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Short communication

Development and validation of a high performance liquid chromatographic method for the determination of oxcarbazepine and its main metabolites in human plasma and cerebrospinal fluid and its application to pharmacokinetic study

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Abstract

An isocratic reversed-phase HPLC-UV procedure for the determination of oxcarbazepine and its main metabolites 10-hydroxy-10,11-dihydrocarbamazepine and 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine in human plasma and cerebrospinal fluid has been developed and validated. After addition of bromazepam as internal standard, the analytes were isolated from plasma and cerebrospinal fluid by liquid–liquid extraction. Separation was achieved on a X-TERRA C18 column using a mobile phase composed of 20 mM KH_2PO_4, acetonitrile, and *n*-octylamine (76:24:0.05, v/v/v) at 40 °C and detected at 237 nm. The described assay was validated in terms of linearity, accuracy, precision, recovery and lower limit of quantification according to the FDA validation guidelines. Calibration curves were linear with a coefficient of variation (r) greater than 0.998. Accuracy ranged from 92.3% to 106.0% and precision was between 2.3% and 8.2%. The method has been applied to plasma and cerebrospinal fluid samples obtained from patients treated with oxcarbazepine, both in monotherapy and adjunctive therapy.
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Keywords: Oxcarbazepine; Metabolite; HPLC; Analytical method validation; Plasma/cerebrospinal fluid ratio

1. Introduction

Oxcarbazepine (10,11-dihydro-10-oxo-5*H*-dibenzo[*b*,*f*]azepine-5-carboxamide), a relatively new antiepileptic drug, is a 10-keto analogue of carbamazepine with a similar therapeutic profile, but with less adverse effects and less clinical relevant pharmacokinetic drug interactions [1–3]. Oxcarbazepine is indicated as first-line drug in monotherapy or polytherapy for the treatment of partial seizures with or without secondarily generalized tonic–clonic epileptic seizures [4–6].

Like most anticonvulsants, the precise mechanism by which oxcarbazepine and its pharmacologically active metabolite 10-hydroxy-10,11-dihydrocarbamazepine exert their antiseizure effect is unknown so far, but it is suggested that they produce blockade of voltage-sensitive sodium channels, thereby stabilizing neural membranes, inhibiting repetitive neuronal firing, and diminishing synaptic impulse activity. These actions are thought to be important in the prevention of synaptic neurotransmission and seizure spread in the intact brain [7]. In addition, increased potassium conductance and modulation of high-voltage activated calcium channels may contribute to the anticonvulsant effects of the drug [8].

Following oral administration, oxcarbazepine is completely absorbed and extensively reduced by a non inducible aldo-keto reductase to its pharmacologically active metabolite 10-hydroxy-10,11-dihydrocarbamazepine [9,10], which is further metabolized by glucuronidation and excreted into urine [11]. A small percentage of 10-hydroxy-10,11-dihydrocarbamazepine is oxidized through hepatic *CYP3A4/5* isoenzymes to the inactive metabolite 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine [12].

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A number of LC methods for the quantification of oxcarbazepine and its main metabolites 10-hydroxy-10,11-dihydrocarbamazepine and 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine in biological fluids are already published [13–25]. However, some of these methods are not sufficiently sensitive, some are time-consuming, some require expensive instruments, some are not focused exclusively for the determination of oxcarbazepine and/or its metabolites but are used and for the determination of other antiepileptic drugs and/or their metabolites, and some are enantioselective and require expensive chiral columns and long analysis time. Up today, no method is yet published to quantify oxcarbazepine and/or its main metabolites in cerebrospinal fluid of humans. The described method has been adapted from a previously proposed method by Pienimaki et al. [14], which involves a HPLC-UV assay following liquid–liquid extraction procedure for the determination of carbamazepine and oxcarbazepine and their metabolites in plasma samples.

The aim of the present investigation was the development and validation of a reliable HPLC-UV method for the determination of oxcarbazepine and its main metabolites 10-hydroxy-10,11-dihydrocarbamazepine and 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine in authentic human plasma and cerebrospinal fluid samples. The method was successfully implemented for the quantification of oxcarbazepine and its main metabolites in real plasma and cerebrospinal fluid samples and for the determination of the cerebrospinal fluid/plasma concentration ratio of the pharmacologically active metabolite 10-hydroxy-10,11-dihydrocarbamazepine, in epileptic patients undergoing chronic treatment with oxcarbazepine, either in monotherapy or in adjunctive therapy with other antiepileptic drugs.

2. Experimental

2.1. Chemicals and reagents

Oxcarbazepine, 10-hydroxy-10,11-dihydrocarbamazepine, and 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine were kindly provided by Novartis Pharma (Basel, Switzerland). Bromazepam, used as internal standard, albumin, glucose, *n*-octylamine, potassium dihydrogen phosphate, sodium chloride and sodium bicarbonate were purchased from Sigma–Aldrich (St. Luis, MO, USA). HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). All other chemicals and solvents used were of analytical grade.

2.2. Chromatographic conditions

The development and validation work was carried out on a HPLC system consisted of a Shimadzu LC-20AD isocratic pump, a SPD-20A variable wavelength UV detector, a manual injector with a 20 μl loop (Rheodyne) and an integrator (Varian, model 4290). Separation was performed at 40 °C using a X-TERRA C18 analytical column (150 mm × 4.6 mm, 5 μm) (Waters), equipped with a guard column (20 mm × 4.6 mm) dry packed with pellicular ODS material (37–53 μm).

The mobile phase composed of 20 mM KH_2PO_4, acetonitrile, and *n*-octylamine (76:24:0,05, v/v/v), was filtered through a 0.45 μm pore size nylon filter (Alltech) and degassed by ultrasonic treatment before use. The HPLC system was operated isocratically at a flow rate of 0.7 ml/min and the detector was set at 237 nm.

2.3. Preparation of spiked plasma and cerebrospinal fluid samples

Calibration standards were prepared freshly in 0.5 ml of drug-free human plasma or artificial cerebrospinal fluid by spiking with concentrated standards in order to achieve concentration ranges between 25 to 1000 ng/ml, 1000 to 25 000 ng/ml, and 100 to 4000 ng/ml, for oxcarbazepine, 10-hydroxy-10,11-dihydrocarbamazepine, and 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine, respectively.

Quality control samples were prepared in 25 ml of either drug-free human plasma or artificial cerebrospinal fluid by spiking with concentrated standards. Three levels of quality control concentrations were prepared, a low level at 50, 2000, and 200 ng/ml, a medium level at 500, 10 000, and 2000 ng/ml, and a high level at 1000, 25 000, and 4000 ng/ml, for oxcarbazepine, 10-hydroxy-10,11-dihydrocarbamazepine, and 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine, respectively. The quality control samples were used for the analytical method validation and for the in run validation.

These quality control samples were divided into aliquots of about 2 ml into vials capped tightly, stored at −20 °C and thawed on the day of analysis at room temperature.

The artificial cerebrospinal fluid was prepared according to Ostermann et al. [26], and consisted from 0.8 g/l glucose, 0.2 g/l albumin, 7.3 g/l sodium chloride, and 1.9 g/l sodium bicarbonate (adjusted to pH 7.5 with phosphate buffer).

2.4. Sample preparation

Calibration curve standards, quality control and patients samples of either plasma or cerebrospinal fluid were thawed and allowed to equilibrate at room temperature. The thawed samples were vortexed to ensure complete mixing of contents. Then to 500 μl of each sample spiked with 50 μl of internal standard solution (at 5000 ng/ml of methanol) were added 0.5 ml of 0.1 M NaOH and 5 ml of methyl-*tert*-butyl ether [27]. After vortex mixing for 30 s, the samples were shaken for 30 min, then centrifuged at 3000 rpm for 10 min and the upper aqueous phase was discharged by aspiration. The organic phase was transferred to a clean test tube and evaporated to dryness in a water bath at 40 °C with the aid of a gentle stream of air. The residue was reconstituted in 50 μl of mobile phase and a 20 μl aliquot was injected in the HPLC system for quantification.

2.5. Analytical method validation

The validation of the method was based on the guidelines of the United States FDA [28].

The linearity of the method, for plasma and cerebrospinal fluid samples, was demonstrated over the concentration range of 25–1000 ng/ml, 1000–25 000 ng/ml, and 100–4000 ng/ml for oxcarbazepine, 10-hydroxy-10,11-dihydrocarbamazepine, and 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine, respectively, by assaying six calibration standards and three quality control samples in triplicate on three separate occasions. Calibrations curves were obtained by plotting the peak height ratios of analyte/internal standard versus the analyte concentrations in spiked plasma samples. The concentration of the unknown was calculated using linear regression analysis from the following equation: $y = b + mx$, where y is the peak height ratio of analyte to internal standard, b the y-axis intercept of the calibration curve, m the slope of the calibration curve, and x is the concentration of the analyte.

Intra-assay precision was calculated within a single run as the coefficient of variation (CV%) for six determinations at each level of quality control concentrations and intra-assay accuracy, was determined by calculating the estimated concentrations as the percentage of the nominal concentrations. Inter-assay precision and accuracy were assessed by assaying three quality control samples in triplicate on three separate occasions.

The lower limit of quantification (LLOQ) was experimentally chosen as the lowest concentration at which both accuracy and precision should be within the maximum tolerable CV of 20%.

The absolute recovery was calculated by comparison of the peak heights from the processed plasma and cerebrospinal fluid samples versus those obtained after direct injection of standards of the same amount of the analytes. Recovery of internal standard was determined in the same solutions simultaneously.

2.6. Application

The HPLC method developed was used to investigate the plasma and cerebrospinal fluid concentrations of oxcarbazepine, 10-hydroxy-10,11-dihydrocarbamazepine, and 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine in epileptic patients treated with oxcarbazepine (mean dose: 1800 mg), both in monotherapy and in combination with other antiepileptic drugs. Blood samples were collected into heparinized test tubes and plasma was immediately separated by centrifugation at $3000 \times g$ for 10 min. Cerebrospinal fluid samples were collected through a lumbar puncture. Plasma and cerebrospinal fluid samples were stored at $-20\,^{\circ}C$ pending analysis.

3. Results and discussion

3.1. Chromatographic separation and interferences

The chromatographic conditions described in this assay were arrived at after investigating several mobile phases and internal standards. The addition of *n*-octylamine in the solvent system used as well as the column temperature (40 °C) were found to improve further the separation of the analytes giving symmetrical and sharp peaks. The peaks of all analytes were well separated. The proposed HPLC method enables the quantification of oxcarbazepine and its two main metabolites, 10-hydroxy-10,11-dihydrocarbamazepine, and 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine, in human plasma and cerebrospinal fluid with UV detection at 237 nm.

Typical chromatograms obtained from drug-free plasma and cerebrospinal fluid samples and from real plasma and cerebrospinal fluid samples are illustrated in Fig. 1. No interfering peaks due to the matrix components or to coadministered drugs elute at the retention time of the studied analytes. The retention time of 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine, 10-hydroxy-10,11-dihydrocarbamazepine, oxcarbazepine, and internal standard were 5.04, 6.52, 10.67, and 13.18 min, respectively.

3.2. Linearity

Calibration curves were established on each day of analysis and typical curves from plasma samples had the regression equation of $y = -0.015023 + 0.001040x$ ($r = 0.9998$), $y = -0.405054 + 0.000845x$ ($r = 0.9985$), and $y = 0.087446 + 0.000865x$ ($r = 0.9985$), for oxcarbazepine, 10-hydroxy-10,11-dihydrocarbamazepine, and 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine, respectively. The corresponding regression equations for cerebrospinal fluid samples were: $y = -0.002139 + 0.001088x$ ($r = 0.9992$), $y = 0.125511 + 0.001045x$ ($r = 0.9998$), and $y = -0.192881 + 0.001105x$ ($r = 0.9985$), for oxcarbazepine, 10-hydroxy-10,11-dihydrocarbamazepine, and 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine, respectively. For each calibration standard level, the concentration was back-calculated from the calibration curve equation. Intra-assay precision ranged between 3% and 11% and accuracies were within 9% of the nominal values for the three compounds.

3.3. Recovery from plasma and cerebrospinal fluid

The mean absolute recovery values of the analytes in plasma samples were $79.7 \pm 5.4\%$, $86.4 \pm 5.8\%$, and $79.3 \pm 2.2\%$, for oxcarbazepine, $77.3 \pm 4.3\%$, $81.7 \pm 7.8\%$, and $78.2 \pm 3.6\%$, for 10-hydroxy-10,11-dihydrocarbamazepine, and $51.9 \pm 8.2\%$, $49.1 \pm 6.9\%$, and $45.5 \pm 7.6\%$, for 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine, for the low, medium, and high concentration quality control samples, respectively. The corresponding mean absolute recovery values for the cerebrospinal fluid samples were $79.8 \pm 5.4\%$, $81.3 \pm 5.8\%$, and $79.6 \pm 7.8\%$, for oxcarbazepine, $77.0 \pm 4.3\%$, $73.4 \pm 6.9\%$, and $71.2 \pm 2.1\%$, for 10-hydroxy-10,11-dihydrocarbamazepine, and $58.8 \pm 3.5\%$, $50.1 \pm 7.9\%$, and $53.0 \pm 6.6\%$, for 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine, for the low, medium, and high concentration quality control samples, respectively. Mean recovery of internal standard at 500 ng/ml was $89.6 \pm 5.3\%$ and $89.6 \pm 5.4\%$ for plasma and cerebrospinal fluid samples, respectively.

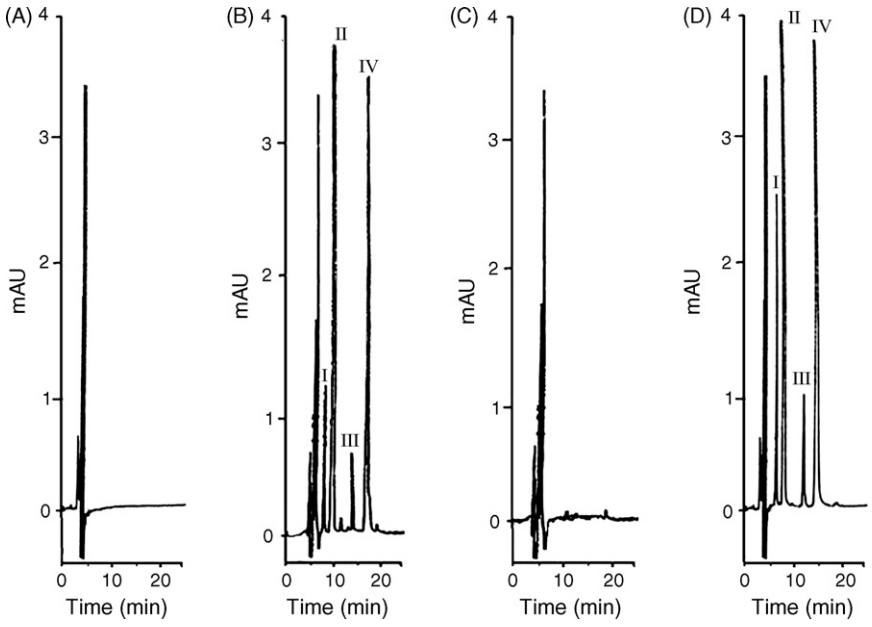

Fig. 1. Examples of chromatograms: (A) extract of a 0.5-ml drug-free cerebrospinal fluid sample; (B) extract of a cerebrospinal fluid sample obtained from a female patient 3 h after taking 1800 mg of oxcarbazepine; (C) extract of a 0.5-ml drug-free plasma sample; (D) extract of a plasma sample obtained from the same patient 3 h after taking 1800 mg of oxcarbazepine. Peaks: (I) 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine; (II) 10-hydroxy-10,11-dihydrocarbamazepine; (III) oxcarbazepine; (IV) internal standard (bromazepam).

3.4. Accuracy and precision

The intra- and inter-assay precision and accuracy results are given in Table 1. Intra- and inter-assay precision for plasma and cerebrospinal fluid samples was found to be less than 8.2%. 6.4%, and 7.3% for oxcarbazepine, 10-hydroxy-10,11-dihydrocarbamazepine, and 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine, respectively. Mean accuracy ranged from 92.8% to 103.6% and from 92.3% to 106%, for plasma and cerebrospinal fluid samples, respectively. The preci-

Table 1
Intra- and inter-assay accuracy and precision for oxcarbazepine, 10-hydroxy-10,11-dihydrocarbamazepine, and 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine, in plasma and cerebrospinal fluid quality control samples

Analyte	Nominal concentration (ng/ml)	Intra-assay accuracy and precision		Inter-assay accuracy and precision	
		Accuracy (%)[a]	Precision (CV%)[b]	Accuracy (%)[a]	Precision (CV%)[b]
Plasma					
Oxcarbazepine	50	93.4	5.6	92.8	6.7
	500	94.8	6.3	95.7	5.8
	1000	100.5	4.9	103.2	4.7
10-Hydroxy metabolite	2000	93.8	6.4	92.9	6.4
	10000	103.2	5.9	102.7	5.3
	25000	97.1	4.2	98.2	4.9
10,11-Dihydroxy metabolite	200	92.8	7.1	93.7	6.7
	1000	103.6	6.4	102.1	7.2
	4000	96.6	4.1	95.2	3.8
Cerebrospinal fluid					
Oxcarbazepine	50	94.6	6.4	92.3	8.2
	500	99.6	6.2	98.7	7.1
	1000	96.8	2.8	95.4	4.2
10-Hydroxy metabolite	2000	94.2	5.8	95.1	5.4
	10000	95.2	3.6	94.2	4.1
	20000	95.8	2.3	93.9	3.4
10,11-Dihydroxy metabolite	100	106.1	6.9	105.2	7.3
	500	98.4	5.1	96.8	6.4
	1000	99.2	3.7	98.3	5.2

[a] Accuracy, found concentration expressed in % of the nominal concentration.
[b] CV, coefficient of variation.

Table 2
Concentration of oxcarbazepine and its two main metabolites, 10-hydroxy-10,11-dihydrocarbamazepine, and 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine, in plasma and cerebrospinal fluid samples obtained from patients treated with oxcarbazepine (mean dose: 1800 mg/day, range: 900–2100 mg/day)

Compound	Plasma concentration (ng/ml)	*n*	Cerebrospinal fluid concentration (ng/ml)	*n*
Oxcarbazepine	45–1225 (301 ± 321)	23	31–96 (60 ± 29)	5
10-Hydroxy metabolite	3731–36811 (18429 ± 8388)	23	2068–10441 (5544 ± 2899)	9
10,11-Dihydroxy metabolite	372–2652 (1358 ± 800)	23	123–589 (262 ± 148)	9

sion and accuracy data satisfactorily met the acceptance criteria: precision lower than 15% and mean accuracy within 85–115% [28].

3.5. Lower limit of quantification

The lower limit of quantification was experimentally determined to be 25, 50, and 50 ng/ml for oxcarbazepine, 10-hydroxyoxcarbazepine, and 10,11-dihydroxyoxcarbazepine, respectively, for both plasma and cerebrospinal fluid samples. The mean precision and accuracy of the lower limit of quantification was found to be less than the maximum tolerable CV of 20% and within 80–120% [28]. This lower limit of quantification is better or comparable to that reported previously by published HPLC methods [14,16,23] and is sufficient to determine plasma and cerebrospinal fluid concentrations of oxcarbazepine, 10-hydroxy-10,11-dihydrocarbamazepine, and 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine, in the conduct of clinical pharmacokinetic studies.

3.6. Clinical application

The present method was successfully applied to perform the determination of concentrations of oxcarbazepine, 10-hydroxy-10,11-dihydrocarbamazepine, and 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine in several authentic plasma and cerebrospinal fluid samples obtained from patients undergoing chronic treatment with oxcarbazepine (mean dose: 1800 mg/day, range: 900–2100 mg), either as monotherapy or in polytherapy with other antiepileptic drugs.

The measured therapeutic plasma concentrations ($n = 20$) ranged from 45 to 1225 ng/ml for oxcarbazepine, from 3731 to 36 811 ng/ml for its pharmacologically active metabolite 10-hydroxy-10,11-dihydrocarbamazepine, and from 372 to 2652 ng/ml for its inactive metabolite 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine (Table 2). These results seem to be in agreement with those reported previously [14,20]. Oxcarbazepine is a prodrug for its active metabolite, 10-hydroxy-10,11-dihydrocarbamazepine and therefore, concentrations of this metabolite were much higher than those of oxcarbazepine in all samples. Intermediate concentrations were measured for the inactive metabolite 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine.

The concentration of oxcarbazepine in cerebrospinal fluid samples was very low and varied between 31 and 96 ng/ml and it was measured in 5/9 samples. The concentration of the two metabolites was measured in all samples and ranged from 2068 to 10 441 ng/ml and from 123 to 589 ng/ml, for 10-hydroxy-10,11-dihydrocarbamazepine and 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine, respectively. The mean cerebrospinal fluid/plasma concentration ratio for 10-hydroxy-10,11-dihydrocarbamazepine was found to be 0.302 ± 0.130 (range: 0.179–0.554). These values are lower than the unbound plasma levels of 10-hydroxy-10,11-dihydrocarbamazepine (approximately 60%) [29], indicating that this metabolite does not appear to cross the blood-brain barrier by simple diffusion and it acts as a substrate of the transporter P-glycoprotein [30].

3.7. Conclusion

In conclusion, an isocratic reversed-phase HPLC-UV assay using liquid–liquid extraction for the simultaneous determination of oxcarbazepine and its two main metabolites, 10-hydroxy-10,11-dihydrocarbamazepine and 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine, in human plasma and cerebrospinal fluid samples was developed and validated. To the best of our knowledge this is the first report describing an assay of oxcarbazepine and its metabolites in cerebrospinal fluid samples. The method was simple, precise, accurate, selective and sufficiently sensitive and seems suitable for the quantitative determination of oxcarbazepine, 10-hydroxy-10,11-dihydrocarbamazepine and 10,11-dihydroxy-*trans*-10,11-dihydrocarbamazepine in plasma and cerebrospinal fluid samples, obtained in the conduct of clinical pharmacokinetic studies, after oral administration of oxcarbazepine, either in monotherapy or in polytherapy.

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