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Simultaneous quantitative analysis of oxcarbazepine and 10,11-dihydro-10-hydroxycarbamazepine in human plasma by liquid chromatography–electrospray tandem mass spectrometry

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Abstract

A fast and sensitive method to quantify oxcarbazepine (OXC) and its active metabolite, 10,11-dihydro-10-hydroxycarbamazepine (MHD) in human plasma using HPLC–MS/MS has been developed. The method involved liquid–liquid extraction (LLE), with diethyl ether–diclhoromethane (60:40 v/v) using deuterade carbamazepine (d10-carbamazepine) as internal standard (IS). The analytes and IS were separated using an isocratic mobile phase (acetonitrile/water (50:50 v/v) + 20 mM acetic acid) on the analytical column Phenomenex® Luna C18 5 μm (150 mm × 4.6 mm) at room temperature. Detection was performed by a Micromass Quatro LC mass spectrometer in the reaction monitoring mode using positive electrospray ionization (ESI+). The MS–MS ion transition monitored were *m/z* 253 > 208 for OXC, *m/z* 255 > 194 for MHD and *m/z* 247 > 204 for IS. Over the range 20–5250 ng/ml for OXC and 40–10,500 ng/ml for MHD, the calibration curves were defined by the following equations: $y = 0.00568 + 0.00296x - 5.70e{-}8x^2$ and $y = 0.00749 + 0.00178x - 5.70e{-}8x^2$ for OXC and MHD, respectively. All coefficient of determination (r^2) were close to unity (0.9986–0.9994). The lower limits of quantification obtained as a result of the LLE procedure was 20 ng/ml for OXC and 40 ng/ml for MHD. The statistical evaluation of the developed method was conducted by examining within-batch and between-batch precision data, which were within the required limits. The suitability of the assay for pharmacokinetics studies was determined by measuring OXC and MHD concentration after administration of a single 10 ml of OXC oral suspension (6%) in plasma human of healthy volunteers.

Keywords: Oxcarbazepine; 10,11-Dihydro-10-hydroxycarbamazepine; Metabolite; HPLC analysis; Validation

1. Introduction

In the last decades several antiepileptic drugs (Gabapentin, Clobazan, Lamotrigine, and Topiramate) have been introduced onto the market, among which oxcarbazepine (10,11-dihidro-10-oxo-5*H*-dibenzo-[*b*,*f*]azepine-5-carboxamide) a structural analog of carbamazepine which even so if does not differentiate in terms of pharmacological efficacy shows fewer unwanted side effect as compared with the traditional antiepileptic ones [1–3].

Oxcarbazepine (OXC) is considered effective not only as drug of first choice, as well as of coadjuvante therapy in the partial epilepsy [4,5]. It acts primarily by promoting stabilization of excitable membranes through the blockade of voltage-dependent sodium canals [6,7]. In human OXC is rapidly metabolized by cytosolic enzymes in the liver to its active non-toxic metabolite

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(10,11-dihydro-10-hydroxycarbamazepine (MHD), which predominates in plasma after oral administrating as compared as OXC [8–11], and show a linear pharmacokinetic [12,13]. Thus, identification, separation and quantification of OXC and MHD, its clinically relevant metabolite, is essential for the biological research related to therapeutic drug monitoring as well-bioequivalence studies.

In the past, the methods more commonly used for the determination of the OXC and MHD in biological samples involved the gas chromatography [14,15]. Currently, the high-pressure liquid chromatography (HPLC) coupled to ultraviolet detection [3,5,8,10,16–22] has been widely utilized for simultaneous measurement of OXC and MHD in biological fluids. However, the more commonly discussed limitation of all these methods for antiepileptic drugs determination is the fact of the existence of the great similarity (physicochemical properties and structures) existing between these compounds [5]. On the other hand, simultaneous determination of OXC and MHD in human plasma using HPLC separation followed by selective mass spectrometry detection has not been usually described. Two methods have been described for simultaneous measurement of OXC and its active metabolite (MHD) in human plasma by liquid chromatography–mass spectrometry with atmospheric pressure chemical ionization [6] or electrospray ionization [23] with a limit of quantification (LOQ) of 100 ng/ml and 300 and 400 ng/ml for OXC and MHD, respectively.

Here, we present a fast and selective LC–MS/MS method which provides reliable and sensitive simultaneous quantification of OXC and MHD in human plasma to a lower LOQ (20 and 40 ng/ml for OXC and MHD, respectively), which in addition combines lower plasma sample, short retention times and a simple one-step sample preparation, sensitivity, precision and linearity.

2. Experimental

2.1. Chemical and reagents

Oxcarbazepine was kindly supplied by Aché Laboratórios Farmacêuticos S/A (São Paulo, Brazil) and its dihydro metabolite (MHD) was purchased from Synfine Research (Canada). Carbamazepine-D10 (internal standard, IS) was obtained from CDN Isotops (Canada). Glacial Acetic Acid, Diethyl ether, Dichloromethane and Acetonitrile (HPLC grades) were obtained from Tedia (USA). Ultra-high quality water was obtained from a Milli-Q system (Millipore, USA). Human blank plasma samples used for the development and validation of the procedure were obtained from the Hematology and Hemotherapy Center of Ceara (Ceara, Brazil).

2.2. Preparation of standard solutions and quality control samples

Stock solutions of OXC (1 mg/ml), MHD (1 mg/ml) and internal standard (d10-carbamazepine (1 mg/ml)) were prepared in acetonitrile. Standard working solutions and quality control samples were obtained from different stock solution by making appropriate dilutions with acetonitrile–water (50:50 v/v). The nominal plasma concentration of calibration standards were prepared by adding appropriate volumes of working solutions to drug-free plasma to give final concentrations of 20/40, 750/1500, 1500/3000, 2250/4500, 3000/6000, 3750/7500, 4500/9000 and 5250/10500 ng/ml for OXC and MHD, respectively. Three levels of quality controls (QC) fixed at 60/120 (low, LQC), 2000/4000 (medium, MQC) and 4000/8000 ng/ml (high, HQC) for OXC and MHD, respectively, were prepared using the same blank plasma utilized to prepare the calibration-standard curves. All solutions were stored at 4 °C and were brought to room temperature before use.

2.3. Extraction produce

A 25 μl aliquot of IS (400 ng/ml) and 100 μl of water were added to 100 μl of human plasma samples and the tubes were briefly shaken. The mixture was vortex-mixed with 1 ml of diethyl ether–dichloromethane (60:40 v/v) for 5 min. Then, the tubes were centrifuged at 2000 × *g* for 5 min at 8 °C. The upper organic layer was carefully removed, transferred to new tubes, and evaporated to dryness under N_2 at 50 °C. The residue was reconstituted in 100 μl of acetonitrile followed by vortex-mixed for 30 s, and the sample transferred to glass autosampler vial.

2.4. Instrumentation and chromatography conditions

The chromatographic analysis were carried out using a Shimadzu (Kyoto, Japan) chromatograph consisting of an LC-10AD pump, a SIL-10AD autosampler. The mobile phase involved a mixture of acetonitrile–water (50:50 v/v) + 20 mM acetic acid pumped at a flow rate of 1.0 ml/min through the analytical column Phenomenex Luna C18 5um (150 mm × 4.6 mm), at room temperature. A split of the column eluant of approximately 1:10 was included so that only approximately 50 μl/min entered the mass spectrometer. The injection volume was 5 μl. The mass spectrometer (Micromass Quattro LC) equipped with an electrospray source using a crossflow counter electrode was run in positive mode (ES+), and set up in multiple reaction monitoring (MRM), monitoring the transitions *m/z* 253 > 208, *m/z* 255 > 194 and *m/z* 247 > 204 for OXC, MHD and IS, respectively. The source and desolvation temperature was of 120 and 350 °C, respectively. The cone and desolvation gas flow (N_2) was 107 and 353 l/h, respectively. The dwell time was set at 0.5 s, the values of the capillary voltage, the cone energy and the collision energy were 3 kV, 15 V, 15 eV for OXC, 3 kV, 20 V, 20 eV for MHD and 3 kV, 23 V, 15 eV for carbazepine-D10, with a gas pressure (argon) of 2.83×10^{-3} mbar. Data acquisition and analysis were performed using the software MassLynx (V 4.0 running under Windows XP on Pentium PC).

2.5. Method validation

The described procedure was validated according to internationally accepted recommendations for bioanalytical methods [24,25]. To define the relationship between concentration and

peak area ratios of analytes to the internal standard, calibration curves were defined in four runs of different lots of plasma based on triplicate assays of the spiked plasma samples (20–5250 ng/ml for OXC and 40–10500 ng/ml for MHD). The lower limit of quantification (LOQ) was determined as the lowest concentration, which could be quantified with a bias value below 20%, and a signal-to-noise ratio of at least 15 ($n = 5$) according to our protocol of validation. Matrix effects were determined from six different lots of blank human plasma [26]. Each blank sample was tested for interference using the proposed extraction procedure and compared with those obtained with aqueous solution of the analyte at a concentration near to the LOQ. The selectivity of the detection system was also tested to evaluate the possibility of cross-talk. This test was performed by the following: (1) blank extracted sample injection and monitoring the response in the OXC, MHD and IS transitions; (2) by injecting separately a plasma sample spiked only with OXC or MHD at the highest standard concentration and monitoring the response in the IS and MHD or OXC transition; (3) by injecting a plasma sample spiked only with IS and monitoring OXC and MHD transitions. Inter- and intra-day precision and accuracy of the method were assessed by performing replicate ($n = 8$) analyses of QC samples (LQC, MQC and HQC) in plasma against a calibration curve. The procedure was repeated on different days ($n = 4$) to determine within-batch accuracy and precision validation data. Within-batch and between-batch precision and accuracy were determined by treating spiked sample in replicate at the same day. Accuracy (percentage relative error (RE (%)) was calculated from the agreement between measured and nominal concentration of the spiked plasma samples. Method precision (within-batch and between-batch analyses) was calculated from the percentage relative standard deviations (R.S.D. (%)) for the repeated measurements.

The extraction efficiencies of OXC and MHD from three different lots of human plasma were determined by analyzing QC sample. The samples were extracted according to the procedure described above. Recovery was determined by comparing the mean of the area or response of five-plasma sample of each QC levels (LQC, MQC and HQC) spiked before extraction with that spiked after extraction ($n = 5$). According to Matuszewski et al. [27], this procedure allows to take into account matrix effect, giving a true recovery. The recovery of IS was also determined using the same procedure. The recoveries have to be higher than 50% according to our protocol of validation.

The stability of OXC and MHD stored at three concentrations of each QC levels (LQC, MQC and HQC) was investigated under distinct timing and temperature conditions. The stability of OXC and MHD in samples extracted with diethyl ether–dichloromethane (60:40 v/v) and stored in the autosampler (8 °C) was determined after 92 h. Freezing and thawing stability study for OXC and MHD in plasma samples was determined in three freeze–thaw cycles during 3 days. Short-term stability of OXC and MHD in QC samples after 15 h of storage at room temperature (20 °C) was also evaluated. In addition, long-term stability of OXC and MHD in QC samples after 30 days of storage at (−20 °C) was also assessed. All samples ($n = 5$) were analyzed using freshly prepared calibration samples. The calculated values at each concentration were averaged and the percentage error was calculated to estimate stability.

3. Results and discussion

For routine clinical investigation and therapeutic drug monitoring a simple and rapid preparation procedure with reproductive and satisfactory selectivity and sensitivity is always desirable.

In this work, a HPLC–MS/MS method for determination of OXC and MHD in human plasma was developed and validated. The chromatographic conditions and MS parameters were optimized such that the resultant extracted MRM chromatograms should all exhibit consistent integrated areas and signal-to-noise-ratios (S/N > 15) to OXC, MHD and IS. Using an analytical column Phenomenex Luna C18 5 μm (150 mm × 4.6 mm), it was possible to obtain satisfactory separation and resolution of peaks for OXC and MHD and its IS in a mobile phase consisting of acetonitrile–water (50:50 v/v) + 20 mM acetic acid.

The chemical structures as well the mass spectrum (precursor and product ion spectra) for analytes and IS are shown in Fig. 1. Several fragment ions were observed in the product ion spectra for analytes and internal standard. The major fragment ions at m/z 208, m/z 194 and m/z 204 are chosen in the MRM acquisition for OXC, MHD and IS, respectively.

The method selectivity was evaluated by comparing chromatograms of six extracted blank plasma samples of different sources with those of plasma samples spiked with OXC, MHD and its IS. Representative MRM chromatograms of blank plasma sample and spiked with OXC and MHD and IS subjected to the LLE procedure were shown in Fig. 2a and b, respectivelly. A chromatogram of a real sample from a subject to whom OXC oral suspension (6%) was administered also can be seen in Fig. 2c. Under optimum method conditions, no interference (matrix effect) was observed from endogenous compounds following the extraction of spiked sample plasma over the time window of elution, and gave retention times of 2.44 ± 0.3, 1.87 ± 0.3 and 2.89 ± 0.2 min for OXC and MHD and IS, respectively. No significant cross-talk effect at the retention time of compound was observed.

Two methods for the simultaneous measurement of OXC and its active metabolite (MHD) in human plasma by liquid chromatography coupled to mass spectrometry have been published [6,23]. The first one [6], has been successfully for the simultaneous measurement OXC and MHD, but involves tedious samples pretreatment of the biological samples and a HPLC method by elution gradient, so that the total analysis time (from pretreatment to equipment analysis) requires longer period, and others disadvantage such as: (1) bigger sample plasma size (0.2 ml) as compared to that required in the present method (0.1 ml). A smaller simple size is especially advantageous when only blood samples are available for quantification, as in the case of pharmacokinetic studies in children; (2) the chromatographic run times is about twice longer (∼6 min) than that verified in our method (3.5 min). Comparing ours results to second one [17], we found that their chromatography analyses also need for gradient elution, a larger sample volume (0.3 ml), and a retention

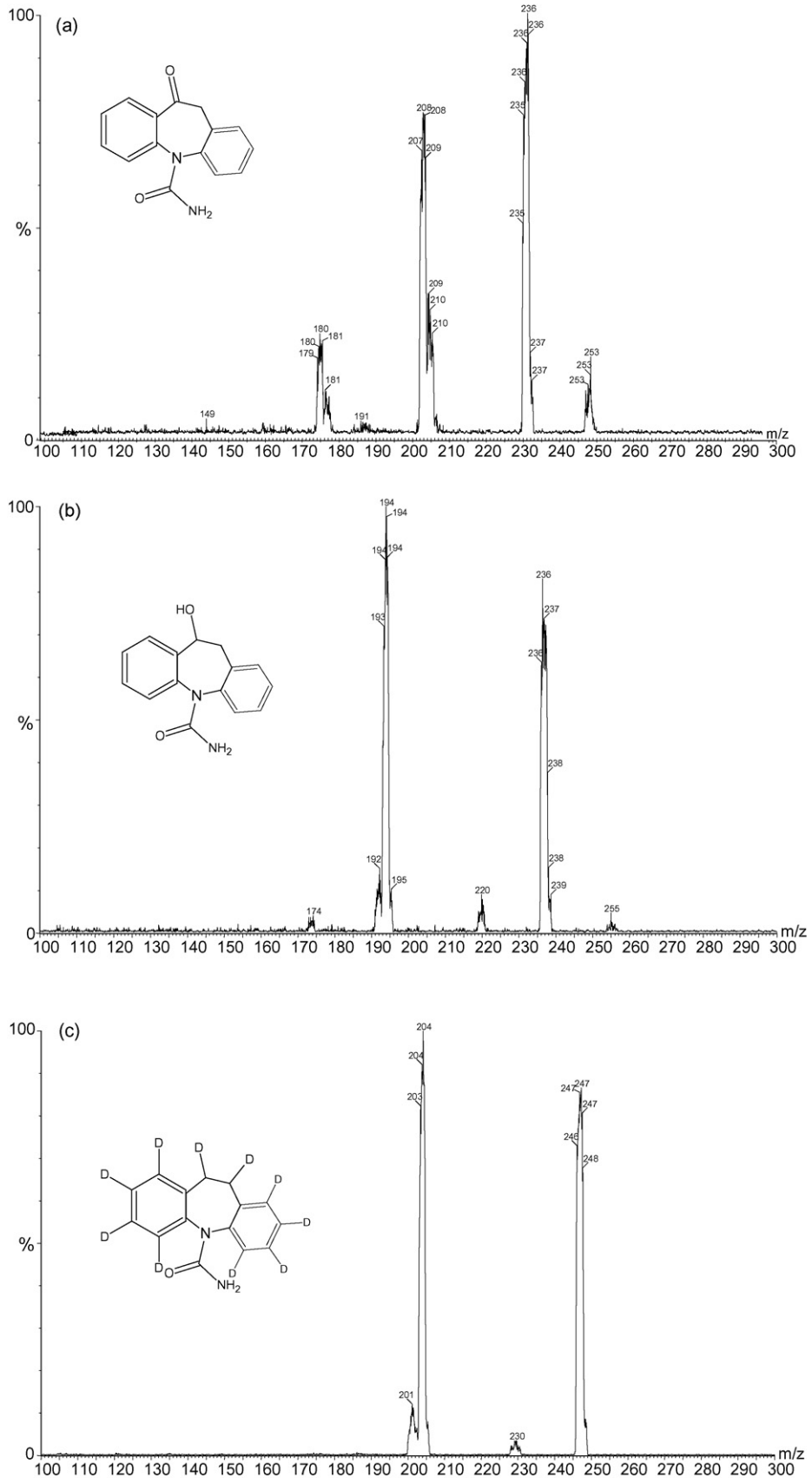

Fig. 1. Full-scan product ion spectra of $[M+H]^+$ and the structures for (a) OXC, (b) MHD and (c) d10-carbamazepine.

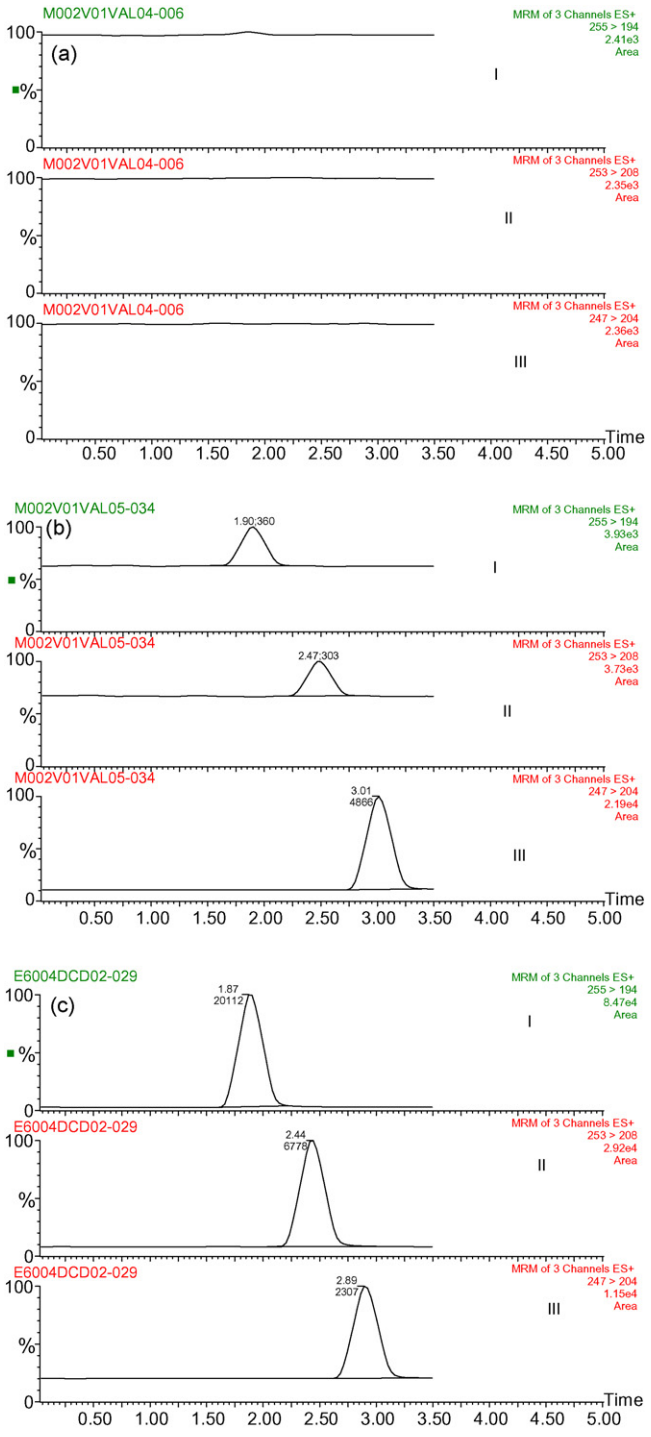

Fig. 2. Representative MRM chromatograms: (a) a blank plasma sample; (b) a blank plasma sample spiked with OXC (20 ng/ml) and MHD (40 ng/ml); (c) a volunteer sample 1.5 h after administration of OXC oral suspension (6%) in healthy volunteers. Peaks I–III refer to the MHD, OXC and IS.

time too long for OXC (∼18 min) and MHD (∼12 min) as compared to those found by our method for OXC (2.44 min) and MHD (1.87 min). Our method utilizes a simple one-step extraction with a diethyl ether–dichloromethane mixture before the run and was performed using an isocratic mobile phase. Thus it was not necessary to wait for the initial condition feedback. It should be considered that a faster extraction process is an important fact for routine analysis. Furthermore, it is well known that an isocratic method is generally preferred because of its convenience, simplicity and reproducibility. Thus, our method has sufficient selectivity and can be successfully applied for measurement of OXC and MHD in human plasma samples. The relatively simple sample preparation procedure and a short chromatographic analysis time presented by the proposed method allows a very high sample throughput (150–200 samples per day).

3.1. Method validation

The quality of bioanalytical data is highly dependent of a well designed and interpreted calibration curve. The test of homoscedasticity is one important step to be taken in the study of the calibration curve to verify variances data [28]. In this study, the test of homoscedasticity was performed by plotting residual versus concentration obtained for the intra-batch assay HPLC–MS/MS data and by applying an *F*-test [29] (data not shown). Since the data showed non-uniform variance, weighted least-squares regression method with different weighing factor ($1/x$, $1/x^2$, $1/y$ and $1/y^2$) was used to chose the best one that presented the minor percentage relative error distribution scatter at the lower end of the calibration curve According to all these circumstances, the chosen weighing factor was $1/x^2$ (Table 1). Thus, the calibration curves were defined by the following equations: $y = 0.00568 + 0.00296x - 5.70e{-}8x^2$ for OXC and $y = 0.00749 + 0.00178x - 5.70e{-}8x^2$ for MHD, where y is the ratio of drug/internal standard peak area and x is the concentration (ng/ml) of analyte. All determination coefficients (r^2) were close to unity (0.9986–0.9994). Within-batch and between-batch precision and accuracy data for standard curves of OXC and MHD are summarized in Table 1. Relative error of the values range between 1.7 and 4.5%, and R.S.D. (%) from 0.15 to 7.1% for the two compounds in standard (Table 1) and QC sample (Table 2) showing the good fit of individual points to the regression line.

The LOQ of the assay based on the R.S.D. (%) and RE (%) was 20 and 40 ng/ml for OXC and MHD, respectively (Table 1). These limits are near to 1% of the anticipated maximal concentration (C_{max}) of OXC and 2% for MHD in average human pharmacokinetics, good enough to perform bioavailability studies. Comparison with the LOQ values formerly obtained with previous published methods using chromatography-atmospheric pressure chemical ionization [6] or electrospray mass spectrometry [23], reveals that our LC–MS/MS method is, about 5 and 20 times more sensitive. Lower LOQ values (1 ng/ml) were reported from published study involving measuring of OXC and MHD by LC–MS/MS in microdialysis samples [30], but it should be kept in mind that dialysates (microdialysis samples) are clean and free of proteins so no sample preparation is necessary for the analysis of dialysate concentration. Pienimaki et al. [31] published a HPLC–UV method that achieved LOQ values (55 and 24 ng/ml for OXC and MHD, respectively) close to ours, but it requires a larger size sample (500 μl) and laborious and time expensive sample pre-treatment. We have also calculated the theorical sensitivity of the proposed method, i.e., drug on column, for OXC and MHD, and it was estimated that with a 100 μl

Table 1
Within-batch and between-batch precision and accuracy of the standard curve

Spiking plasma concentration (ng/ml)	*Within − batch*		
	Back-calculated concentration (mean ± S.D.) (ng/ml)	R.S.D. (%) ($n = 3$)	RE (%)
OXC			
20	19.99 ± 0.26	1.31	−0.05
750	768.82 ± 16.46	2.14	2.51
1500	1558.17 ± 25.50	1.64	3.88
2250	2115.04 ± 62.20	2.94	−6.00
3000	3040.24 ± 198.16	6.52	1.34
3750	3400.62 ± 74.78	2.20	−9.32
4500	4659.85 ± 120.48	2.59	3.55
5250	5469.95 ± 51.35	0.94	4.19
Σ RE (%)			0.1
MHD			
40	39.98 ± 1.90	4.76	−0.05
1500	1529.95 ± 18.82	1.23	2.00
3000	3086.33 ± 56.46	1.83	2.88
4500	4260.31 ± 231.44	5.43	−5.33
6000	6111.70 ± 298.49	4.88	1.86
7500	6999.86 ± 220.81	3.15	−6.67
9000	9123.15 ± 477.68	5.24	1.37
10500	10935.65 ± 200.52	1.83	4.15
Σ RE (%)			0.2

sample, but reconstituted in 100 μl of acetonitrile, 5 μl injection volume and LOQ of 20 ng/ml for OXC and 40 ng/ml for MHD to be 100 pg and 200 pg, respectively. This represent a considerable increase in sensitivity compared to similar methods. Thus, we consider that these points reflect some advantages of our method as compared to previously published data in literature.

3.2. Recovery

The recoveries of OXC and MHD determined at three different concentrations (LQC, MQC and HQC) were 105.4, 89.2 and 92.8%, and 88.4, 88.7 and 90.6, respectively, the overall average recovery being 95.8% for OXC and 86.7% for MHD. Comparable recoveries values (117% for OXC and 81% for MHD) have been registered by Breton et al. [17] at concentration range of 500–20000 ng/ml, but as already mentioned they used a larger size sample (0.3 ml). At concentration range of 10–35 ng/ml, but using a plasma sample (0.2 ml) pre-treatment much more complex than ours, Maurer et al. [6] reported recoveries of 59.6 and 79.2% for OXC and 57.4–63.9% for MHD. Thus, the proposed method showed a very good recovery for the plasma samples by applying a simple one-step sample treatment with a smaller size sample (0.1 ml).

3.3. Stability studies

Assessment of OXC and MHD stability was performed by analyzing working standard solutions as well spiked samples immediately and at subsequent hours or day for the anticipated storage period. All samples ($n = 5$) were analyzed using freshly prepared calibration samples. No changes in OXC and MHD concentration were detected in the working standard solution

Table 2
Precision and accuracy in the quantification of OXC and MHD in human plasma sample (analysis with spiking plasma sample at four different concentrations)

Spiking plasma concentration (ng/ml)	Within-batch			Between-batch		
	Back-calculated concentration (mean ± S.D.) (ng/ml)	R.S.D. (%) ($n = 8$)	RE (%)	Back-calculated concentration (mean ± S.D) (ng/ml)	R.S.D. (%) ($n = 24$)	RE (%)
OXC						
20	**19.86 ± 0.86**	**4.3**	**0.7**	**20.11 ± 0.24**	**1.2**	**0.5**
60	64.3 ± 1.56	2.4	7.1	63.1 ± 3.44	4.2	5.2
2000	2050.9 ± 43.54	2.1	2.5	2050.3 ± 84.12	2.9	2.5
4000	3804.6 ± 114.4	3.0	−4.9	3880.2 ± 282.29	2.3	−3.0
MHD						
40	**38.04 ± 2.34**	**6.2**	**−4.9**	**38.586 ± 2.01**	**5.2**	**−3.5**
120	119.5 ± 4.48	3.7	−0.41	119.7 ± 5.30	4.4	−0.25
4000	3919 ± 67.34	1.7	−2.0	4006.5 ± 137.04	3.4	0.15
8000	7487.4 ± 318.68	4.3	6.4	7736.6 ± 350.89	4.5	−3.29

Table 3
Stability of QC samples of OXC and MHD in human plasma under different storage conditions ($n = 5$)

	OXC (ng/ml)			MHD (ng/ml)		
	60	2000	4000	120	4000	8000
Post-processing stability test (RE (%))						
0 h	3.6	1.0	5.2	6.6	9.1	8.4
137 h	0.8	3.7	3.9	5.1	5.9	6.2
Freeze-and-thaw stability test (RE (%))						
0 cycle	2.6	3.6	3.3	3.6	2.8	2.7
3 cycles	1.7	2.2	3.3	5.2	2.6	2.2
Short-term stability test (RE (%))						
0 h	4.2	2.2	5.7	2.4	2.5	3.1
7 h	3.3	2.7	7.4	1.2	2.1	3.2
Long-term stability test (RE (%))						
0 day	1.2	2.2	2.0	3.5	2.8	1.6
30 days	2.7	1.0	2.8	1.2	1.0	1.0

after 1 month of storage with protection from light at 8 °C. In a post-processing stability study, no significant degradation could be detected in the cooled samples (8 °C) left in the autosampler for 92 h. The results showed that the peak areas of OXC and MHD remained almost unchanged and no extra peaks were observed in the period studied. The analysis of three sets of spiked plasma sample (LQC, MQC and HQC) of OXC and MHD were prepared and stored at −18 °C and subjected to three thaw–freeze cycles. The data show that plasma samples can be frozen and thawed at least three time prior to analyses. In addition, short-term stability of spiked plasma samples processed and stored at room (+23 °C) for 15-h period was also evaluated. No tendency of degradation of OXC and MHD after 15 h of maintenance at room temperature was observed. Finally, to demonstrate the long term stability of OXC and MHD, three sets of spiked plasma samples ((LQC, MQC and HQC) were processed and stored at −20 °C for 30 days. The analysis of spiked plasma samples before and after storage at −20 °C for this period showed no significant loss of analyte ($p > 0.05$). The relative error calculated for the different stability studies are illustrated in Table 3.

After validation, the proposed method was used to analyze plasma sample taken from healthy volunteers immediately before and after 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 10, 12, 24 and 48 h after administration of a single 10 ml of OXC oral suspension (6%). Typical plasma concentration versus time profile is shown in Fig. 3. Plasma concentrations of OXC and MHD were in the standard range and remained above the quantification limits for the entire sampling period.

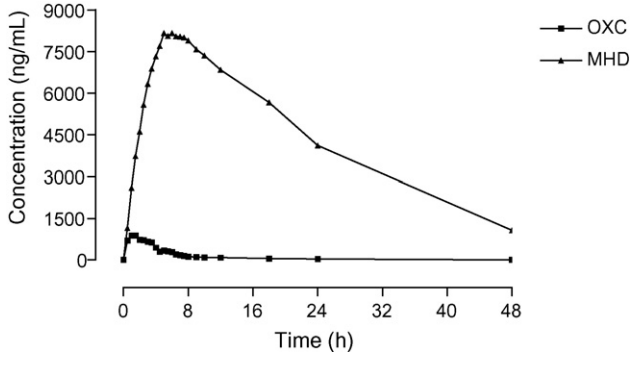

Fig. 3. Mean plasma profiles of OXC and MHD after administration of a single 10 ml of OXC oral suspension. (6%) to healthy volunteers ($n = 8$).

4. Conclusion

In conclusion, the LC–MS/MS method described here for OXC and MHD quantification in human plasma agrees with the concepts of high sensitivity, specificity and precision. The method has proved to be fast and reliable, with each sample requiring less than 4 min of analysis time. It has significant advantages on previous ones published for simultaneous measuring of OXC and MHD in biological fluids. Finally, the suitability of LC–MS/MS method to identify and quantify OXC and MHD in human plasma has been successfully demonstrated.

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