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Development and validation of a LC/MS/MS method for simultaneous quantification of oxcarbazepine and its main metabolites in human serum

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Abstract

A fast, sensitive and specific LC/MS/MS method for the simultaneous analysis of oxcarbazepine (OXC), 10-hydroxycarbazepine (MHD) and *trans*-diol-carbazepine (DHD), in human serum, has been developed and validated. Serum drugs were extracted by C8 solid-phase cartridges (SPE) and separated in less than 3 min on a C18 reverse-phase column using an isocratic elution. A tandem mass spectrometer, as detector, was used for quantitative analysis in positive mode by a multiple reaction monitoring. Calibration curves, obtained on two ranges of concentration (0.78–50 mg/L for MHD and 0.078–5.0 mg/L for OXC and DHD), showed correlation coefficients (r) better than 0.997. Within day and between days quality controls imprecision, as CV%, ranged from 0.3 to 4.6% and from 1.9 to 5.8%, respectively. Cyheptamide (CYE) was used as internal standard. No detectable carry-over and no relevant cross-talk and matrix effect occurred. Samples from 24 treated patients were analysed and drug serum concentrations obtained by this method are in agreement with those of other methods and also are well correlated ($r = 0.88$) in comparison to our routine HPLC-UV method. Based on the analytical results and short run time, the method is suitable to support routine analysis of therapeutic drugs monitoring from human serum of treated patients or for pharmacokinetic studies.

Keywords: Oxcarbazepine; 10-Hydroxycarbazepine; Therapeutic drug monitoring; LC/MS/MS

1. Introduction

Oxcarbazepine (OXC), 10,11-dihydro-10-oxo-5H–dibenz [*b*,*f*] azepine-5-carboxamide, is an antiepileptic drug that is quickly replacing the carbamazepine (CBZ), 5H-dibenz [*b*,*f*] azepine-5-carboxamide, in the recent therapeutic protocols. OXC is the ketoanalogue of carbamazepine, with a ketonic group in position 10 and with a similar action mechanism. It is often used for patients who are intolerant to CBZ [1] which gives many unpredictable adverse reactions associated with high mortality rates [2]. The OXC is essentially a pro-drug in humans in fact, after the administration, it is completely absorbed by the gastrointestinal tract and completely (96–98%) converted by an arylketon reductase, a cytosolic enzyme of hepatic cells, into its main and pharmacologically active metabolite monohydroxy derivative (MHD), 10,11-dihydro-10-hydroxy-5H-dibenz [*b*,*f*] azepine-5-carboxamide [3]. The MHD is subsequently metabolised by two different ways. The predominant one is the MHD-glucuronide conjugation mediated by the UDP-glucuronyltransferase, while a minor amount (approximately 4%) is oxidised into its pharmacologically inactive metabolite, the 10,11-*trans*-hydroxycarbazepine (DHD), 10,11-dihydro-10,11-*trans*-dihydroxy-5H-dibenz [*b*,*f*] azepine-5-carboxamide [4]. Both OXC and MHD do not show self-induction characteristics and, moreover, do not seem to influence, by inhibition or by induction, the P450 cytochrome system, catalyst for the metabolic ways of many drugs, avoiding problems of interactions with other substances in eventual multiple therapies [5]. Because the OXC has a minor tendency to interact with other drugs it can be administered both as monotherapy and

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polytherapy in association with other antiepileptic drugs (AEDs) [6]. The OXC is detectable in the blood at very low concentrations and just for some hours before its conversion in MHD [7]. After a single dose (600 mg) of oxcarbazepine, the OXC C_{max} is from 0.4 to 1 mg/L after 1 h, while the MHD C_{max} is about from 5 to 15 mg/L in a time range of 5–12 h [4]. The therapeutic range has not been very well defined, but clinical studies have approximately determined that this range is from 3 to 50 mg/L for patients in monotherapy and from 5 to 40 mg/L for patients in multiple therapy [8,9].

As well known, it is important to monitor blood drugs concentration to establish the therapeutic drug amount or to avoid toxic effects due to an overdose. Moreover, during a therapeutic treatment physician could request the drug bioavailability in a particular patient with a gastro-intestinal or pancreatic alteration or with hepatic or renal pathologies. In addition, it is important to evaluate if the drug metabolism is altered by the contemporary consumption of other drugs or substances such as alcohol or tobacco. To date, some analytical methods reported for OXC and its metabolites determination have been performed using mainly LC or LC/MS procedures. It is well known that these methods are less specific than LC/MS/MS methods and also are time consuming because of long chromatographic analysis and/or complex sample preparation procedures [10–16].

Liquid chromatography tandem mass spectrometry method is considered the gold standard to implement procedures to analyse drugs in biological samples; in fact, LC/MS/MS detection permits high specificity for qualitative and quantitative analysis of several compounds. Moreover, MS/MS detection allows a reduction of time in sample clean-up and chromatographic separation [17]. Besides, an isocratic method is generally preferred because of its convenience, simplicity and reproducibility and tandem mass spectrometry techniques, as multiple reaction monitoring (MRM), are highly specific and almost independent from chromatographic resolution [18]. In addition, phase I therapeutic drug monitoring and pharmacokinetic studies require highly selective and sensitive analytical methods to quantify drugs accurately. To date, a fast, quantitative and detailed method, based on a standard LC/MS/MS technique, to analyse OXC and its metabolites in biological fluids is still lacking. Recently, OXC and MHD have been also analysed on rat brain microdialysis and human plasma samples using tandem mass spectrometry. In these works the authors used a microbore and nanobore LC/MS/MS with a total run time over than 15 and 20 min, respectively, in rat brain samples [19,20] and a method with a run time of 3 min for the analysis of OXC and MHD in human plasma [21].

This study is aimed to develop a fast, sensitive and accurate method for the simultaneous quantification of OXC and its two metabolites, MHD and DHD, in human serum using CYE, 10,11-dihydro-5H-dibenz [*a,d*] cycloheptene-5-carboxamide, as internal standard. The assay method has been optimised on a triple quadrupole mass spectrometer coupled with a liquid chromatograph with a total run time of 3 min. Based on the analytical results the method is suitable to support routine analysis of therapeutic drugs monitoring from human serum of treated patients or for pharmacokinetic studies.

2. Experimental

2.1. Materials and reagents

OXC, MHD and DHD were supplied from NOVARTIS PHARMA (Basel, Switzerland); CYE was from SIGMA–ALDRICH (Steinheim, Germany). The analytical solvents of HPLC grade, acetonitrile, methanol, formic acid and ammonium acetate, were from J.T. BAKER (Deventer, Netherlands). Purified water was generated by a Milli-Q reagent water system (Millipore corporation, Bedford, MA). Cartridges SPE Isolute C8 (200 mg, 3 ml) were purchased from StepBio (Bologna, Italy). A sample concentrator dry-block (DB-3D, TECHNE, Cambridge, UK) was used to concentrate the samples.

2.2. Standards and samples

Aliquots of sera, obtained by centrifugation at $1000 \times g$ for 10 min from drug-free blood samples, were collected at the Diagnostic Laboratory of the Hospital (A.O.U. OO.RR. of Foggia). For the method validation sera were pooled, aliquoted and frozen at −20 °C, then used during the study for the preparation of calibration standards and quality control (QC) samples.

The positive samples were obtained from patients already treated with oxcarbazepine. The specimens were collected after 12 h from the consumption of the drug and the sera were aliquoted and stored at −80 °C until the analysis.

Stock standard solutions were prepared in methanol to provide solutions of 1 g/L for OXC, DHD and MHD while a solution of 2 g/L was prepared for CYE. Stock solutions were stored at −80 °C until use. Working solutions were prepared fresh daily by diluting stock solutions with the mobile phase to provide a concentration of 10 mg/L for MS tuning.

2.3. Preparation of calibration standards and quality control samples

Calibration standards (calibrators) were prepared by spiking drug-free human serum with standard solutions containing OXC, MHD and DHD to give MHD concentrations of 0.78, 1.563, 6.25, 12.5, 25.0, 50.0 mg/L and OXC and DHD concentrations of 0.078, 0.156, 0.625, 1.25, 2.5, 5.0 mg/L. The calibration points were prepared freshly for each assay and extracted along with serum samples and quality controls. The added volume was always less than 4% of total volume of the samples, so that the integrity of the matrix was maintained. Serum quality control samples, used in the validation study, were prepared by spiking pooled human drug-free serum at final concentrations of 1 mg/L, 10 mg/L and 25 mg/L, for MHD, and of 0.1 mg/L, 1 mg/L and 2.5 mg/L, for OXC and DHD, aliquoted and stored at −20 °C with the clinical samples until required.

2.4. Preparation of calibrators and serum samples

Calibrators, quality controls and subjects samples (0.15 mL of serum) were extracted after the addition of 600 μL ammo-

nium acetate saturated solution containing IS solution (10 μL of IS [2 g/L] in 20 mL of ammonium acetate saturated solution). The tubes were briefly vortex-mixed and were allowed to stand at room temperature for 15 min. A single-step solid phase extraction (SPE) was carried out on a vacuum box reported as follows: (i) cartridges were conditioned with 3 mL of methanol and were washed twice with 3 mL of water; (ii) calibrators, quality controls and subjects samples were loaded on cartridges; (iii) the cartridges were washed twice with 3 mL of water and the excess of water was eliminated by vacuum at −0.15 bar; (iv) the drugs were eluted with 3 mL of methanol as completely as possible. The eluate was removed by a gentle stream of nitrogen in a dry bath at 40 °C and redissolved with 0.15 mL of the HPLC mobile phase. The solution was transferred to a micro vial, then capped and placed into the HPLC autosampler rack.

2.5. Liquid chromatography–mass spectrometry

Chromatographic conditions were experimented to obtain the best drugs separation with a total run time as short as possible (3 min). An HPLC Alliance 2695 system with autosampler (Micromass Quattromicro, Waters, USA) equipped with an analytical column (Symmetry C18 3.5 μm 2.1 mm × 100 mm i.d. – Waters, USA) without guard column was used. The column was maintained at a temperature of 40 °C. The mobile phase consisted of acetonitrile 40% containing 0.02% formic acid. The mobile phase was pumped through the column with a flow rate of 350 μL/min. A split was included so that only approximately 1/3 of the column eluent entered into ESI probe. The temperature of the autosampler was maintained at 8 °C and 10 μL were automatically injected into the HPLC.

MS analysis was performed using a triple quadrupole mass spectrometer (Micromass Quattromicro, Waters, USA) equipped with an electrospray source ionization (ESI) operating in the positive-ion mode. The mass spectrometer was calibrated with NaIRb solution in the range from (*m*/*z*) 18 to 1077 in static and from (*m*/*z*) 20 to 1120 in scanning mode, according to the instrument specifications. The data were processed with Masslynx® software (version 4.0-Micromass, Waters) running on an IBM personal computer. The mass spectra (MS or MS/MS) were obtained as background-subtracted sums of 13 scans in continuum mode scanning the range from (*m*/*z*) 50 to 300, scan time of 1 s and interscan delay of 0.1 s. The daughter ion spectra were obtained using the following cone voltage, collision energy and gas collision pressure (argon) parameters: 25 V, 25 eV and 3.1×10^{-3} mbar, for OXC and MHD; 5 V, 25 eV and 3.1×10^{-3} mbar for DHD; 25 V, 30 eV and 3.1×10^{-3} mbar for CYE.

From daughter scan spectra of OXC, of its metabolites and of CYE were selected the following most abundant fragments: (*m*/*z*) 180 for OXC and DHD, (*m*/*z*) 194 for MHD and (*m*/*z*) 178 for CYE. Then, the quantitative analysis was performed using the multiple reaction monitoring mode (MRM, dwell time 0.1 s) by following reactions (*m*/*z*): 253 > 180 for OXC, 255 > 194 for MHD, 271 > 180 for DHD and 238 > 178 for CYE.

Calibration curves were constructed using Masslynx from data generated with the calibration samples plotting the analyte to IS peak area ratios (PARs) against concentration by a weighted ($1/x$) least-squares linear regression. The PARs of unknown samples were interpolated on the calibration curve to obtain the OXC, MHD and DHD concentrations.

An HPLC-UV method, according to Franceschi and Furlanut [14], already implemented in our Diagnostic Laboratory of the Hospital and routinely used to monitor OXC and its metabolites in patients sera, was employed as method of comparison. This method has been performed on a Bio-Rad System CROMAT-2-HPLC equipped with a UV–VIS detector set to 214 nm. Chromatographic separations were performed with a Zorbax SB-CN column (Hewlett-Packard, USA) of 250 mm × 4.6 mm i.d., 5 μm particle size, connected to a pre-column, and maintained at 50 °C. The mobile phase was a mixture of water/acetonitrile/methanol/acetic acid/triethylamine (725/150/125/1/0.6 by volume) with a pH adjusted at 4.20 with acetic acid. The imprecision of HPLC-UV ranged from 1.2 to 12.9% for OXC, from 1.0 to 5.4% for MHD and from 1.3 to 8.2% for DHD. The accuracy ranged from 87.2 to 103.6% for OXC, from 92.2 to 112% for MHD and from 86 to 104% for DHD.

3. Results and discussion

In this paper is described the implementation of a sensitive, specific and fast method for simultaneous quantification of OXC and its metabolites in human serum using liquid chromatography coupled with tandem mass spectrometry. This method requires a simple preparative step with a total chromatographic run time of 3 min, so it is less time consuming compared with other LC/MS and LC/MS/MS methods reported in literature [10–16,19,20].

3.1. Method development and validation

3.1.1. MS development

The MS tuning and MS/MS dissociation study was optimised, for each single standard compound, by varying the cone voltage and collision energy, using the flow injection analysis (FIA) of working solutions in mobile phase at a flow rate of 0.01 mL/min directly through the electrospray probe. MS/MS tuning experiments displayed daughter scan spectra of OXC and its metabolites in according to those reported in literature [10,12,19,21]. The most abundant ions from daughter ion experiments were selected to obtain MRM transitions as more sensitive as possible. Specific Ion Chromatograms for each analyte obtained using the conditions reported in methods are shown in Fig. 1.

3.1.2. MS specificity and selectivity

Extracted blank serum monitored as total ion chromatogram does not show any peak. Specific DHD (named DHD_1), MHD and CYE ion chromatograms show just one peak at relative retention time (RRT) of 0.97 min, 1.08 min and 2.51 min, respectively. Specific OXC ion chromatogram, at (*m*/*z*) 253 > 180,

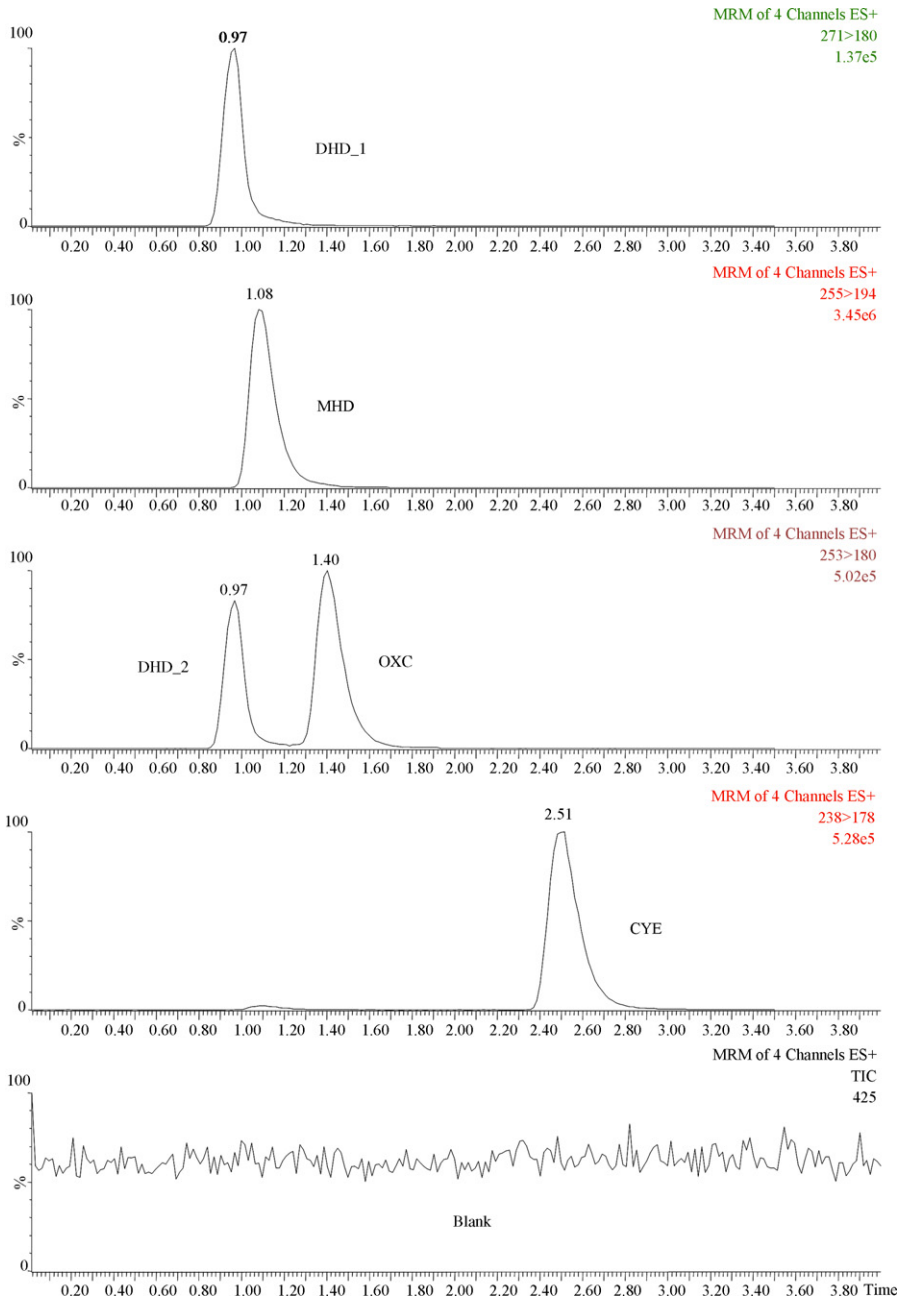

Fig. 1. Total ion chromatogram (TIC) and MRM ion chromatograms of blank, CYE, OXC, MHD and DHD_1. The OXC ion chromatogram (*m/z* 253 > *m/z* 180) shows an extra peak corresponding to DHD_2. For experimental conditions see Section 2.5.

shows one peak at RRT of 1.40, due to the elution of OXC, and another peak at RRT of 0.97 min, due to elution of DHD (named DHD_2) fragmented on cone and generating a main fragment at (*m/z*) 253. In fact, in an our previous paper [22] we have demonstrated that there is an interference in the OXC channel due to the ion source fragmentation of DHD at a cone voltage of 25 V. Despite MRM guarantees high specificity in drug assay, it is known that mass selective detection may produce compromised results due to interferences [17]. To avoid the fragmentation of DHD in source it is possible to use a cone voltage of 5 V obtaining just the (*m/z*) 271 molecular ion [22], but during the chromatographic run time the 4 MRM channels were acquired continuously and even if DHD voltage was set at 5 V, the voltages of other channels (OXC, MHD and CYE) were set at 25 V. So, in these conditions, during the assay there is also the generation of the DHD fragment of (*m/z*) 253 when the MS method operates with a cone voltage of 25 V. To avoid this interference we have increased the chromatographic resolution to separate DHD from OXC, completely. Data comparison about the determination of DHD, following both reactions at (*m/z*) 271 > 180 (DHD_1) and (*m/z*) 253 > 180 (DHD_2), showed a good quantitative response for DHD_1 and DHD_2 with better results for the latter; thus we have used the (*m/z*) 253 > 180 MRM reaction for DHD determination in patients samples. In addition, in this work the cross-talk phenomenon among MS/MS channels was evaluated injecting OXC and MHD, separately, at the high-

est concentrations of standard curves (50 mg/L for MHD and 5.0 mg/L for OXC) and monitoring the response in the other channels including the IS one. Samples spiked with the IS CYE alone were also injected to monitor its potential interference on other drug channels. From these experiments no "cross-talk" was observed.

Moreover, the assay selectivity and interferences were also assessed by analysing extracts from three different sera pools (blanks); endogenous peaks at the RRT of the analytes of interest were not observed in any of the sera evaluated (data not shown).

3.2. Assay performance

3.2.1. Solid phase extraction

The extraction recovery was assessed by two sample preparation methods, using a sample pre-treatment with acetone to precipitate the proteins and a solid phase extraction (SPE) on C8 cartridge. The recovery was evaluated on QC serum samples spiked with 1, 10 and 25 mg/L of MHD and with 0.1, 1 and 2.5 mg/L of OXC and DHD. The resulting peak areas of both acetone pre-treatment and SPE C8 cartridges extraction, at the same concentration, were expressed as relative percent recovery and relative standard deviation compared to standard solutions in mobile phase without sample preparation. The recovery data were obtained from three QC samples extracted three times for each point. SPE recovery as percentage was 60 ± 4% for OXC, 88 ± 1% for MHD and 62 ± 15% for DHD (mean ± SD) compared to acetone sample pre-treatment recovery that was 33 ± 29% for OXC, 42 ± 27% for MHD and 7 ± 5% for DHD. We chose the SPE for sample preparation and our results are in agreement with those reported by Klys and by Mandrioli which consider SPE treatment as a fast and feasible alternative to other sample preparations [11,13].

3.2.2. Calibration curves and quality controls

Validation study was obtained by calibration curves and quality controls. Table 1 shows the slopes, intercepts, and correlation coefficients from five different calibration curves; each level was analysed in duplicate along the investigated ranges. The linearity of calibration curves was estimated by the correlation coefficients (r); the results of r were at least 0.997 and the intercept values were not significantly different from zero. Data from calibration curves and quality control samples revealed that the proposed method shows adequate specificity, sensitivity, accuracy and precision. The concentration points of calibration curves, as reported in experimental section, were chosen because they cover pharmacokinetic ranges of these anti-epileptic drugs [10,13].

Table 1
Slopes, intercepts, and correlation coefficients as average values ± SD from five different calibration curves

Drug	n	Slope ± SD	Intercept ± SD	r ± SD
MHD	5	0.548 ± 0.010	0.026 ± 0.019	0.9999 ± 0.0001
OXC	5	1.020 ± 0.012	0.070 ± 0.013	0.9997 ± 0.0003
DHD_1	5	0.117 ± 0.002	0.025 ± 0.024	0.9968 ± 0.0013
DHD_2	5	0.374 ± 0.003	0.051 ± 0.005	0.9986 ± 0.0008

For the investigated concentration points (mg/L) see the Section 2.3.

Quality control samples were analysed after a sequence of calibration and unknown samples. Three levels of serum quality controls were prepared to evaluate inaccuracy and imprecision of method. Each level was extracted and analysed five times together with calibration and patient samples for each batch of analysis. The within day results are reported in Table 2. Table 3 summarizes the between days inaccuracy (%) and imprecision (CV%) calculated as cumulative average of results obtained from 3 days experiments. Between days imprecision ranges from 1.9 to 5.8%. Inaccuracy ranges from −5.3 to +5.8%. Data from quality control samples reveal that the proposed method shows adequate specificity, sensitivity, accuracy and precision.

3.2.3. Limit of detection and carry-over

The signal to noise ratio calculated for MHD, OXC, and DHD at the lowest concentration of the calibration curves gave the following average (±SD) values: 1441(±12):1; 223(±5):1; 103(±5):1, respectively. Since these ratios are abundantly over the minimal values of LOD (signal to noise ratio = 3:1) and

Table 2
Within day imprecision (CV%) calculated on three levels of serum quality controls

Drug	Amount added (mg/L)	Day 1 (n = 5)		Day 2 (n = 5)		Day 3 (n = 5)	
		Calculated concentration (mg/L)	CV%	Calculated concentration (mg/L)	CV%	Calculated concentration (mg/L)	CV%
MHD	1	0.937 ± 0.020	2.2	0.959 ± 0.019	1.9	0.944 ± 0.009	1.0
	10	9.512 ± 0.085	0.9	9.305 ± 0.482	5.2	9.922 ± 0.033	0.3
	25	24.622 ± 0.404	1.6	24.821 ± 0.181	0.7	25.653 ± 0.356	1.4
OXC	0.1	0.098 ± 0.002	2.3	0.110 ± 0.003	1.9	0.099 ± 0.003	0.7
	1.0	0.943 ± 0.018	2.5	0.988 ± 0.008	0.8	0.996 ± 0.010	3.8
	2.5	2.398 ± 0.017	3.3	2.380 ± 0.090	1.0	2.401 ± 0.083	3.5
DHD_2	0.1	0.093 ± 0.004	4.0	0.098 ± 0.004	4.2	0.098 ± 0.005	4.6
	1.0	0.964 ± 0.020	2.1	0.974 ± 0.029	2.9	1.061 ± 0.010	0.9
	2.5	2.514 ± 0.025	1.0	2.668 ± 0.032	1.2	2.750 ± 0.030	1.1

Table 3
Between days inaccuracy (%) and imprecision (CV%) calculated as cumulative average ± SD of results obtained from 3 days experiments of serum quality controls

Drug	Amount added (mg/L)	Calculated concentration (mg/L)	Imprecision CV%	Inaccuracy%
MHD	1	0.947 ± 0.018	1.9	−5.3
	10	9.580 ± 0.373	3.9	−4.2
	25	25.03 ± 0.553	2.1	0.1
OXC	0.1	0.103 ± 0.006	5.8	−2.6
	1.0	0.976 ± 0.027	2.7	−2.4
	2.5	2.393 ± 0.067	2.8	−4.3
DHD_2	0.1	0.096 ± 0.005	4.8	−3.8
	1.0	1.001 ± 0.052	5.1	0.1
	2.5	2.644 ± 0.105	4.0	5.8

LOQ (signal to noise ratio = 10:1), we are able to quantify by this method drug concentrations lower than those examined. In fact, based on the above signal to noise ratios, the extrapolated LOQ for MHD, OXC and DHD are 5.4 ng/mL, 3.9 ng/mL and 7.8 ng/mL, respectively. These results are not so far from those obtained by Lanckmans et al. considering that they used protein free serum samples and micro and capillary LC columns (MHD LOQ was 1 and 0.2 ng/mL, respectively) [19,20].

Moreover, it is well known that highly sensitive methods could be affected by carry-over, more visible in MS/MS methods compared to less sensitive methods as LC/MS or LC-UV detection [17]. In this study carry-over was evaluated analysing mobile phase after serum samples with the highest analyte concentrations, 5.0 mg/L for OXC and DHD and 50 mg/L for MHD. No detectable carry-over occurred in mobile phase runs after serum samples determinations. The results of cumulative carry-over values ranged from 0.007 to 0.17%.

3.2.4. Matrix effect

The matrix effect (ME) for OXC, MHD and DHD was also evaluated by comparing the results of the analysis of two sets of samples (A and B) in according to Matuszewski et al. [18]. The results of matrix effects on OXC, MHD and DHD_2 are summarized in Table 4. In the first set (A), two levels of standards, dissolved in HPLC mobile phase, were analysed directly in triplicate (six samples). In the second set (B), samples from three different serum pools were extracted and then spiked with two levels of OXC, MHD and DHD standards. For both sets, A and B, the peak area ratios were calculated between analyte and IS and the ME values were calculated as follows: ME (%) = $B/A \times 100$. The matrix effect evaluation shows no relevant effect; in fact ME as mean value was of 120.5, 87 and 98.5% for MHD, OXC and DHD_2, respectively.

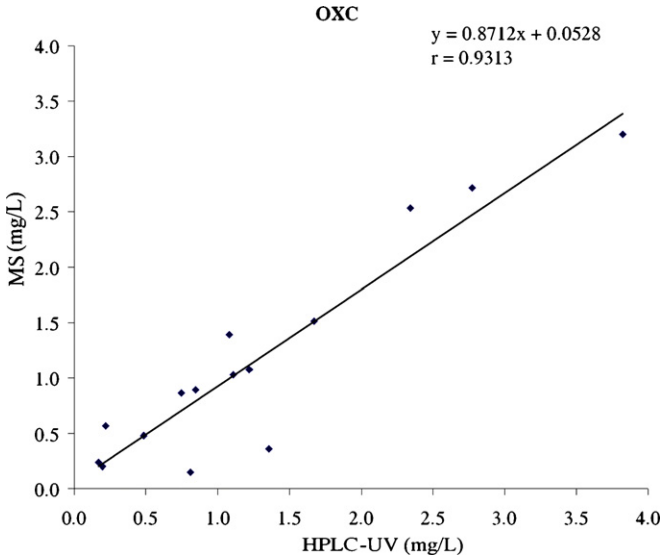

Fig. 2. Method comparison between LC/MS/MS and HPLC-UV routine method using 24 samples of patients treated with OXC. For the correlations of other drugs see Section 3.3.

3.3. Therapeutic drug monitoring of patients treated with OXC

Serum samples of patients treated with OXC ranging from 600 to 1800 mg/day, in mono or in polytherapy, were obtained from our Diagnostic Laboratory of the Hospital where OXC

Table 4
Matrix effect (ME) was evaluated comparing two experiments

Drug	mg/L	Standard solutions (A) Ratio (drug/IS) (mean ± SD) $n = 3$	Serum spiked after extraction (B) Ratio (drug/IS) (mean ± SD) $n = 6$	ME%
MHD	10	1.172 ± 0.007	1.472 ± 0.035	126
	25	2.857 ± 0.045	3.276 ± 0.075	115
OXC	1.0	0.315 ± 0.002	0.269 ± 0.014	85
	2.5	0.731 ± 0.004	0.653 ± 0.007	89
DHD_2	1.0	0.197 ± 0.006	0.199 ± 0.028	101
	2.5	0.420 ± 0.007	0.405 ± 0.046	96

(A): Two concentration points of standards were dissolved in HPLC mobile phase and analysed in triplicate. (B): Three different serum pools were extracted and spiked with two levels of OXC, MHD and DHD standards. Each pool was analysed in duplicate. ME values were calculated as follows: ME (%) = $B/A \times 100$.

and its metabolites are measured routinely ($n = 24$). The concentrations obtained by this LC/MS/MS method from serum samples ranged from 0.15 to 3.20 mg/L for OXC, from 6.76 to 24.27 mg/L for MHD and from 0.13 to 3.26 mg/L for DHD; the results are in agreement with those reported by others [10,13]. Results were also compared with those measured by our routine HPLC-UV method according to Franceschi and Furlanut [14]. Methods comparison (HPLC-UV versus LC/MS/MS) shows a good correlation (r) between two methods, even if lower slopes for MHD (0.5845) and especially for DHD (0.3328) demonstrated that our method produces lower values than HPLC-UV method (Fig. 2). These results could be due to the well known less specificity of HPLC-UV method in comparison to LC/MS methods caused by co-elution of drugs and/or endogenous impurities that can interfere as described by Matuszewski et al. [18]. In addition, using MRM acquisition mode we analyse just specific (m/z) drug ions of interest. Finally, lower values in LC/MS/MS could be due to ion suppression, too, but it does not occur in our method.

4. Conclusion

This is the first paper describing widely a fast, sensitive and specific method, for simultaneous serum quantification of OXC and its two metabolites, MHD and DHD, by high-performance liquid chromatography coupled with tandem mass spectrometry. By this method OXC and its metabolites are quantified accurately in less than 3 min and this aspect is very important for a routine therapeutic monitoring of these drugs. Both sample preparation and analytical run time of the OXC method described in this paper are faster and more simple than other chromatographic methods, using UV or MS as detectors, previously reported [10–16]. Moreover, because just 10 μL are injected into LC/MS/MS system, this method allows us to work with small amounts of biological material, including that coming from experimental animals.

In conclusion, it is our opinion that the developed LC/MS/MS method to analyse OXC, MHD and DHD is suitable to support routine analysis in human serum from treated patients to monitor their therapeutic or toxic levels or for pharmacokinetic studies and also for drugs determination on animal blood.

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