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GAS CHROMATOGRAPHIC ASSAY FOR OXCARBAZEPINE AND ITS MAIN METABOLITES IN PLASMA

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SUMMARY

The new anti-epileptic drug oxcarbazepine is temperature-labile and decomposes under the conditions of gas chromatography, even when injected into a cooled, inert, fused-silica capillary column. In contrast, the trimethylsilyl derivative of oxcarbazepine is stable. The bis-trimethylsilyl derivatives of the enol of oxcarbazepine and of its active metabolite, 10-hydroxycarbazepine, and the tris-trimethylsilyl derivative of carbazepine-10,11-*trans*-diol can be synthesized easily at room temperature. Using the readily available carbamazepine as internal standard, a simple gas chromatographic assay was developed for the simultaneous routine measurement of these three compounds at therapeutic levels. This assay is ten times more sensitive to oxcarbazepine than the previously described high-performance liquid chromatographic assays. It involves a single-step solvent extraction, uses a fused-silica capillary column and a flame ionization detector. On processing 0.5 ml of plasma, limits of detection of 10 ng/ml were obtained for oxcarbazepine and 10-hydroxy-carbazepine and a limit of detection of 25 ng/ml for carbazepine-10,11-*trans*-diol.

INTRODUCTION

Oxcarbazepine (OCB) is a promising new anti-epileptic drug [1, 2], structurally related to carbamazepine (CBZ). OCB contains a keto and a methylene group instead of the ethylene bridge between the two benzene rings. It is a 10,11-dihydro-10-oxocarbamazepine (for formulae see Fig. 1). Its anti-epileptic potency is comparable to that of carbamazepine, but subjective side-effects are fewer [1, 2]. The metabolism is strikingly different. The main metabolic pathway of CBZ in patients is the epoxide-diol pathway. The double bond between C-10 and C-11 of CBZ is oxidatively attacked by an epoxidase. This pathway is especially important in patients because of the autoinductive properties of CBZ [3]. Unwanted side-effects are at least in part related to the

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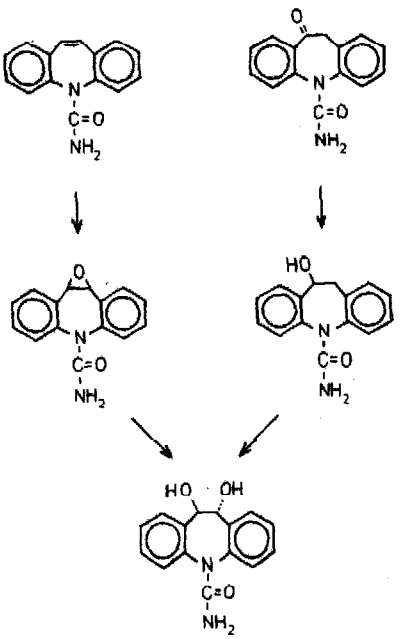

Fig. 1. Formulae of CBZ, molecular weight 236.26 (left, top row) and OCB, molecular weight 252.26 (right, top row), their pharmacologically active metabolites carbamazepine epoxide and 10-hydroxycarbazepine, and the common inactive *trans*-10,11-dihydroxy-carbazepine, molecular weight 270.26.

CBZ epoxide [4]. This oxidative attack is not possible on OCB. The main metabolic reaction of OCB is the reduction to the corresponding alcohol [5], 10,11-dihydro-10-hydroxycarbamazepine (10-hydroxycarbazepine) (see Fig. 1). This metabolite possesses an anticonvulsive activity comparable to the parent compound [5], whereas the 10,11-diol is inactive. In spite of a similar enzyme-inducing potential of OCB for oxidative enzymes, the metabolism of OCB itself appears virtually unaffected. In patients under treatment, the plasma concentration of the 10-hydroxy metabolite (range 5—30 μg/ml) is ca. 10—50-fold higher than the concentration of OCB (range 0.05—1 μg/ml).

High-performance liquid chromatographic (HPLC) assays have been developed for the simultaneous analysis of OCB and its main metabolites [2, 6—9]. HPLC assays have been used for all serum level controls and for the pharmacokinetic studies so far.

A gas chromatographic (GC) assay might be expected to provide a more sensitive alternative but, to our knowledge, all attempts to develop such an assay have failed so far. OCB is considerably more temperature-labile than CBZ. Therefore, all our attempts to develop a gas chromatographic—mass spectrometric (GC—MS) assay for underivatized OCB failed, in spite of considerable experience with CBZ analysis [3] and recent improvements by replacing the packed-column GC method by a procedure using a fused-silica capillary column and a cooled on-column injector. Trying derivatives, we found a simple procedure for the simultaneous GC analysis of OCB, its clinically important reduced metabolite 10-hydroxycarbazepine and the other known metabolite *trans*-10,11-dihydroxycarbazepine. This procedure is fast and simple

enough for routine analysis. For the clinician, only the concentrations of the pharmacologically active compounds OCB and 10-hydroxycarbazepine are of interest. Therefore, we shall concentrate on these two compounds in this paper. The GC—MS analysis and the quantitative determination of 10,11-dihydroxycarbazepine analysis is described in detail elsewhere [10].

OCB reacts in its enol form with N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) at room temperature to form the bis-trimethylsilyl derivative. One trimethylsilyl (TMS) group replaces the hydrogen of the enol group, the other replaces one hydrogen of the amide group, the latter being a well-known reaction of CBZ [11]. OCB-bis-TMS does not decompose appreciably into the iminostilbene analogue under the conditions used (injected on column at 140°C oven temperature in MSTFA as solvent using the cooled on-column injector). The TMS derivatives of 10-hydroxycarbazepine and of *trans*-10,11-dihydroxycarbazepine (see Fig. 2) are also formed under these mild reaction conditions and behave similarly. The peaks of the three decomposition products (the iminostilbene derivatives) were about one-tenth of the hight of the peaks of the undecomposed compounds; their retention time was only 55—60% of that of the undecomposed compounds. A procedure with pyrolytic generation of the iminostilbene derivatives does not seem feasible because their retention times are within the range of peaks originating from blank plasma components.

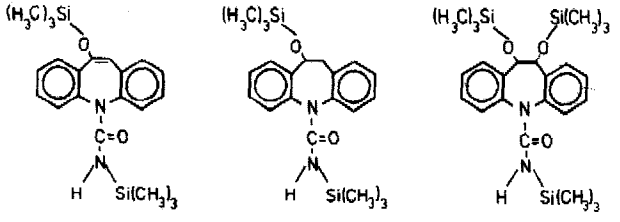

Fig. 2. Formulae of the MSTFA reaction products of OCB, 10-hydroxycarbazepine and *trans*-10,11-dihydroxycarbazepine.

MATERIALS AND METHODS

Patients

All patients were participants in clinical phase 3 studies with oxcarbazepine, conducted by the epilepsy ambulance of the Department of Neurology, University of Bonn (F.R.G.).

Materials

The reference compounds were a gift from Ciba-Geigy (Basel, Switzerland). MSTFA for GC was purchased from Macherey-Nagel & Co. (Düren, F.R.G.). Hydrochloric acid and methylene chloride were analytical grade reagents.

Work-up

An aliquot of 0.5 ml of plasma with 20 μl of internal standard solution (0.25 μg/μl methanol) was acidified with 0.25 ml of 1 *M* hydrochloric acid. The aqueous phase was mixed with the aid of a whirlmix and extracted with 4 ml of methylene chloride by shaking for 0.5 min on a whirlmix. The samples were

centrifuged at 2500 *g* for 10 min. The aqueous phase was aspirated and discarded, and the methylene chloride extract was transferred into a clean glass tube with a pointed tip. This extract can be stored at −20°C for at least one month. Before analysis, the methylene chloride was blown down with nitrogen at room temperature. To the dry extract 30 μl of MSTFA were added. The sample was redissolved by agitating for 0.5 min on a whirlmix and allowed to react for 20 min at room temperature.

Gas chromatographic conditions

A Carlo Erba Fractovap Series 4160 capillary gas chromatograph was used with a cooled on-column injector. For plasma level monitoring, normal flame ionization detection (FID) is sufficient. The detector should be cleaned regularly from the silicon dioxide formed. The capillary was a 30 m × 0.31 mm I.D. fused-silica high-performance capillary from Hewlett-Packard (1 μm cross-linked methyl silicone phase). Carrier gas pressure was 1.4 kg/cm^2, make-up gas pressure was 1 kg/cm^2, and detector temperature was 300°C for the chromatogram shown in Fig. 3. Carrier and make-up gas was hydrogen. Hydrogen, however, is not required. Helium may be used to give equally satisfactory results. (In the GC—MS system we used helium with the same capillary, see Fig. 4.) A 1-μl volume of the solution was injected into the cooled on-column injector, oven temperature 140°C (the injection part of the capillary having a temperature below the boiling point of MSTFA of 130°C), temperature programme ballistic to 200°C, 2 min after injection 30°C/min to

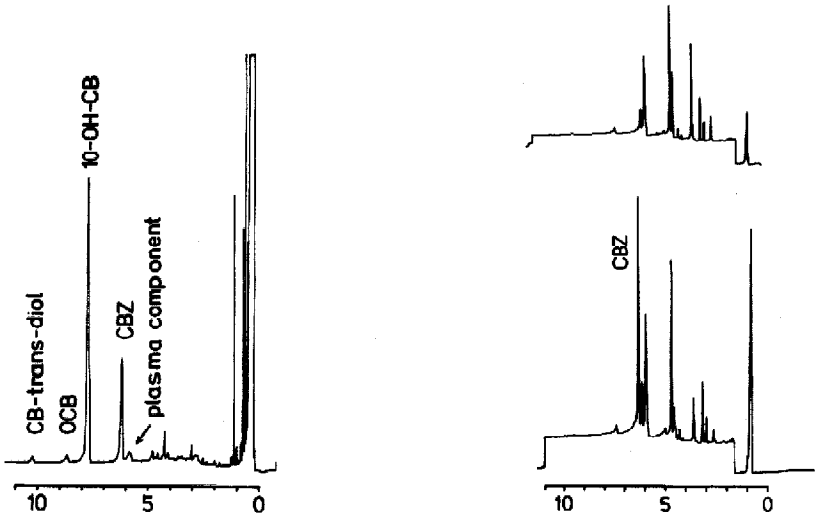

Fig. 3. Gas chromatogram of the silylated extract of a plasma sample of a patient receiving 6 × 300 mg of OCB, 2 mg of clonazepam and 5 × 300 mg of valproic acid sodium salt; attenuation 128, 0.5 μl injected. CB-*trans*-diol = *trans*-10,11-dihydroxycarbazepine; 10-OH-CB = 10-hydroxycarbazepine.

Fig. 4. Gas chromatogram of a silylated blank plasma extract displaying two large interfering peaks (top) and the corresponding chromatogram from the same plasma spiked with the normal amount of internal standard. These two traces are total ion chromatograms.

240°C. Retention times: the derivatives of CBZ (internal standard), 6 min 20 s; 10-hydroxycarbazepine, 7 min 45 s; OCB, 8 min 45 s; carbazepine-*trans*-diol, 10 min 25 s. Recording was done with a strip-chart recorder.

RESULTS

Extraction efficiency

The extraction efficiencies for OCB, 10-hydroxycarbazepine and *trans*-10,11-dihydroxycarbazepine, under the conditions reported for the work-up, were 73 ± 4%, 65 ± 5% and only 16 ± 3%, respectively.

Sensitivity

The limit of detection for OCB and for 10-hydroxycarbazepine was 10 ng/ml (40 nmol/l plasma) and that for *trans*-10,11-dihydroxycarbazepine was 25 ng/ml (90 nmol/l plasma), if 0.5 ml of plasma was used.

Stability of derivatives

The stability of the derivatives formed is good if humidity and hydroxyl-group-containing solvents are carefully excluded. One sample containing 30 μg CBZ, 30 μg 10-hydroxycarbazepine and 30 μg OCB was derivatized with 300 μl MSTFA (ten times the normal amount) and frequently injected over 24 h. The ratios obtained are given in Table I. The data were obtained with a

TABLE I

RESPONSE RATIOS AS A FUNCTION OF TIME

Time*	10-Hydroxycarbazepine/CBZ	OCB/CBZ
3 min	1.10	0.74
10 min	1.25	0.87
20 min	1.20	0.82
27 min	1.03	0.76
35 min	1.09	0.76
45 min	1.16	0.86
52 min	1.18	0.76
1 h	1.08	0.82
2 h	1.19	0.80
2 h 30 min	1.21	0.82
2 h 37 min	1.20	0.85
2 h 45 min	1.08	0.82
2 h 50 min	1.12	0.80
3 h	1.03	0.87
5 h	1.09	0.81
—**		
24 h	1.17	0.83
24 h 7 min	1.25	0.93
Mean	1.143	0.819
S.D.	0.0707	0.0486
C.V.	6.2%	5.9%

*Time of injection after addition of MSTFA.
**Stored at − 20°C.

slightly faster temperature programme in order to follow the derivatization reaction yield [11]. We found no striking differences over the period of one day. Conclusions of this experiment: (i) carefully stoppered samples may be left at room temperature for several hours without giving misleading results; (ii) the reaction time of only 3 min may be too short (smallest OCB/CBZ ratio of the series), the 20 min given in the procedure include a safety margin; (iii) exclusion of values obtained before 20 min and after 5 h improves the coefficients of variation from 6.2 and 5.9% to 5.8 and 4.5% for the 10-hydroxycarbazepine/CBZ and OCB/CBZ ratios, respectively.

If a normal sample derivatized with 30 μl MSTFA is repeatedly injected, the solution may already start to solidify after six injections, depending on the humidity of the air. Ratios obtained from such samples are no longer reliable.

Calibration

Known amounts of 10-hydroxycarbazepine and OCB in methanolic solution and the usual amount of internal standard solution were added to blank plasma samples to prepare the calibration curve. After analysing the calibration samples, peak-height ratios of 10-hydroxycarbazepine/CBZ and OCB/CBZ were calculated and plotted against concentration. The calibration curves were linear in the ranges 0—40 and 0—3 μg/ml for the two compounds. Calibration data are given in Table II.

TABLE II

CALIBRATION DATA

Concentration 10-hydroxycarbazepine (μg/ml)	10-Hydroxycarbazepine/CBZ*	Concentration OCB (μg/ml)	OCB/CBZ*
0.0	0.000	0.00	0.000
5.0	0.172	0.10	0.009
10.0	0.378	0.50	0.029
20.0	0.723	1.00	0.057
30.0	1.178	2.00	0.108
40.0	1.500	3.00	0.165
$y = -0.009 + 0.38\,x$		$y = 0.002 + 0.054\,x$	
$r = 0.9991$		$r = 0.9997$	

*Ratios are mean values from two samples, each injected twice.

Reproducibility

The precision of the overall assay was determined for the pharmacologically active compounds by analysing five 0.5-ml plasma samples containing 30 μg 10-hydroxycarbazepine and 0.5 μg OCB per millilitre of plasma; the data are given in Table III. The precision of the GC quantification (within-run precision) was determined by analysing the last of the aforementioned plasma samples ten times. The concentrations found were 30.316 ± 1.2808 and 0.538 ± 0.0202 (mean ± S.D.) and the coefficients of variation were 4.2 and 3.8%, respectively. Subsequently, we learned to improve the precision of the assay by heating the

capillary to 300°C for 15 min whenever the peaks started to tail. The heating reduced the number of outlayers. The precision could also be improved by use of an integrator.

TABLE III

PRECISION OF THE ASSAY

Concentrations (μg/ml) found (mean from two injections) in five spiked plasma samples; amounts added: 30.0 and 0.50 μg/ml.

Sample No.	10-Hydroxycarbazepine	OCB
1	29.67	0.478
2	26.46	0.484
3	30.44	0.480
4	29.54	0.504
5	28.69	0.533
Mean	28.96	0.496
S.D.	1.529	0.0232
C.V.	5.3%	4.7%

Accuracy

Ten 0.5-ml spiked plasma samples containing 10-hydroxycarbazepine and OCB in concentrations representative for the range of patient plasma levels were analysed; mean data are given in Table IV.

TABLE IV

ACCURACY

Concentrations found (μg/ml) are mean values from two samples, each injected twice.

Added		Found	
10-Hydroxycarbazepine	OCB	10-Hydroxycarbazepine	OCB
30.0	0.05	28.76	0.047
20.0	0.10	21.90	0.102
10.0	0.25	10.47	0.261
20.0	1.00	19.22	1.036
5.0	1.25	5.48	1.201
25.0	0.40	23.68	0.392
20.0	0.20	18.86	0.216
10.0	1.50	9.88	1.512
30.0	2.00	28.13	2.001
10.0	0.80	10.03	0.790

Specificity

The following anti-epileptic drugs and their metabolites did not interfere with the assay as determined in plasma from patients treated with these drugs: carbamazepine (if the above-mentioned *cis*-diol is used as the internal standard), clonazepam, phenobarbital, phenytoin, primidone, valproic acid.

In order to evaluate the specificity of this assay in more detail, twelve patient plasma samples were remeasured by GC—MS (see Table V). At the time of analysis, the medication of the patients was unknown to us. All patients were participants of a double blind study CBZ versus OCB. Three samples were from the CBZ phase of the study and neither OCB nor 10-hydroxycarbazepine was found. For the eight samples out of the OCB phase of the study (numbers 1—8), the coefficients of correlation of the measured concentrations were 0.991 and 0.981 for 10-hydroxycarbazepine and OCB, respectively. The last sample (No. 9) presented a special case. It was from the morning of the first day of the OCB phase after CBZ treatment, and CBZ was still present in the plasma. Therefore, the use of this drug as the internal standard for the FID analysis produced an internal standard peak which was too high and consequently a concentration which was too low. The 10-hydroxy metabolite was now only present at a concentration equal to the limit of detection. This low concentration was not expected and therefore the recording was not sensitive enough to measure this small amount. These analytical errors were apparent from the GC—MS results using *cis*-10,11-dihydroxycarbazepine as the internal standard.

TABLE V

CONCENTRATIONS (μg/ml) OF 10-HYDROXYCARBAZEPINE AND OXCARBAZEPINE IN PLASMA OF PATIENTS UNDER OXCARBAZEPINE THERAPY

Samples were worked-up separately for the GC—FID and the GC—MS measurements.

Sample No.	10-Hydroxycarbazepine		OCB	
	FID	GC—MS	FID	GC—MS
1	27.78	25.72	0.708	0.798
2	5.79	4.78	0.129	0.134
3	26.87	27.30	0.581	0.523
4	10.82	9.28	0.208	0.200
5	10.06	8.59	0.387	0.360
6	10.00	8.71	0.201	0.219
7	29.00	27.00	0.702	0.650
8	18.57	20.00	0.667	0.640
9	N.D.*	0.01	0.070	0.080

*N.D. = Not detected at the attenuation used.

DISCUSSION

The procedure described is a superior alternative to an HPLC assay. It is ten times more sensitive to OCB than the HPLC assays. The times for the work-up and the run-times are comparable or shorter. OCB and its main metabolites can be quantified simultaneously if desired. In patients who receive no CBZ, the readily available CBZ can be used as the internal standard. If patients receive both CBZ and OCB, *cis*-10,11-dihydroxycarbazepine, which in man is neither a metabolite of CBZ nor of OCB, has to be used as the internal standard. The electron-impact mass spectrum of the reaction product of OCB

with MSTFA shows a small molecular ion for the bis-TMS derivative, indicating the formation of the enol derivative. The base peak in the spectrum is the fragment ion corresponding to the substituted iminostilbene formed by the electron-impact-induced loss of the silylated iminoketene. This ion of mass *m/z* 281 is used for selective ion monitoring. The mass spectra of the reaction products of the above-mentioned hydroxy metabolites show the corresponding ions (for details, see ref. 10).

The requirements on the chromatographic separation power of the column are determined by the endogenous plasma peak eluting just before the CBZ peak. Because of this peak (for an example of a large peak see Fig. 4, blank plasma GC without and with CBZ; for an example of a normal peak see Fig. 3), the low temperature and the resulting long analysis times had to be selected. If *cis*-10,11-dihydroxycarbazepine (the other internal standard) is used, the aforementioned interference is no longer important. But the derivatives of *cis*- and *trans*-10,11-dihydroxycarbazepine have to be separated completely. This separation also requires either a thick film or a 50-m capillary, also resulting in a long analysis time.

The extraction procedure (with methylene chloride from acidified plasma) was selected because of the cleanness of the resulting extracts and because the number of interfering GC peaks was minimal. In the literature, the extraction has been described from acidified [1], neutral [2, 7—9] or alkaline [6] plasma. The pH dependence of the extraction efficiency was therefore measured and found to be virtually constant for OCB and 10-hydroxycarbazepine in the pH range 1—10. The yield of extraction of 10,11-dihydroxycarbazepine was clearly lower from an acidified than from an alkaline aqueous phase. It is, however, sufficient for simultaneous measurement (see Fig. 3). For a clean extract and a good extraction efficiency for *trans*-10,11-dihydroxycarbazepine, the charcoal extraction procedure [3, 10] has to be used. As an extraction solvent, methylene chloride was found to be superior to cyclohexane, diethyl ether, and ethyl acetate, with respect to ease of sample handling and number of interfering peaks.

The high sensitivity — 1 ppm of the mean 10-hydroxycarbazepine level and one fiftieth of the mean OCB level can be detected — and the simplicity and speed of work-up make this procedure a good candidate for routine determination of OCB plasma levels. Now it is possible to investigate the importance of OCB plasma-level determinations for patient management. If it turns out that knowledge of the 10-hydroxycarbazepine level is sufficient for the treating physician, the GC analysis time can be shortened considerably. This procedure is also well-suited for OCB quantification in samples from single-dose pharmacokinetic studies.

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