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Short communication

Simultaneous stereoselective high-performance liquid chromatographic determination of 10-hydroxycarbazepine and its metabolite carbamazepine-10,11-*trans*-dihydrodiol in human urine

Andrew Volosov[a], Meir Bialer[a,c], Sun Xiaodong[d], Emilio Perucca[e], Amnon Sintov[f], Boris Yagen[b,c,*]

[a]*Department of Pharmaceutics, School of Pharmacy, The Hebrew University of Jerusalem, Jerusalem, Israel*
[b]*Department of Natural Products, School of Pharmacy, The Hebrew University of Jerusalem, Jerusalem, Israel*
[c]*The David R. Bloom Center for Pharmacy, Jerusalem, Israel*
[d]*Institute of Clinical Pharmacology, Great Chinese Wall Hospital, Beijing, China*
[e]*Clinical Pharmacology Unit, Department of Internal Medicine and Therapeutics, University of Pavia, Pavia, Italy*
[f]*Institute of Applied Research, Ben-Gurion University, Beer-Sheva, Israel*

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Abstract

An enantioselective HPLC method for the simultaneous determination of the concentration of the enantiomers of the oxcarbazepine metabolites 10-hydroxycarbazepine (MHD) and carbamazepine-10,11-*trans*-dihydrodiol (DHD) in human urine is described. The method is based on extraction with *tert*.-butylmethyl ether–dichloromethane (2:1, v/v) under alkaline conditions, separation and evaporation of the organic phase and dissolution of the residue in the mobile phase. Enantiomers are resolved on a Diacel Chiralcel OD column (250 mm×4.6 mm I.D.) under isocratic conditions using as mobile phase *n*-hexane–ethanol–2-propanol (18:2:1, v/v/v) with addition of glacial acetic acid (0.1%). The enantiomers are detected by UV at 215 nm. The method allows reliable determination of the MHD and DHD enantiomers in human urine with limits of quantification of 0.2 mg/l and 0.4 mg/l, respectively.

Keywords: 10-Hydroxycarbazepine; Carbamazepine-10,11-*trans*-dihydrodiol

1. Introduction

Oxcarbazepine (OXC) (Fig. 1) is a novel antiepileptic drug, which was developed as a second-generation and follow-up compound to carbamazepine (CBZ) [1,2]. Unlike CBZ, which in humans is metabolized by CYP3A4 and CYP2C8 to a stable CBZ-10,11-epoxide (CBZ-E) [3], OXC undergoes rapid presystemic 10-keto-reduction by cytosolic enzymes in the liver to its monohydroxylated derivative 10-hydroxycarbazepine (MHD), which is then partly conjugated with glucuronic acid and partly converted to carbamazepine-10,11-*trans*-dihydrodiol (DHD) prior to excretion in the urine [4–6]. MHD possesses antiepileptic activity similar to that of OXC and it is essentially responsible for the pharma-

*Corresponding author. Department of Natural Products, School of Pharmacy, The Hebrew University of Jerusalem, P.O. Box 12065, Jerusalem, Israel. Tel.: +972-2-6758-606; fax: +972-2-6436-246.
E-mail address: yagen@cc.huji.ac.il (B. Yagen)

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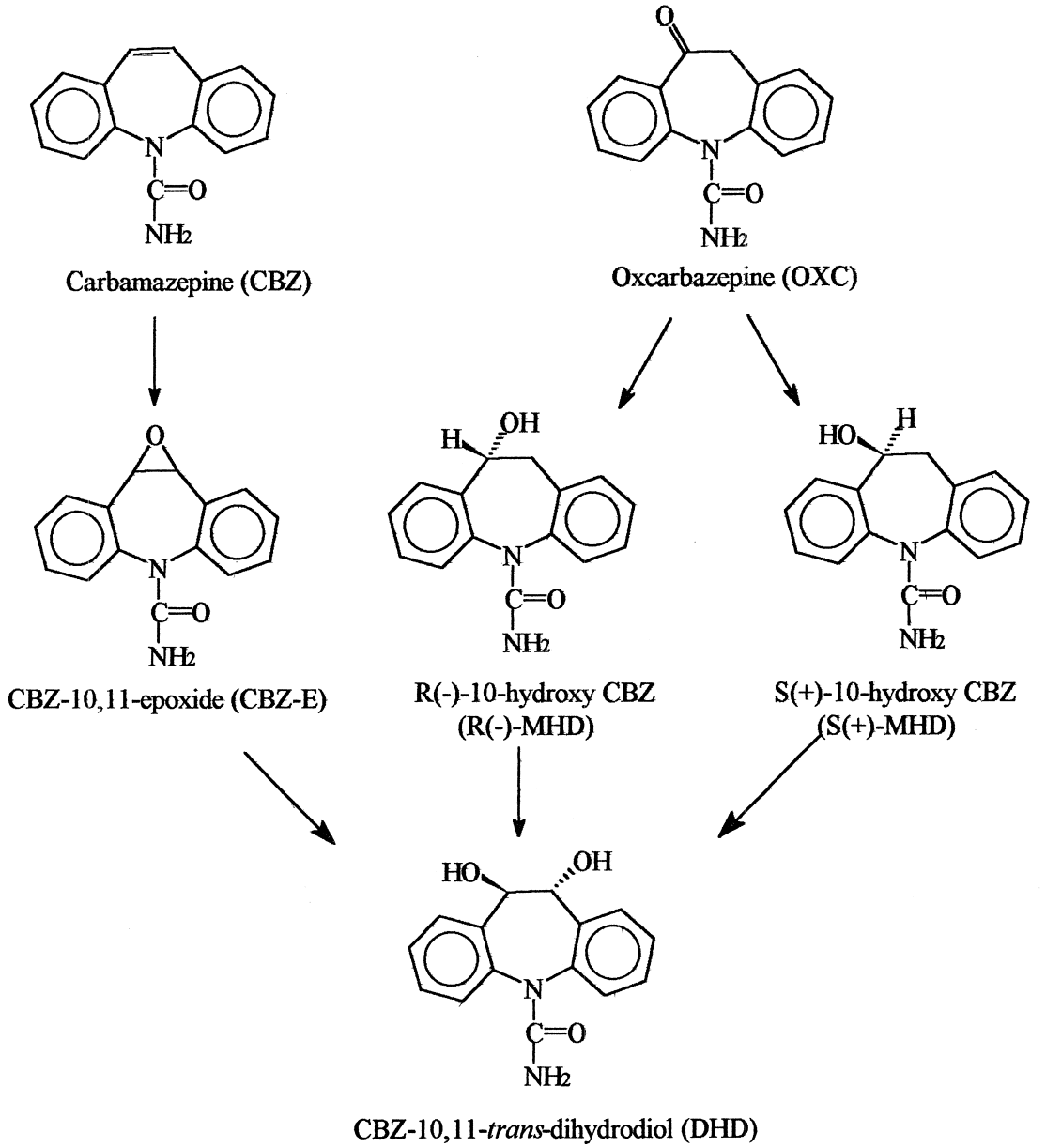

Fig. 1. Chemical structures and metabolic pathways of OXC and CBZ.

cological effects observed after administration of OXC in humans [7].

The molecule of MHD has a chiral center at position 10 and, accordingly, it exists in two enantiomeric forms [5]. Previous reports indicated that the first-pass reduction of OXC to MHD in humans is stereoselective, resulting in approximately a 1:4 area under concentration–time curve (AUC) ratio between the *R*-(−) and the *S*-(+) enantiomer [5,8,9]. The issue of stereoselectivity in formation of the secondary metabolite DHD, however, has not been addressed in the literature, partly due to the lack of an assay for the analysis of individual DHD enantiomers. Since DHD has two chiral centers at positions

10 and 11, its *trans*-configuration exists in two enantiomeric forms. Although DHD reportedly is devoid of pharmacological activity, differentiation of its enantiomers in body fluids may provide additional insights into the stereoselectivity of OXC metabolism. As DHD is also one of the main urinary metabolites of CBZ (Fig. 1), the availability of an assay method for its enantiomers could also be applied to investigation of chiral factors involved in CBZ metabolism and may provide additional information for comparison between metabolic pathways of OXC and CBZ (Fig. 1).

The present article describes a sensitive and reliable high-performance liquid chromatography (HPLC) method for the simultaneous stereoselective determination of the two enantiomers of MHD and DHD in human urine samples using a chiral column and UV detection.

2. Experimental

2.1. Chemicals

MHD (racemate and its enantiomers) and racemic DHD were obtained from Novartis. Other chemicals and solvents were purchased from Sigma and Merck and were of the highest grade available. Oxcarbazepine oxime, which was used as internal standard was synthesized according to the procedure described below.

2.2. Synthesis of oxcarbazepine oxime

A mixture of 50 mg of oxcarbazepine and 100 mg of hydroxylamine hydrochloride in 5 ml of ethanol and 1 ml of pyridine were refluxed for 30 min. The alcohol was evaporated and the residue was dissolved in dichloromethane. The organic solution was washed with cold hydrochloric acid (5%) and then with water, dried with magnesium sulphate, filtered and evaporated. The residue was recrystallized from ethyl acetate. The identity of the product (m.p. 231°C, with decomposition) was confirmed by nuclear magnetic resonance (NMR), IR and mass spectrometry.

2.3. Samples and solutions

Stock solutions of MHD, DHD and the internal standard (oxcarbazepine oxime) were prepared by dissolving each compound in methanol to obtain concentrations of 5 mg/l for the enantiomers and 2 mg/l for the internal standard. Aliquots from these stock solutions were taken to prepare spiked plasma samples for calibration. To test the applicability of the assay, the urine samples for analysis were collected from a healthy male volunteer over 12 h after oral administration of 600 mg of OXC [9].

2.4. Sample preparation

After adding the internal standard (10 μl of 2 mg/ml oxcarbazepine oxime in methanol), 200 μl of urine was mixed with 100 μl of 2 *M* KOH solution, extracted with 5 ml of *tert*.-butylmethyl ether–dichloromethane (2:1, v/v) mixture, vortexed vigorously for 30 s and centrifuged for 10 min at 1000 *g* (2300 rpm). The upper (organic) layer was separated and evaporated under vacuum (25 mmHg at 35°C), using the Buchler Instruments vortex evaporator (1 mmHg=133.322 Pa). The residue was dissolved in 200 μl of the mobile phase and a 20-μl aliquot was injected into the chromatograph.

2.5. Chromatographic conditions

The separation of MHD and DHD enantiomers was performed using a Shimadzu HPLC system with UV detector (Model 10A) equipped with a chiral Diacel Chiralcel OD column (250 mm×4.6 mm I.D., Shanir, Jerusalem, Israel). The detector was set at 215 nm. The mobile phase consisted of *n*-hexane–ethanol–2-propanol (18:2:1, v/v/v), with addition of glacial acetic acid (0.1%), and the flow-rate was 1.0 ml/min. Chromatography was performed at ambient temperature. Retention times were 10.6 min for *R*-(−)-MHD and 13.3 min for *S*-(+)-MHD. The two enantiomers of DHD eluted at 11.7 min and 16.2 min, and the internal standard at 14.6 min (Fig. 2B).

2.6. Calibration

Calibration curves with concentration range of 1–150 μg/ml were prepared by spiking 0.5 ml of

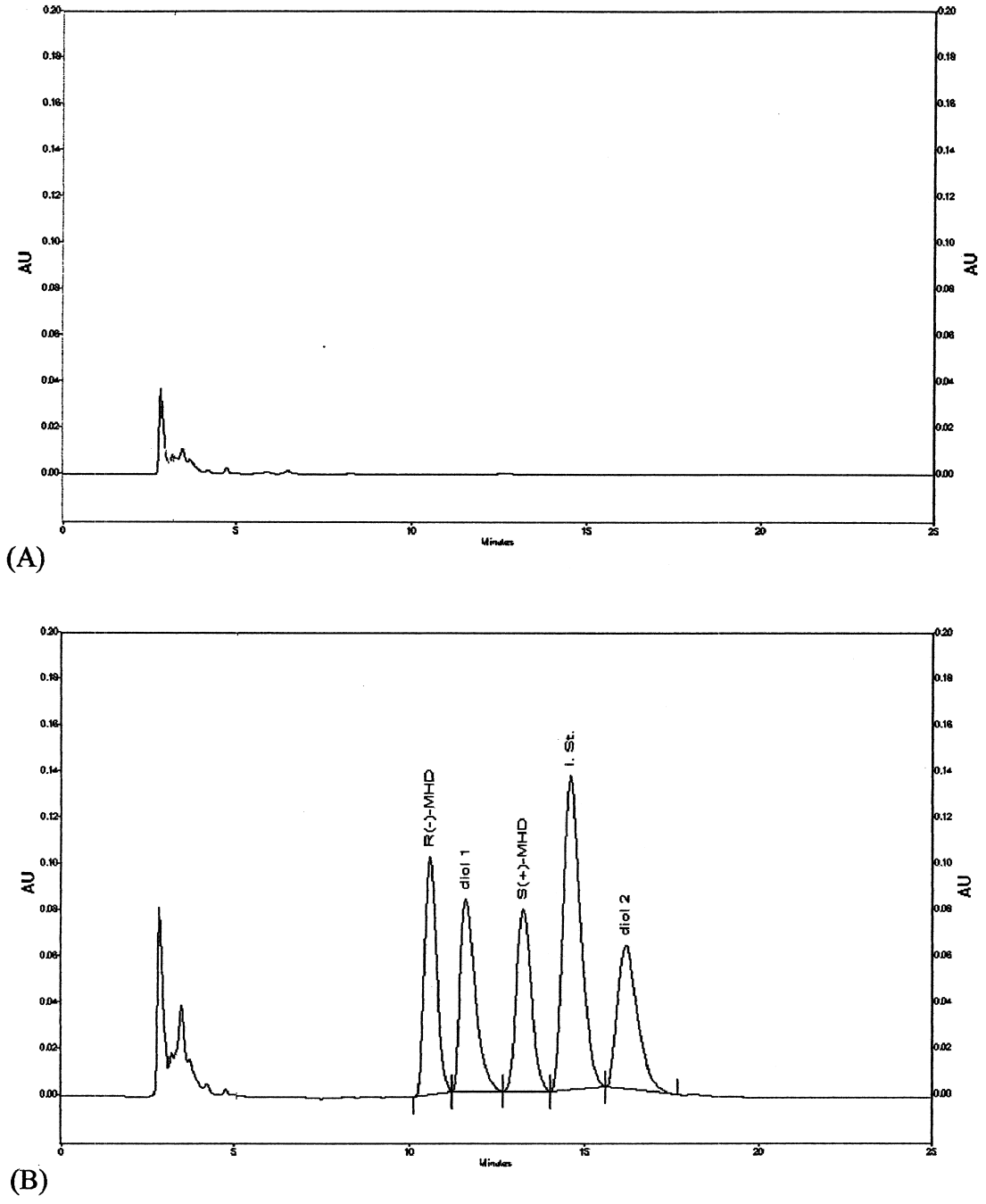

Fig. 2. Chromatograms of extracted blank urine (A), urine spiked with internal standard, MHD and DHD (B), and urine collected from a healthy male volunteer over the first 12 h following oral administration of OXC 600 mg (C).

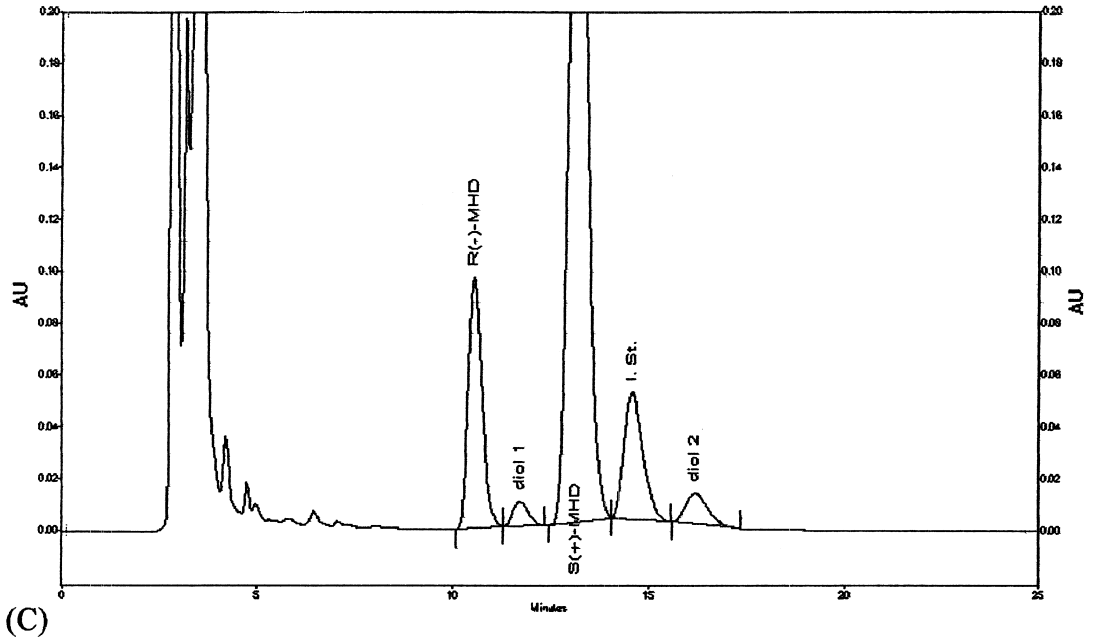

Fig. 2 (*continued*).

drug-free human urine with known amounts of MHD and DHD. The samples were then processed as described above and 20 μl of the reconstituted extract was injected into the chromatograph. Peak height ratios (PHRs) between the test compounds and the internal standard were plotted as a function of the concentration of each analyte in the sample. Calibration curves were constructed by method of least-squares linear regression with $1/x$ weighting.

3. Results and discussion

Fig. 2 shows chromatograms of extracted blank urine (A), urine spiked with internal standard, MHD and DHD (B), and urine collected from a healthy male volunteer over the first 12 h following oral administration of OXC 600 mg (C). No parent or related compounds, or other interfering substances were detected. The retention time of the parent compound OXC under the same chromatographic conditions is 18.3 min. The related compound CBZ elutes after 13.8 min. CBZ-E is not detectable under the described conditions because of its instability in acidic environment. Although CBZ is not sufficiently resolved from *S*-(+)-MHD, the assay may also be used for determination of the enantiomers of DHD in urine after administration of CBZ, since in this case the MHD enantiomers are not produced.

Calibration curves for all compounds were linear over the concentration range of 1–150 mg/l. The linear regression parameters for the equation (PHR= slope×concentration+intercept) are presented in Table 1. Since standards for the DHD enantiomers were not available, the absolute configurations of these enantiomers could not be determined and therefore they are referred to as diol 1 and diol 2 throughout the text.

3.1. Recovery

Extraction efficiencies of MHD and DHD from human urine were determined at the low, medium and high levels of the concentration range used for calibration, i.e., 1, 75 and 150 mg/l. Absolute peak heights obtained after injection of standards of known concentrations were compared with those obtained after injection of spiked urine samples that underwent the extraction procedure described above. The absolute extraction efficiency was determined as

Table 1
Linear regression parameters of typical calibration curve (concentration range 1–150 μg/ml, $n=6$)

	R-MHD	*S*-MHD	Diol 1	Diol 2
Slope (ml/μg)	0.0406	0.0308	0.0263	0.0191
Standard error of the slope	0.0003	0.0003	0.0004	0.0003
Intercept	−0.0028	−0.0073	−0.0147	−0.0094
Standard error of the intercept	0.0027	0.0051	0.0130	0.0070
Standard error of the estimate[a]	0.0362	0.0416	0.0517	0.0428

[a] Also known as standard deviation around the regression line.

the sample/standard peak height ratio. In all cases extraction efficiency was over 90%, with relative standard deviation (RSD) not exceeding 5%.

3.2. Accuracy and precision

Accuracy (ratio between the measured concentration and the spiked concentration) and precision (expressed as RSD) were determined by analyzing on four different days spiked urine samples with concentrations ranging from 1 to 150 mg/l. Results are presented in Table 2. As can be seen from the table, the RSDs ranged from 0.8 to 5.1% and the measured concentrations were between 94.8 and 109.0% of the nominal (spiked) concentrations, indicating good precision and accuracy of the assay.

Table 2
Precision and accuracy of the assay for MHD and DHD enantiomers in human urine

Spiked concentration (mg/l)	Measured concentration (mg/l)					RSD (%)	Accuracy (%)
	Day 1	Day 2	Day 3	Day 4	Mean		
R-(−)-MHD							
1	1.07	1.08	1.12	1.00	1.07	4.7	106.8
10	10.08	9.95	10.02	9.69	9.94	1.7	99.4
20	20.56	20.24	19.85	20.48	20.28	1.6	101.4
60	58.13	58.37	59.71	59.53	58.94	1.4	98.2
100	94.81	98.41	98.08	97.25	97.14	1.7	97.1
150	152.49	147.82	152.13	156.96	152.35	2.5	101.6
S-(+)-MHD							
1	1.06	1.11	1.13	1.06	1.09	3.3	109.0
10	10.00	9.67	9.84	9.63	9.79	1.7	97.9
20	20.32	19.86	19.53	20.16	19.97	1.7	99.8
60	58.00	58.04	59.53	58.99	58.64	1.3	97.7
100	95.25	98.59	98.58	97.03	97.36	1.6	97.4
150	155.00	149.72	154.25	158.12	154.27	2.2	102.8
Diol 1							
1	1.05	1.03	1.07	0.95	1.03	5.1	102.5
10	10.27	10.05	10.15	9.90	10.09	1.6	100.9
20	19.56	19.26	19.02	19.45	19.32	1.2	96.6
60	57.73	58.06	59.22	58.75	58.44	1.1	97.4
100	93.98	97.54	97.08	95.94	96.14	1.7	96.1
150	156.20	151.82	155.42	159.74	155.80	2.1	103.9
Diol 2							
1	1.09	1.02	1.09	1.02	1.06	3.8	105.5
10	10.02	9.80	9.99	9.74	9.89	1.4	98.9
20	19.22	18.89	18.65	19.08	18.96	1.3	94.8
60	57.53	57.73	58.60	58.14	58.00	0.8	96.7
100	94.41	97.56	97.13	95.91	96.25	1.5	96.3
150	158.74	153.72	157.13	161.03	157.66	1.9	105.1

3.3. Limit of quantification

The limit of quantification (LOQ) was determined based on the approach described by Jelliffe and co-workers [10,11]. For each compound, standard deviations (SDs) of replicates were plotted against the measured concentration and the relationships were fitted by a polynomial equation of third order. The equations were then used to calculate the LOQ, defined as the lowest concentration at which the RSD does not exceed 15%. LOQ values calculated by this procedure were 0.2 mg/l for *R*-MHD (RSD 11.5%) and *S*-MHD (RSD 11.1%) and 0.4 mg/l for diol 1 (RSD 13%) and diol 2 (RSD 15%). These values were confirmed by analyzing on four different days samples spiked with each of the analytes at the LOQ concentrations calculated above. For all samples, RSD values did not exceed 15% and accuracy was within the acceptable 80–120% range, thus confirming the theoretically calculated values.

3.4. Stability

All reference solutions were stable for at least a month if stored at 4°C. No change occurred in the 1:1 ratio of the enantiomers in the solutions of racemates. Both MHD and DHD enantiomers remained stable and no enantiomeric interconversion was observed after incubation of spiked urine samples for 48 h at 37°C with and without the presence of β-glucuronidase (110 350 units/ml, pH 5.0, Sigma).

4. Conclusions

The analytical method described is suitable for the simultaneous quantitative determination of the two MHD enantiomers and the two DHD enantiomers in human urine with quantification limits of 0.2 mg/l and 0.4 mg/l, respectively. The proposed procedure is fast, simple and may be usefully applied in pharmacokinetic and metabolic studies involving OXC and/or MHD. In addition, the method may be used in the CBZ studies, since DHD is the main urinary metabolite after administration of CBZ.

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