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Improved detection and determination of carbamazepine and oxcarbazepine and their metabolites by high-performance liquid chromatography

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Abstract

An HPLC assay for carbamazepine or oxcarbazepine (OXC) and six of their metabolites in one run was applied to 35 clinical samples from patients receiving monotherapy. This rapid and economical method, utilizing a simple one-step extraction with methyl *tert.*-butyl ether before the run, showed recoveries of 77–108%, except for 43% for 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine, from 500-μl samples, with detection limits of 8–12 ng/ml and limits of quantification of 14–55 ng/ml depending on the compound. Indication of a new OXC metabolite, 3-hydroxycarbamazepine, was found in 2/12 patients.

1. Introduction

Carbamazepine (5-carbamoyl-5*H*-dibenz[*b,f*]-azepine) is a tricyclic neutral lipophilic compound, which is widely used in partial epilepsy, trigeminal neuralgia and as an adjunct to neuroleptic therapy in psychosis [1]. It is nearly completely metabolized in the body [2], forming over 30 metabolites [3], and its metabolism is inducible, e.g., by antiepileptic drugs (AED). The main route is oxidation (40%), mainly to carbamazepine-10,11-epoxide (CBZ-E) [2], which is then hydrolysed by epoxide hydrolase. The end product of the epoxy pathway is 10,11-dihydroxy-10,11-dihydrocarbamazepine (10,11-D), which is considered to be pharmacologically inactive [2]. 10,11-D is found in plasma in concentrations exceeding those of CBZ-E [4]. Other metabolites described in humans include phenols and sulfate and glucuronide conjugates of the primary metabolites [2].

The main metabolite of CBZ, CBZ-E, has anticonvulsant properties approaching that of the parent drug in animal studies [5]. CBZ-E can occur in significant amounts in plasma (more so in children than in adults), and it is thought to produce side-effects [6]. Hence it is important to measure plasma levels of CBZ-E in addition to the parent drug.

Oxcarbazepine (OXC), a 10-keto analogue of CBZ, is a new antiepileptic compound, which has a similar therapeutic profile to CBZ but seems to be tolerated better. Less allergic reactions, tiredness, headache, dizziness and ataxia have been

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described [7,8]. In man, OXC is almost immediately and completely metabolized to 10-hydroxy-10,11-dihydrocarbamazepine (10-OH-CBZ), which is the major active substance in plasma [9]. The antiepileptic effects of OXC and 10-OH-CBZ are comparable in animals and man [7]. 10-OH-CBZ is further metabolized to 10,11-D [10,11]. Although OXC is a potent enzyme inducer in rats [12], it did not induce hepatic monooxygenase enzymes in eight volunteers [13]. The metabolism of OXC presumably does not depend on cytochrome P450 [9]. The keto group is reduced by aldo-keto reductase, which is not inducible [14], and the resulting monohydroxy metabolite is cleared by glucuronidation (UDP-glucuronyltransferase) [9].

The thermal instability of CBZ and OXC makes HPLC more attractive to use than gas chromatography [15,16], which in addition, requires elaborate sample preparation. Cross-reactivity produces problems in immunoassays [4]. Several HPLC methods have been developed to measure the parent drug, CBZ, and its main metabolites, CBZ-E and/or 10,11-D, in serum and urine samples [17–31]. In addition, detection of 2-hydroxycarbamazepine (2-OH-CBZ) [32] and the simultaneous determination of CBZ or OXC with 10-OH-CBZ [33,34] has been reported. Mostly reversed-phase chromatography has been used and separations have been carried out with columns packed with 3–5-μm particles.

Since all the published methods so far have included only up to three metabolites of CBZ, and many more metabolites are known to exist both in animals and in man [3], further method development for accurate human studies are needed. We have improved the HPLC methodology to measure a total of six CBZ metabolites. The same method is also applicable to OXC and its metabolites.

2. Experimental

2.1. Clinical samples

Serum samples were obtained from 23 patients (thirteen males, ten females) on CBZ and from twelve patients (six males, six females) on OXC without other medication for epilepsy. The mean age of the patients was 39 ± 13.4 years. The samples were stored at −20°C before analysis.

2.2. Chemicals

Carbamazepine (CBZ), 2-hydroxycarbamazepine (2-OH-CBZ), 3-hydroxycarbamazepine (3-OH-CBZ), 9-hydroxymethyl-10-carbamoylacridan (9-AC), carbamazepine-10,11-epoxide (CBZ-E), 10-hydroxy-10,11-dihydrocarbamazepine (10-OH-CBZ), 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine (10,11-D) and oxcarbazepine (OXC) were all gifts from Ciba-Geigy (Basle, Switzerland). Potassium dihydrogenphosphate (KH_2PO_4), acetonitrile, dichloromethane and methanol were obtained from Merck (Darmstadt, Germany), methyl *tert.*-butyl ether from Rathburn Chemicals (Walkerburn, UK), triethylamine from Fluka (Buchs, Switzerland) and Seronorm from Nycomed (Oslo, Norway). The reagents were of analytical-reagent grade and the solvents were of HPLC grade.

2.3. Extraction of samples

The starting point for sample preparation used in the study was the method of Chelberg et al. [32]. A standard or a serum sample was extracted first with an organic solvent. Samples were vortex mixed for 30 s with the solvent, shaken and then centrifuged for 5 min at 800 *g*. The organic phases were evaporated to dryness under a stream of nitrogen in a warm water-bath. The residues were dissolved in organic solvent–water and 20 μl were injected into the HPLC column.

2.4. Instruments and accessories

CBZ, OXC and their metabolites were determined by HPLC modified from the method of Turcant et al. [35] and Chelberg et al. [32]. The HPLC system consisted of a Merck–Hitachi L-6200 gradient pump and L-4250 UV–Vis detector set at 212 nm. The data were processed with a personal computer (Ektar EW-286PC-02

Monitor DM-3114), which incorporated a STAR Multi-Font LC-10 printer using Merck–Hitachi HPLC software. The mobile phase was a mixture of an organic solvent and water or buffer. The HPLC analysis was isocratic, carried out at room temperature at a flow-rate used of 1.0 ml/min. A LiChrospher 100 RP-18 precolumn (4 × 4 mm I.D., 5 μm) was used in all instances and LiChrospher 100 RP-18 (125 × 4 mm I.D., 5 μm), LiChrospher 60 RP-select B (250 × 4 mm I.D., 5 μm), LiChrospher 60 RP-select B (125 × 4 mm I.D., 5 μm), Superspher 60 RP-select B (250 × 4 mm I.D., 4 μm) and Superspher 60 RP-select B (125 × 4 mm I.D., 4 μm) reversed-phase columns from Merck were compared for analysis.

2.5. Working standards

Calibration graphs for CBZ, OXC and their metabolites were established using an identical extraction procedure with the standards and with the samples. Known amounts of each metabolite (CBZ-E, 2-OH-CBZ, 3-OH-CBZ, 10-OH-CBZ, 9-AC, 10,11-D) and parent compounds were added to drug-free serum (Seronorm) to give concentrations of 0.63–16.0 μg/ml. Peak areas were used for calculations. The slopes of the graphs were determined by linear regression analysis.

3. Results

3.1. Extraction

Methyl *tert.*-butyl ether [32], dichloromethane [10] and acetonitrile [36] were tried as described in the respective publications as the organic solvent for the extraction. The best yields for all metabolites and also the parent drug (CBZ) were obtained using methyl *tert.*-butyl ether, which was adopted in all subsequent extractions (data not shown).

Because it has been suggested that alkalinization of the solution is beneficial for the yield of CBZ-E [32], 0.05 *M* or 0.1 *M* of NaOH was added to 250- or 500-μl samples. The recoveries of CBZ and OXC and of 10,11-D, 10-OH-CBZ and CBZ-E increased with addition of the alkali, regardless of the sample size and the amount of NaOH added. However, alkali clearly decreased the yields for 2-OH-CBZ and 3-OH-CBZ (data not shown), as suggested also by Chelberg et al. [32]. We tried to overcome this by performing the first extraction without the alkali, and adding alkali in the second extraction. With this system the yields increased to 95% for 2-OH-CBZ and 101% for 3-OH-CBZ. Increasing the shaking time of the alkalinized samples from 5 to 30 min did not further improve the extraction yields of 2-OH-CBZ and 3-OH-CBZ. Acidification of the sample was also tried, to establish the effect of lower pH on the yield. However, neither 0.1 *M* HCl nor 0.1 *M* citrate–phosphate buffer (pH 3.5) gave better yields than the original one-extraction procedure.

Although the recoveries were best using two consecutive extractions of a 250-μl sample, with the second extraction alkalinized (87–112%, except for 59% for 10,11-D) compared with one extraction of a 500-μl sample without alkalinization (77–108%, except for 43% for 10,11-D), the total amount of metabolites was higher using the latter. The latter procedure was selected also based on the detection of 3-OH-CBZ and 2-OH-CBZ only from non-alkalinized 500-μl samples. All clinical samples were analysed by both the one-step extraction of 500 μl and the two-step extraction of 250 μl to obtain the maximum amount of information.

3.2. HPLC method

Selection of conditions

With a LiChrospher RP-18 column and NaH_2PO_4–MeOH (49:51) as the mobile phase [31], 3-OH-CBZ, CBZ-E and 9-AC were separated, but poorly, and there were problems with other metabolites regardless of the length of the column, flow-rate or the percentage of the organic solvent. Changing the mobile phase to 28–30% of acetonitrile in water, as used by Chelberg et al. [32], did not lead to a better separation of the metabolites.

Reducing the acetonitrile concentration to 20% and using either a LiChrospher or a

Table 1
The HPLC method selected to determine CBZ, OXC and their metabolites in serum

Sample size	500 μl
Sample treatment	One extraction with 6 ml of methyl *tert.*-butyl ether, evaporated organic phase dissolved in 40 μl of methanol–water (5:2, v/v), 20 μl injected into the column
Column	Superspher 60 RP-select B (125 × 4 mm I.D., 4 μm)
Mobile phase	Acetonitrile–20 m*M* KH_2PO_4 (20:80) containing 0.05% of triethylamine (pH 6.30)
Flow rate	1.0 ml/min
Temperature	Ambient
Detection wavelength	UV, 212 nm

Superspher 60 RP-select B column made a remarkable difference: in 60 min all six metabolites and the parent drugs could be separated. Potassium phosphate buffer (20 m*M* with 0.05% triethylamine, pH 6.30) [35] was used to stabilize the pH. A higher flow-rate shortened the retention time in both columns. However, with LiChrospher (5-μm particles) this led to insufficient separation. Superspher (4 μm particles) was therefore selected as the column to be used in subsequent studies. With a shorter column (125 mm), the total analysis time decreased to 30 min and the peaks nearest to each other (3-OH-CBZ and OXC) were separated by 1 min.

A gradient was tried in order to shorten the retention time of CBZ, but it caused variations in the retention times of some metabolites and isocratic conditions were used for the rest of the study. A better shape for the metabolite peaks was obtained when the sample was dissolved in methanol–water (5:2, v/v) compared with 100% methanol or acetonitrile–water (2:8, v/v) [35].

The method selected on basis of these experiments is shown in Table 1 and Fig. 1.

Validation of the method

For the calibration graphs, drugs and metabolites dissolved in Seronorm were used. Calibration graphs for CBZ and OXC and their metabolites were linear from 0.01–0.08 μg/ml up to at least 10 μg/ml (16 μg/ml for CBZ) (Table 2 and Fig. 2). Correlation coefficients and slopes were determined from peak areas for both the drug

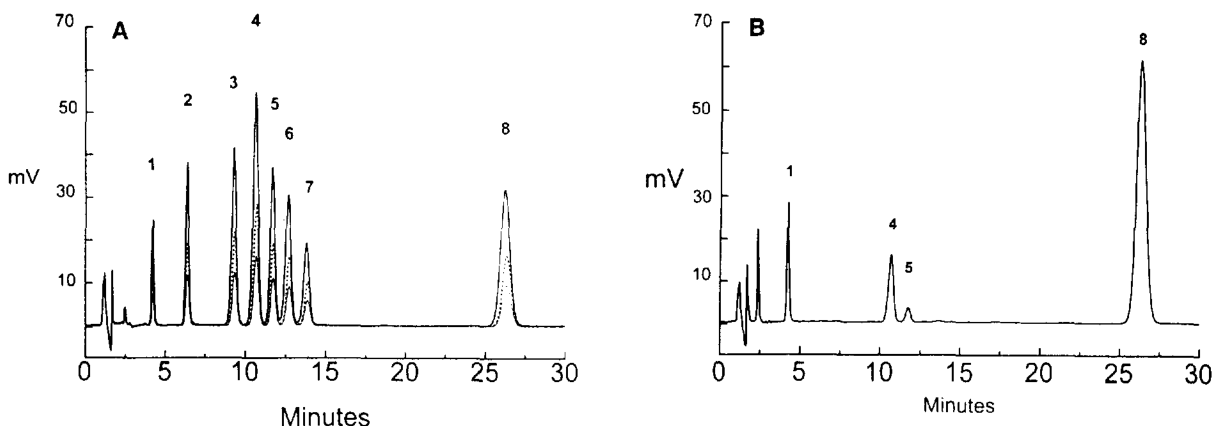

Fig. 1. HPLC of (A) standard compounds and (B) a sample from a carbamazepine-treated patient. For details of the HPLC, see Table 1. For carbamazepine overlaid profiles from concentrations of 1, 2 and 4 μg/ml are shown, and for other compounds 0.625, 1.25 and 2.5 μg/ml (A). Peaks: 1 = 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine (retention time 4.38 min); 2 = 10-hydroxy-10,11-dihydrocarbamazepine (6.63 min); 3 = 2-hydroxycarbamazepine (9.86 min); 4 = carbamazepine-10,11-epoxide (11.22 min); 5 = 9-hydroxymethyl-10-carbamoylacridan (12.28 min); 6 = 3-hydroxycarbamazepine (13.34 min); 7 = oxcarbazepine (14.49 min); 8 = carbamazepine (27.43 min).

Table 2
Data for the HPLC method selected

Compound	Range (μg/ml) (n = 5–6)	Intercept	r^2	Limit of detection (ng/ml; ng) (mean ± S.D., n = 4–5)	Limit of quantification (ng/ml; ng) (mean ± S.D., n = 4–5)	Recovery (%) (mean ± S.D., n = 5)
CBZ	1.0–16.0	0.0209	0.9974	10 ± 0; 0.2	39 ± 0; 0.8	108.2 ± 13.0
	0.039–0.625	−0.0127	0.9927			
CBZ-E	0.625–10.0	0.0316	0.9974	8 ± 3; 0.2	14 ± 5; 0.3	81.8 ± 8.8
	0.039–0.625	−0.0194	0.9955			
10,11-D	0.625–10.0	0.0136	0.9980	8 ± 3; 0.2	24 ± 15; 0.5	42.6 ± 2.4
	0.078–0.0625	−0.0345	0.9897			
9-AC	0.625–10.0	0.0329	0.9970	8 ± 3; 0.2	22 ± 11; 0.4	102.4 ± 9.1
	0.039–0.625	−0.0086	0.9940			
2-OH-CBZ	0.625–10.0	0.0323	0.9974	8 ± 3; 0.2	24 ± 15; 0.5	91.0 ± 3.1
	0.039–0.625	−0.0169	0.9943			
3-OH-CBZ	0.625–10.0	0.0378	0.9974	9 ± 2; 0.2	33 ± 13; 0.7	97.0 ± 5.5
	0.039–0.625	−0.0165	0.9924			
OXC	0.625–10.0	0.0132	0.9965	12 ± 5; 0.2	55 ± 21; 1.1	96.6 ± 13.3
	0.078–0.625	0.0330	0.9827			
10-OH-CBZ	0.625–10.0	−0.0456	0.9980	8 ± 3; 0.2	24 ± 15; 0.5	76.8 ± 5.1
	0.039–0.625	−0.0239	0.9961			

and the metabolites. The recoveries of all metabolites in serum varied very little from one measurement to another. Quantitative results calculated using external standardization (comparison with the calibration graphs) were close to those obtained with internal standardization and the values with external standardization were consistently higher (8.8–9.5%) than those with internal standardization (Table 3). We therefore considered it unnecessary to search for a new internal standard. Metabolites used earlier as internal standards [24,33] were naturally out of the question because the aim was to detect as many metabolites as possible and the retention time of 2-methylcarbamazepine [32] was too long compared with those for the analyte compounds. That an internal standard is not necessary in the HPLC of CBZ is also supported by the literature [20,29].

The sensitivity and reproducibility of the selected method were at least as good as those of published methods [31,32]. The reproducibility of the retention times for CBZ, OXC and their metabolites, as judged by the coefficients of variation (C.V.), was good: 0.1–0.3% within one day and 0.2–3.8% within one week (see also Fig. 1). The column needed to be washed on average once a week with acetonitrile–water containing a few drops of phosphoric acid per litre. This inhibited the increases in retention times and pressure seen otherwise.

The day-to-day variation in quantification was assessed as the C.V. of six repeated measurements of a standard (CBZ 8 μg/ml and OXC and metabolites 5 μg/ml) at 1–4 day intervals and was 5%. When the same clinical sample was thawed eight times over a period of 24 days, the C.V. was 8% for CBZ and about 11% for the metabolites. The very low concentrations of metabolites in the clinical sample probably explain the higher C.V. compared with the measurement of standards.

The limits of detection and quantification were derived from multiple measurements in the low concentration range. The limit of detection is defined as the level three times the noise level,

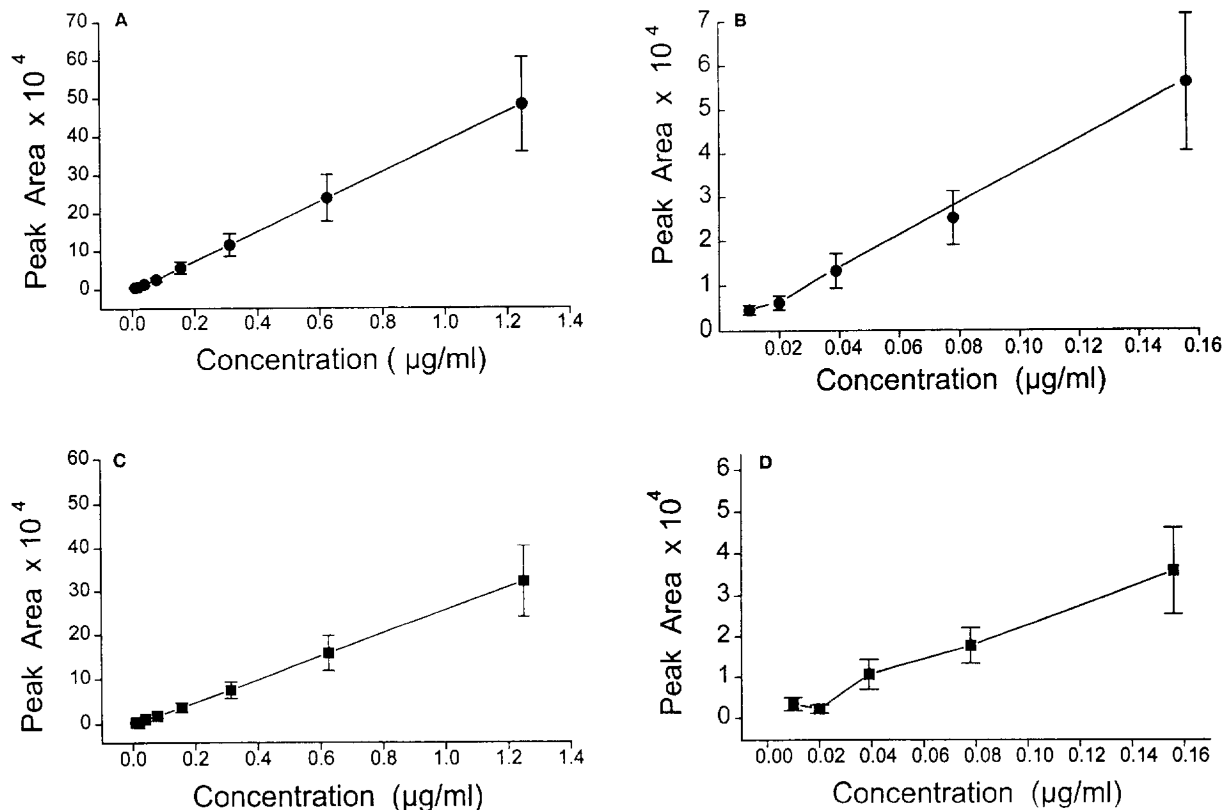

Fig. 2. Calibration graphs with error bars (± S.E.M., $n = 4$–5) for CBZ-E and CBZ with external standardization. (A) CBZ-E 0.01–1.25 μg/ml; (B) CBZ-E 0.01–0.156 μg/ml; (C) CBZ 0.01–1.25 μg/ml; (D) CBZ 0.01–0.156 μg/ml.

according to the IUPAC convention. Similarly, the limit of quantification is defined as the level ten times the noise level. The limits of detection and quantification varied depending on the compound (Table 2). The stability of the samples was tested at 4 and −20°C. After 10 days at −20°C

Table 3
Comparison of the results with or without 10-OH-CBZ as an internal standard

Compound	0.625 μg/ml		1.25 μg/ml		2.5 μg/ml	
	Internal	External	Internal	External	Internal	External
CBZ	0.55 ± 0.03 (4.4)	0.58 ± 0.03 (5.1)	1.28 ± 0.03 (2.0)	1.36 ± 0.05* (3.9)	2.55 ± 0.04 (1.6)	2.90 ± 0.12** (5.6)
CBZ-E	0.63 ± 0.02 (2.3)	0.66 ± 0.03* (4.3)	1.28 ± 0.04 (3.2)	1.36 ± 0.05** (3.6)	2.53 ± 0.04 (1.5)	2.87 ± 0.12** (5.4)
10,11-D	0.67 ± 0.02 (2.1)	0.71 ± 0.04 (6.0)	1.38 ± 0.03 (2.3)	1.47 ± 0.06** (3.7)	2.13 ± 0.02 (0.9)	2.43 ± 0.12** (4.8)
9-AC	0.62 ± 0.02 (3.0)	0.65 ± 0.03 (4.7)	1.24 ± 0.04 (3.2)	1.32 ± 0.02* (3.6)	2.43 ± 0.04 (1.4)	2.75 ± 0.06** (5.5)
2-OH-CBZ	0.60 ± 0.03 (4.4)	0.64 ± 0.03 (5.2)	1.26 ± 0.04 (3.2)	1.33 ± 0.05* (3.6)	2.61 ± 0.05 (2.0)	2.97 ± 0.18** (6.0)
3-OH-CBZ	0.61 ± 0.03 (4.4)	0.64 ± 0.02* (3.4)	1.25 ± 0.04 (3.1)	1.32 ± 0.05* (3.8)	2.63 ± 0.03 (1.2)	2.98 ± 0.17** (5.5)
OXC	0.46 ± 0.10 (8.5)[a]	0.48 ± 0.13 (8.6)[a]	1.09 ± 0.06 (5.6)	1.15 ± 0.04 (3.5)	3.04 ± 0.06 (2.0)	3.44 ± 0.21** (6.0)

Results are means ± S.D. with C.V. (%) in parentheses; $n = 6$ except where indicated. Significance: $*p < 0.05$; $**p < 0.01$.
[a] $n = 5$.

the samples contained 96–100% of CBZ, OXC or metabolites. The concentrations decreased to one third after 3 days at 4°C.

The following antiepileptic drugs were studied and found not to interfere with the determination of CBZ, OXC or metabolites: phenytoin, sodium valproate, clobazam, clonazepam, phenobarbital and primidone.

3.3. Clinical samples

Twenty-three clinical samples from patients on CBZ monotherapy were studied using the developed HPLC method (Table 4). The CBZ concentration in serum samples was 2.1–10.3 μg/ml, which was within the published therapeutic range (3.5–10 μg/ml) [37]. The concentration of the active metabolite of CBZ, carbamazepine-10,11-epoxide (CBZ-E), varied between 0.11 and 1.07 μg/ml and it was found in all samples from patients receiving CBZ. 10,11-D and 9-AC were also found in all samples from patients receiving CBZ, the concentration ranging from 0.56 to 2.8 μg/ml for 10,11-D and 0.03 to 0.28 μg/ml for 9-AC. In addition, 2-OH-CBZ was found in 1/23 and 3-OH-CBZ in 4/23 samples.

OXC is a pro-drug for the active metabolite, 10-OH-CBZ, and the individual therapeutic level of OXC has been shown to be very low, 0–2.1 μg/ml [7]. In our twelve samples, the range of serum concentrations of OXC was 0.09–0.69 μg/ml. The concentration of the active metabolite of OXC, 10-OH-CBZ, was between 1.38 and 11.29 μg/ml. In addition, 3-OH-CBZ, not described as an OXC metabolite in humans before, was detected in 2/12 samples. The detection was reproducible and the peaks were symmetrical.

One of the 23 samples from patients on the CBZ monotherapy contained an unknown peak. This patient was not taking any other medication. The peak was close to 10-OH-CBZ and it may represent an unknown metabolite of CBZ.

Table 4
CBZ or OXC and their metabolites found in serum from 23 patients on CBZ and 12 patients on OXC monotherapy

Monotherapy	Compound	Concentrations in this study (μg/ml) (mean ± S.D.)	Concentrations reported in literature (μg/ml) (range or mean ± S.D.)
CBZ	CBZ	6.15 ± 0.19	3.5–10[a]
	CBZ-E	0.44 ± 0.23	1.55 (± 1.24)[b]
	10,11-D	1.35 ± 0.65	2.50 (± 1.35)[c]
	9-AC	0.13 ± 0.09	present
	3-OH-CBZ[d]	0.005 ± 0.01[e]	present
	2-OH-CBZ[f]	Present; <0.02[g]	present
OXC	OXC	0.30 ± 0.20	0–2.1[h]
	10-OH-CBZ	11.05 ± 5.59	2.1–9.9[h]
	10,11-D	0.63 ± 0.53	0.9–3.0[h]
	3-OH-CBZ[i]	0.007 ± 0.02	Not found

[a] [37].
[b] [25].
[c] [33].
[d] Found in 4/23 samples; one value above quantification level.
[e] For the calculation of the mean value, a median value between the detection and quantification levels was used for detectable levels below the quantification level.
[f] Found in 1/23 samples.
[g] In one sample; below the quantification level.
[h] [7].
[i] Found in 2/12 samples.

4. Discussion

Using the described HPLC method, CBZ, OXC and six of their metabolites can be analysed in 30 min after a simple preparative step compared with a maximum of three metabolites in the methods published earlier [32]. Using this method, a new metabolite of OXC in humans, 3-OH-CBZ, was detected in 2/12 samples from patients on OXC monotherapy. However, the identity of 3-OH-CBZ needs to be confirmed by another method.

Most of the other HPLC methods measure only CBZ and its principal metabolites (CBZ-E and/or 10,11-D) (e.g., [30,31]). In addition to these 2-OH-CBZ can be determined by an HPLC method published by Chelberg et al. (1988) [32]. For OXC, traditionally only its active metabolite, 10-OH-CBZ, is measured in addition to the parent drug [38–40]. This is clearly insufficient, because other metabolites also exist in humans. Some methods even include CBZ metabolites such as 9-AC [33] and 10-OH-CBZ [24] as internal standards. This has naturally compromised any determination of 9-AC and 10-OH-CBZ as metabolites. For pharmacokinetic studies, our method thus provides a significant improvement. The results with external standardization were consistently higher than those with internal standardization. We conclude that the results are reliable with both methods, but one ought to use the same standardization for calibration graphs and samples (either internal or external).

The recovery, although good for the parent drugs and other metabolites, was low for 10,11-D (maximum 59%). This is also the case, however, with other methods; for instance, Bonato et al. [24] reported a 62% and Riad and Sawchuk [23] a 53% recovery for this metabolite.

The solvent extraction method proposed here is simple, inexpensive, rapid and concentrates the sample. These are all advantages compared with the methods published earlier [26–28,34]. As in the literature, alkalinization of the sample gives a better yield [24,30,32,33]. The benefit of two extractions of 250 μl compared with one extraction of 500 μl in sample preparation was clear when comparing the yields. However, 500 μl still gives a higher total amount of metabolites and was thus a reasonable choice for the final method. Further, more metabolites in clinical serum samples were detected by the method of one extraction of a 500-μl sample without alkalinization; 2-OH-CBZ and 3-OH-CBZ were not detectable in any of the 250-μl samples.

The procedure described demonstrated both the good reproducibility and sensitivity required for pharmacokinetic studies. The quantification limit of CBZ was remarkably low (39 ng/ml) compared with the previous levels (0.1 μg/ml [31] and 0.27 μg/ml [30]).

An interesting aspect raised by our studies is the metabolism of CBZ during pregnancy. In previous work, in a series of plasma samples taken at delivery from sixteen pregnant women treated with CBZ, 2-OH-CBZ was found in four (25%) and 3-OH-CBZ in six (38%) of the cases [41]. These metabolites were found less frequently in this study (2-OH-CBZ in 4% and 3-OH-CBZ in 17% of the cases). Moreover, 10-OH-CBZ was found in two samples in the earlier series whereas no certain positive samples were found among the present series. There are discrepant views as to whether pregnancy influences drug metabolism [42]. It remains to be studied further whether the differences we have seen are due to pregnancy or to some other factors.

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