Analysis of unbound plasma concentration of oxcarbazepine and the 10-hydroxycarbazepine enantiomers by liquid chromatography with tandem mass spectrometry in healthy volunteers

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ABSTRACT

This study describes the development and validation of a method for the analysis of unbound plasma concentrations of oxcarbazepine (OXC) and of the enantiomers of its active metabolite 10-hydroxycarbazepine (MHD) [S-(+)- and R-(-)-MHD] using liquid chromatography with tandem mass spectrometry (LC-MS/MS). Additionally, the free fraction of the drug is described in healthy volunteers (n=12) after the oral administration of 300 mg OXC/12 h for 5 days. Plasma aliquots of 200 µL were submitted to ultrafiltration procedure and 50 µL of the ultrafiltrate were extracted with a mixture of *tert*-butyl methyl ether:dichloromethane (2:1, v/v). OXC and the MHD enantiomers were separated on a OD-H chiral phase column. The method was linear in the range of 4.0 to 2.0 µg/mL for OXC and of 20.0 to 6.0 µg/mL plasma for the MHD enantiomers. The limit of quantification was 4 ng for OXC and 20 ng for each MHD enantiomer/mL plasma. The intra- and inter-day precision and inaccuracy were less than 15%. The free fraction at the time of peak plasma concentration of OXC was 0.27 for OXC, 0.37 for S-(+)-MHD and 0.42 for R-(-)-MHD. Enantioselectivity in the free fraction of MHD was observed, with a higher proportion of R-(-)-MHD compared to S-(+)-MHD.

Key words oxcarbazepine, 10-hydroxycarbazepine, free fraction, LC-MS/MS, enantiomers.

1. INTRODUCTION

Oxcarbazepine (OXC) is considered a prodrug and part of its anticonvulsant effect depends on its active 10-hydroxycarbazepine (MHD) metabolite which is formed by the rapid presystemic reduction of OXC. MHD contains a chiral center at position 10, but the R-(-)- and S-(+)-MHD enantiomers exert similar anticonvulsant effects in animal models [1–5]. The kinetic disposition of the MHD metabolite is enantioselective in healthy volunteers after administration of a single oral dose of OXC, with an area under the plasma concentration versus time curve (AUC) S-(+)/R-(-) ratio of 3.8 [6].

The binding of drugs to plasma proteins affects different pharmacokinetic and pharmacodynamic parameters since only the free concentration is available for distribution, elimination and receptor interaction [7,8]. Plasma protein binding of chiral drugs can be enantioselective, affecting the pharmacological activity and pharmacokinetic profile of these drugs [9]. In patients with trigeminal neuralgia, the percentage of plasma protein binding was approximately 59% for OXC and 39% for the MHD metabolite [10]. Plasma protein binding of MHD administered as enantiomeric mixture to epileptic patients was 40% using equilibrium dialysis and 45% using ultrafiltration [11], while *in vitro* studies report values of 30% for both MHD enantiomers in rat and human plasma [12].

The methods for the separation of the unbound concentration of OXC and MHD described so far have used equilibrium dialysis or ultrafiltration [11-12], followed by HPLC with ultraviolet (UV) detection, for OXC and MHD as enantiomer mixture [10,11] or for the MHD enantiomers [12]. Regarding the methods for analysis of total concentration of oxcarbazepine and MHD enantiomers in plasma using LC-MS/MS, the

quantification limit reported ranged from 12.5 to 50.0 ng for OXC and of 31.5 ng to 50 ng for each MHD enantiomer/mL plasma [13-15].

There are no clinical data on the plasma protein binding of individual MHD enantiomers. The present study describes the development and validation of a method for the sequential analysis of the unbound concentration of OXC and MHD enantiomers in plasma using ultrafiltration and liquid chromatography coupled to mass spectrometry (LC-MS/MS). The method showing a quantification limit of 4.0 ng for OXC and of 20.0 ng for each MHD enantiomer/mL plasma, so far the most sensitive one, was used for analysis of the free fraction of the drug in plasma samples collected at the time of peak plasma concentration (t_{max}) from healthy volunteers after the oral administration of 300 mg OXC/12 h for 5 days.

2. MATERIALS AND METHODS

2.1. Analysis of the unbound concentration of OXC and of the MHD enantiomers

2.1.1. Standard solutions and reagents

Oxcarbazepine (99.6%) was purchased from USP (Rockville, USA) and the racemic MHD metabolite (98%) from Toronto Research Chemicals (North York, Canada). 4-Methylprimidone (internal standard, IS) was purchased from Sigma (St. Louis, MO, USA). The solvents dichloromethane and *tert*-butyl methyl ether were obtained from Mallinckrodt Baker (Phillipsburg, NJ, USA), hexane, methanol and ethanol from Panreac Química SAL (Barcelona, Spain), and isopropanol from Tedia Way (Fairfield, USA). All solvents were of chromatographic grade. Ammonium acetate was

obtained from Mallinckrodt Baker (Phillipsburg, Xalostoc, Mexico). The water used in the experiment was purified with the Sinergy UV® system (Millipore, Molsheim, France).

Stock solutions were prepared in methanol at a concentration of 100 μg OXC/mL and 200 μg MHD/mL. Dilutions were then prepared to obtain the working solutions at concentrations of 0.008, 0.02, 0.04, 0.2, 0.4, 0.8, 1.6, 2.4 and 4 μg OXC/mL methanol and of 0.08, 0.2, 0.4, 2.0, 4.0, 8.0, 16, 24 and 40 μg MHD/mL methanol. The 4-methylprimidone solution was prepared at a concentration of 200 μg/mL methanol and diluted to 40 μg/mL.

2.1.2. Sample preparation

Aliquots (200 μL) of blank plasma or plasma samples were transferred to a Centrifree® ultrafiltration device (Millipore, Carrigtwohill, Ireland). The plasma ultrafiltrate was obtained by centrifugation of the samples at 1,875 *g* for 40 min in a centrifuge with a fixed-angle rotor (angle of 36º) (model NT 825, Nova Técnica, Piracicaba, Brazil) refrigerated at 4ºC.

Aliquots (50 μL) of the ultrafiltrate were spiked with 25 μL of the IS and extracted with 2 mL of a mixture of *tert*-butyl methyl ether:dichloromethane (2:1, v/v). The tubes were shaken for 40 min in a horizontal shaker (Marconi desktop reciprocating shaker, model MA 139/CTF) and then centrifuged for 10 min at 1,275 *g* (Hitachi® refrigerated centrifuge, model CF8DL, Tokyo, Japan). The organic phases were separated and concentrated in a vacuum evaporation system (Christ®, model RVC 2-25 CD plus, Funkentstörungsgrad, Germany). The residues obtained were resuspended in 250 μL of the mobile phase and 15 μL was used for chromatographic analysis.

2.1.3. Chromatographic analysis

Chromatographic analysis of unbound plasma concentrations of OXC and of the MHD enantiomers in plasma was conducted as previously described by our research group regarding the analysis of the drugs as total concentration [13]. The LC-MS/MS system consisted of a Waters 1525µ binary gradient pump, 2777 automatic injector, TCM/CHM column oven, and XEVO TQ-S triple quadrupole mass spectrometer (Waters, Milford, USA).

2.1.4. Method validation

The method was validated according to the recommendations of the National Health Surveillance Agency (Agência Nacional de Vigilância Sanitária – ANVISA) (Resolution RDC No. 27 for May 17, 2012) for bioanalytical methods.

The calibration curves were constructed in triplicate at concentrations 4.0, 10, 20, 100, 200, 400 and 800 ng and 1.2 and 2 µg OXC/mL plasma, and 20, 50, 100 and 500 ng and 1.0, 2.0, 4.0, 6.0 and 10.0 µg of each MHD enantiomer/mL plasma. The linear regression equations and correlation coefficients were obtained from the standard/IS peak area ratios plotted against the respective plasma concentrations.

The limit of quantification was obtained by the analysis of 10 replicates of ultrafiltrate samples spiked with OXC and MHD at concentrations of 4.0 ng OXC/mL plasma and of 20.0 ng of each MHD enantiomer/mL plasma.

The precision and accuracy of the method were evaluated by intra- and inter-assay studies. Five aliquots of each quality control (QC) of OXC (4.0 and 12 ng and 0.6, 0.96 and 1.6 µg OXC/mL plasma) and MHD (20.0 and 60.0 ng and 3, 4.8 and 8.0 µg of each enantiomer/mL plasma) were analyzed in the same analytical run and in three days.

The matrix effect was evaluated by direct comparison of the peak areas of OXC, MHD enantiomers and IS injected directly into the mobile phase with the peak areas of the standard solutions (12.0 ng and 1.0 μg OXC/mL plasma, 60.0 ng and 5 μg of each MHD enantiomer/mL plasma, and IS) added to the blank plasma ultrafiltrates obtained from eight different volunteers, including four normal plasma samples, two lipemic samples and two hemolyzed samples.

For stability analysis, OXC (12.0 ng and 0.96 μg OXC/mL plasma) and MHD (60 ng and 4.8 μg of each enantiomer/mL plasma) QC samples were submitted to 6 h short-term room temperature, three freezes–thaw (-20 to 25°C) cycles and 24 h in the autoinjector at 4ºC stability tests. The samples were also kept frozen at -70ºC for 6 months to evaluate long-term stability. The results of the quality control analysis were compared with the nominal value.

2.2. Clinical protocol

The clinical protocol was performed as described in a previous study from our group [16]. Briefly, the project was approved by the Ethics Committees of the School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, and of the University Hospital of the Ribeirão Preto School of Medicine, University of São Paulo (Protocol No. 214). All subjects agreed to participate in the study by signing the free informed consent form. Twelve adults, non-obese healthy volunteers (22 to 45 years), non-smokers, with hepatic, renal and cardiac functions in the normal range, were included in the study.

The subjects received 300 mg OXC/12 h (Trileptal®, 300-mg tablets, Novartis, Basel, Switzerland) for 5 days. On the fifth day, after administration of the 9th dose of OXC with 200 mL water, serial blood samples (5 mL) were collected through an

intravenous catheter into heparinized syringes (5,000 IU Liquemine®, Roche) over a period of 12 h (zero, 0.25, 0.5, 0.45, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, and 12 h).

The plasma samples for chromatographic analysis were obtained by centrifugation (850 *g*, 10 min) of the blood samples and stored at -70ºC until the time of analysis.

2.3. Pharmacokinetic and statistical analyses

The total plasma concentrations of OXC and of the MHD enantiomers obtained in a previous study from our group [16] were used to determine the time to peak plasma concentration (t_{max}) for each volunteer (n=12). The unbound plasma concentration of OXC and MHD enantiomers was only evaluated at t_{max} for each volunteer.

The free fraction (f_u) in plasma of OXC and of the MHD enantiomers was determined as follows:

$$f_u = \frac{\text{unbound plasma concentration}}{\text{total plasma concentration}}$$

Statistical analysis was performed using the GraphPad Instat® software for the calculation of means and 95% confidence intervals. The Student *t*-test for paired data was used to evaluate enantiomer ratios different from unity for MHD. The level of significance was set at 5% in all tests.

3. RESULTS AND DISCUSSION

The present study describes the sequential analysis of unbound plasma concentrations of OXC and MHD enantiomers in healthy volunteers (n=12) treated with

300 mg OXC at intervals of 12 h for 5 days. It should be noted that there are no *in vivo* clinical data regarding the free fraction of MHD enantiomers in plasma.

This method was developed based on a previous study from our group [13] analyzing total concentration; however, in this study an ultrafiltration step was added using the Centrifree® ultrafiltration device to separate the free drug from that bound to plasma proteins. During the ultrafiltration process, small molecules (free drug) present in the aqueous component of plasma are forced by the pressure of the gradient to pass through the selectively permeable membrane and are collected in the ultrafiltrate. The protein-bound drug does not cross the membrane because of its large size and consequently does not reach the ultrafiltrate [17]. OXC and the MHD enantiomers were separated on a Chiralcel® OD-H chiral phase column (150 x 4.6 mm) using hexane:ethanol:isopropanol (80:15:5, v/v/v) as the mobile phase and a run time of approximately 15 min (Figure 1).

The method was linear over the concentration range of 4.0 ng - 2.0 µg/mL plasma for OXC and 20.0 ng - 6.0 µg of each enantiomer/mL plasma for MHD, with correlation coefficients higher than 0.99 (Tables 1 and 2). The wide intervals comprise all concentrations tested.

The present method is more sensitive than those reported in the literature, with limits of quantification of 4.0 ng OXC/mL plasma (Table 1) and of 20.0 ng of each MHD enantiomer/mL plasma (Table 2) using aliquots of only 50 µL of the ultrafiltrate [10-12]. Low limits of quantification are fundamental for the evaluation of the free fraction of OXC, a drug that exhibits low plasma concentrations as a result of its short elimination half-life (approximately 1.5 h) and of the dose interval of 12 h [13].

The coefficients of variation obtained in the precision studies and the percent inter- and intra-assay accuracy were less than 15%, guaranteeing reproducibility and repeatability of the results (Tables 1 and 2).

There is practically no matrix effect in the ionization of free OXC or MHD enantiomers in human plasma. Values close to 100% (97. 26, 94.27 and 98.87 for OXC, R-(-)-MHD and S-(+)-MHD, respectively) with coefficients of variation lower than 15 % (13.31, 4.55 and 5.74 % for OXC, R-(-)-MHD and S-(+)-MHD, respectively) were observed when the responses of OXC, MHD enantiomers and IS added to blank plasma ultrafiltrates (4 normal plasma samples, 2 lipemic samples, and 2 hemolyzed samples) were compared to the responses of OXC, MHD enantiomers and IS in methanol solution.

OXC and the MHD enantiomers were stable in plasma ultrafiltrates during three freeze-thaw cycles, when kept for 4 h at room temperature, after processing for 24 h in the autoinjector, and after storage at -70ºC for 6 months, as indicated by the relative standard errors of less than 15% in all analyses (Table 3).

The method developed and validated for the analysis of the unbound concentration of OXC and of the MHD enantiomers showed validation parameters compatible with the analysis of samples collected at t_{max} of OXC.

The free fraction of OXC and of the MHD enantiomers in plasma samples collected at t_{max} from each volunteer (n=12) after oral treatment with 300 mg OXC/12 h for 5 days are presented in Table 4. Figure 2 shows the correlation between total and unbound plasma concentrations of OXC, R-(-)-MHD and S-(+)-MHD, with r^2 higher than 0.54. The free fraction evaluated at t_{max} of OXC was 0.27 (0.22-0.31) for OXC, 0.42 (0.36-0.49) for R-(-)-MHD, and 0.37 (0.36-0.39) for S-(+)-MHD in the investigated healthy volunteers, showing enantioselectivity in the plasma protein binding of MHD. However, in an *in vitro* study of eslicarbazepine, Fortuna et al. [12] reported rates of about

30% for both enantiomers and the absence of enantioselectivity in the binding of the MHD enantiomers to human and rat plasma proteins. These differences between the results of the present study, a clinical study, and the *in vitro* findings might be explained by the fact that in *in vitro* protein-binding studies the compounds are added to plasma outside the organism, a method that may not necessarily reflect the *in vivo* situation [18]. Furthermore, the sample size of that study was small (n=3).

4. CONCLUSION

The method for the sequential analysis of unbound plasma concentrations of OXC and MHD enantiomers using a Chiralcel® OD-H chiral phase column coupled to an LC-MS/MS system shows confidence limits that are compatible with the application to a clinical study of the free fraction in plasma samples collected at t_{max} after treatment of healthy volunteers with OXC (300 mg/12 h) for 5 days. The free fraction of MHD is enantioselective in healthy volunteers, with higher proportion of R-(-)-MHD (0.42) when compared to S-(+)-MHD (0.37).

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Figures captions and tables

Figure 1 Chromatograms obtained in the analysis of oxcarbazepine and the MHD enantiomers unbound in plasma. (A) Ultrafiltrate from blank plasma, (B) Ultrafiltrate from blank plasma spiked with oxcarbazepine (0.2 µg/mL), MHD (0.1 µg of each enantiomer /mL) and internal standard (IS - 4-methylprimidone - 40 µg/mL) and (C) Ultrafiltrate from plasma of a healthy volunteer obtained 1 h after the last oral dose of 300 mg of oxcarbazepine.

Figure 2 Correlation between total and unbound plasma concentration of OXC, R-(-)-MHD and S-(+)-MHD. r^2= coefficient of determination.

Table 1 Validation of the analysis method of unbound oxcarbazepine in plasma.

	Unbound oxcarbazepine
Linearity	4.0 ng/mL - 2.0 µg/mL
Equation of the line	Y=2.2194x+-0.0009
r	0.9981
Limit of quantitation (ng/mL)	4.0
Precision (CV %, n = 10)	10.52
Accuracy (% Inaccuracy)	8.06
Intra-assay precision (CV %)	
4.0 ng/mL (n = 5)	9.4
12.0 ng/mL (n = 5)	5.79
0.6 µg/mL (n = 5)	3.40
0.96 µg/mL (n = 5)	7.93
1.6 µg/mL (1:1) (n = 5)	9.69
Interassay precision (CV %)	
4.0 ng/mL (n = 5)	9.18
12.0 ng/mL (n = 5)	7.70
0.6 µg/mL (n = 5)	9.50
0.96 µg/mL (n = 5)	7.59
1.6 µg/mL (1:1) (n = 5)	9.16
Intra-assay accuracy (RSE %)	
4.0 ng/mL (n = 5)	9.37
12.0 ng/mL (n = 5)	-12.17
0.6 µg/mL (n = 5)	-9.8
0.96 µg/mL (n = 5)	-12.62
1.6 µg/mL (1:1) (n = 5)	-14.16
Interassay accuracy (RSE %)	
4.0 ng/mL (n = 5)	10.17
12.0 ng/mL (n = 5)	-10.82
0.6 µg/mL (n = 5)	-3.55
0.96 µg/mL (n = 5)	-13.48
1.6 µg/mL (1:1) (n = 5)	-11.66

Coefficient of variation (CV) = [(SD/mean) x 100]; r = linear correlation coefficient; % Relative Standard Error (RSE) = $[(C_{obs}-C_{nominal})/C_{nominal}] \times 100$.

Table 2 Validation of the analysis method of unbound MHD enantiomers in plasma.

	Unbound R-(-)-MHD	**Unbound S-(+)-MHD**
Linearity	20.0 ng/mL - 6.0 µg/mL	20.0 ng/mL - 6.0 µg/mL
Equation of the line	y=0.4039x+0.0019	y=0.4286x+0.0012
r	0.9988	0.9992
Limit of quantitation (ng/mL)	20.0	20.0
Precision (CV %, n = 10)	6.84	9.20
Accuracy (% Inaccuracy)	-9.83	-3.28
Intra-assay precision (CV %)		
20.0 ng/mL (n = 5)	7.71	10.20
60 ng/mL (n = 5)	14.52	8.48
3.0 µg/mL (n = 5)	7.52	8.09
4.0 µg/mL (n = 5)	12.3	8.74
8.0 µg/mL (1:1) (n = 5)	12.29	8.16
Interassay precision (CV %)		
20.0 ng/mL (n = 5)	1.89	1.90
60 ng/mL (n = 5)	5.44	1.25
3.0 µg/mL (n = 5)	3.05	2.37
4.0 µg/mL (n = 5)	13.69	1.42
8.0 µg/mL (1:1) (n = 5)	4.19	4.53
Intra-assay accuracy (RSE %)		
20.0 ng/mL (n = 5)	7.71	10.20
60 ng/mL (n = 5)	14.52	4.45
3.0 µg/mL (n = 5)	-7,61	-5.91
4.0 µg/mL (n = 5)	-6.98	-2.93
8.0 µg/mL (1:1) (n = 5)	-13.51	-12.76
Interassay accuracy (RSE %)		
20.0 ng/mL (n = 5)	1.27	6.50
60 ng/mL (n = 5)	9.23	9.91
3.0 µg/mL (n = 5)	2.89	2.59
4.0 µg/mL (n = 5)	13.50	10.94
8.0 µg/mL (1:1) (n = 5)	1.55	-1.31

Coefficient of variation (CV) = [(Standard deviation/mean) x 100]; r = linear correlation coefficient; % Relative Standard Error (RSE) = $[(C_{obs}-C_{nominal})/C_{nominal}] \times 100$.

Table 3 Study of the stability method of analysis of unbound oxcarbazepine (OXC) and the MHD enantiomers in plasma.

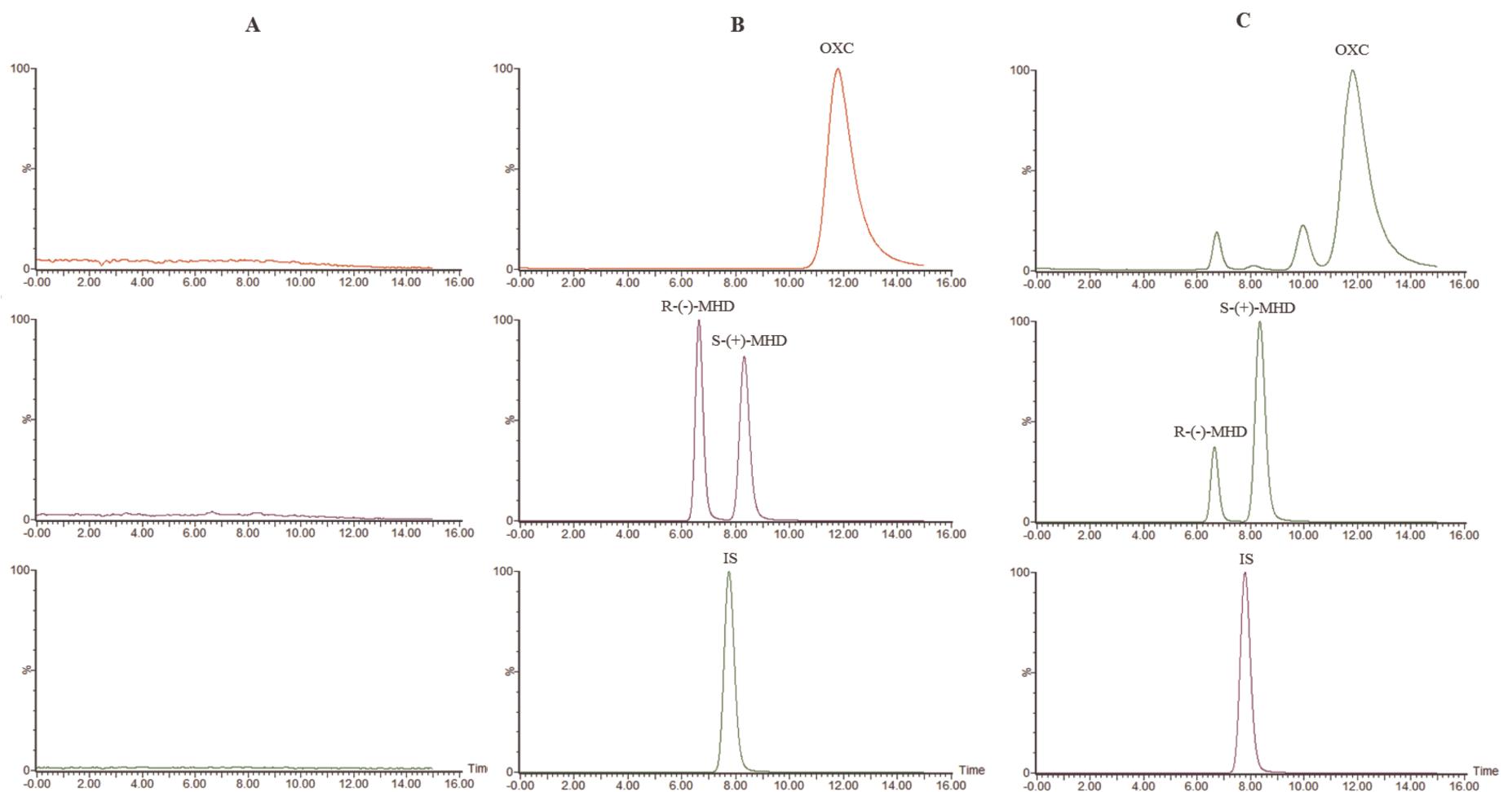
Concentration (µg/mL)	Short term (4 h)		Freezing/thawing (3 cycles)		Post-processing (24 h)		Long term (6 months)	
	Precision (CV %)	Accuracy (RSE %)	Precision (CV %)	Accuracy (RSE %)	Precision (CV %)	Accuracy (RSE %)	Precision (CV %)	Accuracy (RSE %)
OXC								
12.0 ng/mL	11.53	-11.40	2.70	-14.60	5.26	-12.40	2.81	4.78
0.96 µg/mL	8.22	-13.21	8.30	-9.45	10.06	-12.97	-14.40	-10.78
R-(-)-MHD								
60.0 ng/mL	1.90	-2.70	5.23	2.57	2.65	14.50	8.77	5.87
4.0 µg/mL	13.37	9.10	5.85	11.52	9.50	14.07	6.16	14.04
S-(+)-MHD								
60.0 ng/mL	1.95	-0.90	4.57	3.47	2.79	13.83	8.43	6.20
4.0 µg/mL	6.16	10.13	5.71	13.40	8.50	12.53	4.14	14.80

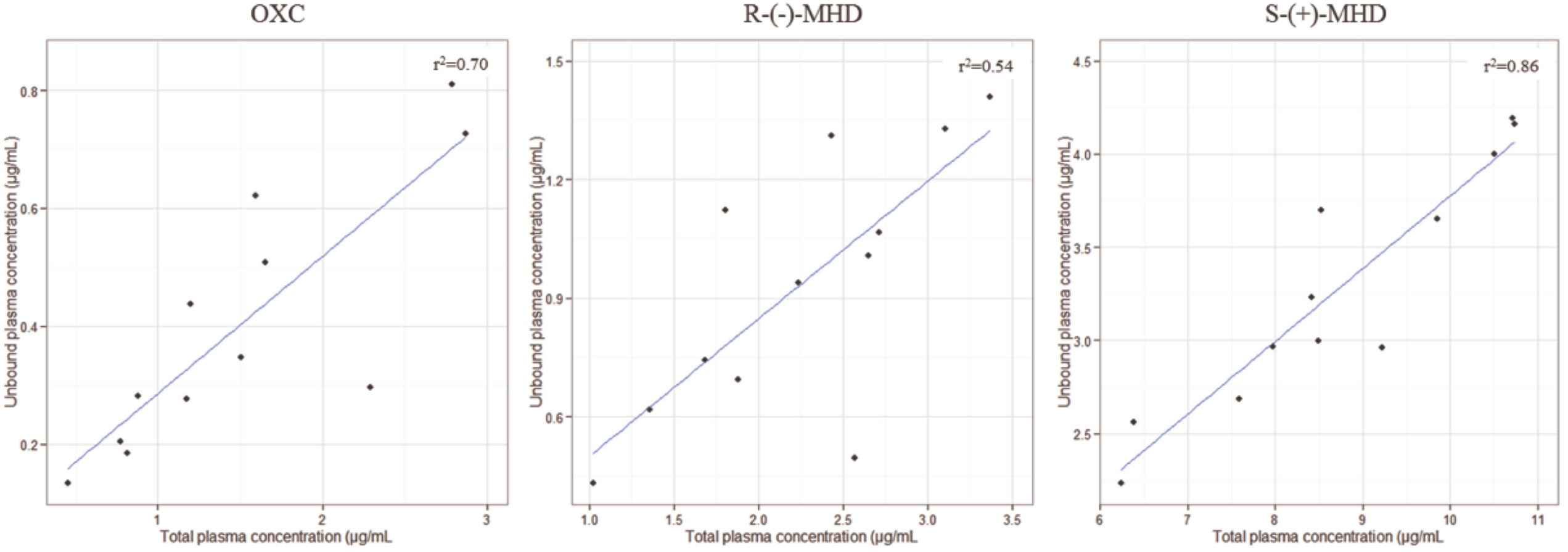
Coefficient of variation (CV) = [(Standard deviation/mean) x 100]; % Relative Standard Error (RSE) = $[(C_{obs}-C_{nominal})/C_{nominal}] \times 100$.

452 **Table 4** Free fraction of oxcarbazepine (OXC) and the MHD enantiomers in healthy
453 volunteers treated with 300 mg/12 h oxcarbazepine orally analysed at the time to reach
454 the maximum plasma concentration (t_{max}).

Subject	t_{max}	OXC	R-(-)-MHD	S-(+)-MHD
1	0.5	0.18	0.39	0.37
2	1.0	0.23	0.43	0.38
3	0.5	0.39	0.54	0.43
4	1.0	0.32	0.42	0.40
5	0.75	0.37	0.44	0.38
6	1.0	0.25	0.62	0.37
7	1.5	0.23	0.38	0.35
8	2.0	0.26	0.19	0.39
9	0.5	0.23	0.45	0.35
10	2.5	0.30	0.42	0.36
11	1.0	0.13	0.37	0.32
12	2.0	0.31	0.42	0.39
Mean	1.19	0.27	0.42	0.37*
(95% CI)	(0.76-1.61)	(0.22-0.31)	(0.36-0.49)	(0.36-0.39)

455 *Paired Student's t-test, $p < 0.05$ (R-(-)-MHD *vs* S-(+)-MHD); t_{max}: time to reach
456 maximum plasma concentration; 95% CI = 95% confidence interval.


A
B
C
OXC
OXC
R-(-)-MHD
S-(+)-MHD
S-(+)-MHD
R-(-)-MHD
IS
IS
100
%
0
-0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
Time


OXC
r²=0.70
Unbound plasma concentration (μg/mL)
Total plasma concentration (μg/mL
R-(-)-MHD
r²=0.54
Unbound plasma concentration (μg/mL)
Total plasma concentration (μg/mL
S-(+)-MHD
r²=0.86
Unbound plasma concentration (μg/mL)
Total plasma concentration (μg/mL