromazine	ND	Trimetropim	10.323
Cimetidine	10.328	Lidocaine (I.S.)	13.360		

Eletrophoretic conditions as in Fig. 3. The analyses were performed using a capillary with 48 cm of effective length.
a) t_m, migration time in minutes; ND, not detected by the electrophoretic method up to 20 min of analysis.

3.3 Method validation

Under the optimized conditions, recovery values higher than those recently reported by our group [13, 14] were obtained for the enantiomers of MRT (75.9 and 80.0%), DMR (23.4 and 23.9%) and 8-OHM (23.6 and 23.8%) with CV% values lower than 10.6% (Table 1). DMR recovery could be increased by increasing the pH of the

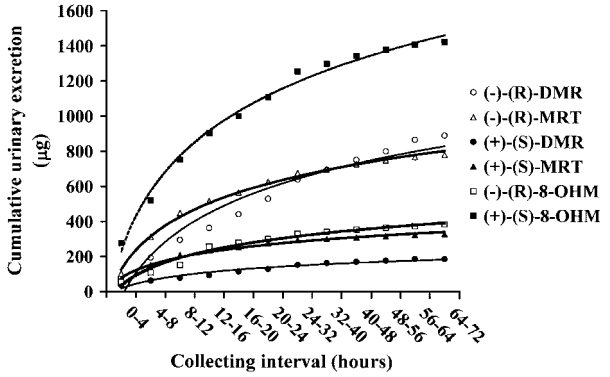

Figure 5. Cumulative urinary excretion of *R* and *S* forms of MRT, DMR, 8-OHM after single oral doses of 45 mg rac-MRT to an healthy volunteer.

donor phase but, in this case, lower recoveries were observed for the metabolite 8-OHM. Suitable extraction for both 8-OHM and DMR without losing the MRT LPME extraction capacity was observed at pH 8. In addition, the inclusion of a protein precipitation step before the extraction procedure provided cleaner samples, which resulted in low quantification limits.

The method proved to be linear over the concentration range of 62.5–2500 ng/mL for each MRT and 8-OHM enantiomer and 62.5–1250 ng/mL for each DMR enantiomer with typical calibration curve equations and determination coefficients as shown in Table 1. Suitable within- (Table 2) and between-day assay (Table 3) CVs for both precision and accuracy of all analytes enantiomers analyzed were below 15%. In addition, it was possible to quantify the analytes within their therapeutically relevant concentration levels in human urine (LOQ = 62.5 ng/mL) for each enantiomer with very good precision and accuracy (Table 2).

The method developed here proved to be selective, since the t_m for most drugs analyzed under the established electrophoretic conditions was not similar to those obtained for the MRT, its metabolites and for the internal standard. In addition, when some drugs analyzed had shown coincident t_m with MRT, its metabolites and with internal standard, they were no longer detected after being submitted to the three-phase LPME procedure (Table 4). The blank urine collected immediately before the drug administration showed no interference in the migration times of the analytes (Fig. 4).

Stability test demonstrated no statistically significant difference between freeze–thaw cycle and short-term room temperature stability studies with p values ≥ 0.05.

3.4 Method application

Finally, the method was used for the analysis of urine samples collected from a volunteer after a single oral administration of *rac*-MRT (Fig. 4). The cumulative urinary excretion *versus* collecting intervals for each MRT and its metabolite enantiomers were plotted on a graph. As shown in Fig. 5, the results obtained in this preliminary cumulative urinary study were in agreement with published data [9]. According to these authors, less than 4% of the administered dose was excreted unchanged and MRT is predominantly metabolized to 8-hydroxy analogues. In addition, previous works [11, 12, 15] have shown that the pharmacokinetics of MRT appears to be enantioselective. These findings are in accordance with the results in Fig. 5, in which the differences in the cumulative urinary excretion profile of MRT and its metabolite enantiomers are shown.

4 Concluding remarks

This paper describes for the first time a single and selective LPME-CE method, with negligible organic solvent consume, for the simultaneous chiral determination of MRT and its two major metabolites in human urine samples within 18 min. The developed and validated method allows the determination of MRT and its metabolites with LOQ of 62.5 ng/ml for all enantiomers. Finally, this method proved to be suitable for a preliminary cumulative urinary excretion study.

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