Journal of Chromatography, 343 (1985) 369—377
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2689

DETERMINATION OF CLOBAZAM, N-DESMETHYLCLOBAZAM AND THEIR HYDROXY METABOLITES IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received March 7th, 1985; revised manuscript received April 19th, 1985)

SUMMARY

Owing to the pharmacological and clinical importance of the determination of plasma and urine levels of the hydroxy metabolites of clobazam and N-desmethylclobazam in healthy volunteers and in epileptic patients, a high-performance liquid chromatographic (HPLC) method was developed that permits the determination of all these compounds in the same plasma or urine sample. The method involved ether extraction at pH 13 with diazepam as internal standard for the measurement of clobazam and N-desmethylcobazam, followed by ether extraction at pH 9 with nitrazepam as internal standard for the measurement of the hydroxy derivatives. The limit of detection was about 10—20 ng/ml for each of these compounds. Applications to patients were limited by chromatographic interferences between the hydroxy metabolites and some medications currently associated with clobazam in the treatment of epilepsy. The only interference in clobazam and N-desmethylclobazam analysis was from N-desmethyldiazepam. Despite these inconveniences, this HPLC procedure appears to be the only available method for the simultaneous quantification of clobazam and its three main metabolites.

INTRODUCTION

Clobazam (I), a 1,5-benzodiazepine synthesized by Rossi et al. [1], shows considerable anti-anxiety activity [2] but weak hypnotic properties [3]. It has been used as a second drug in the treatment of various forms of epilepsy [4, 5]. The two most important chemical changes that clobazam undergoes during metabolism are dealkylation and hydroxylation [6, 7], the main metabolites being N-desmethylclobazam (II), 4'-hydroxyclobazam (III) and

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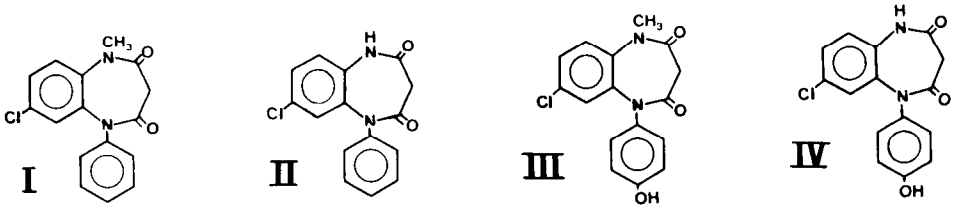

Fig. 1. Structures of clobazam and its metabolites.

4'-hydroxy-N-desmethylclobazam (IV) (Fig. 1). II is pharmacologically less active than the parent drug [8]; no information about relative efficiencies in man of III and IV has been published.

Several methods for the quantification of I and/or II in biological fluids have been described, involving gas—liquid chromatography (GLC) with flame-ionization [9], thermionic [10—13] or electron-capture detection [14—19], high-performance liquid chromatography (HPLC) [20—22], gas chromatography—mass spectrometry [12, 23], thin-layer chromatography [6, 24], spectrofluorimetry [14, 25, 26], immunoenzymology [13], radioimmunoassay [27] and radioreceptor assay [28]. However, none of these methods allows the specific quantification of the individual hydroxy derivatives of I and II.

From a fundamental point of view, the precise determination of the relative plasma and urine concentrations of I, II and free and conjugated III and IV in man after clobazam intake would be of great interest. During long-term antiepileptic treatments in patients, a loss of clobazam efficiency may be observed, with a correspoding decrease in the plasma concentrations of I without increased plasma levels of II [29]. Therefore, such clinical observations might be related to hydroxylation processes which are quantitatively more important. Lastly, the association of clobazam with enzymatic inducers such as phenobarbital or other drugs such as valproic acid, as usually performed in antiepileptic therapy, leads to a decrease in plasma concentrations of I without an increase in II [30, 31]. Here too, a metabolic deviation might occur with intensification of hydroxylation processes.

In order to elucidate these phenomena, we developed an HPLC method for the determination of I and II and their hydroxy metabolites in the same plasma or urine sample. The method was applied to a preliminary study of healthy volunteers under a chronic dosage regimen.

EXPERIMENTAL

Materials

Chromatographic analysis. HPLC analyses were performed on an HP 1080 A apparatus (Hewlett-Packard, Les Ulis, France), equipped with an autosampler, a variable-wavelength UV detector and a data acquisition system. μBondapak C_{18} columns (30 cm × 3.9 mm I.D.) with 10-μm particles (Waters Assoc., Paris, France) were used at ambient temperature. The mobile phase was acetonitrile—water (47:53, v/v) at a flow-rate of 1 ml/min for I and II and 0.6 ml/min for III and IV. The detector was operated at 230 nm. Under such conditions, the retention times for I, II and the internal standard V were 6.5, 8.7 and 11.4

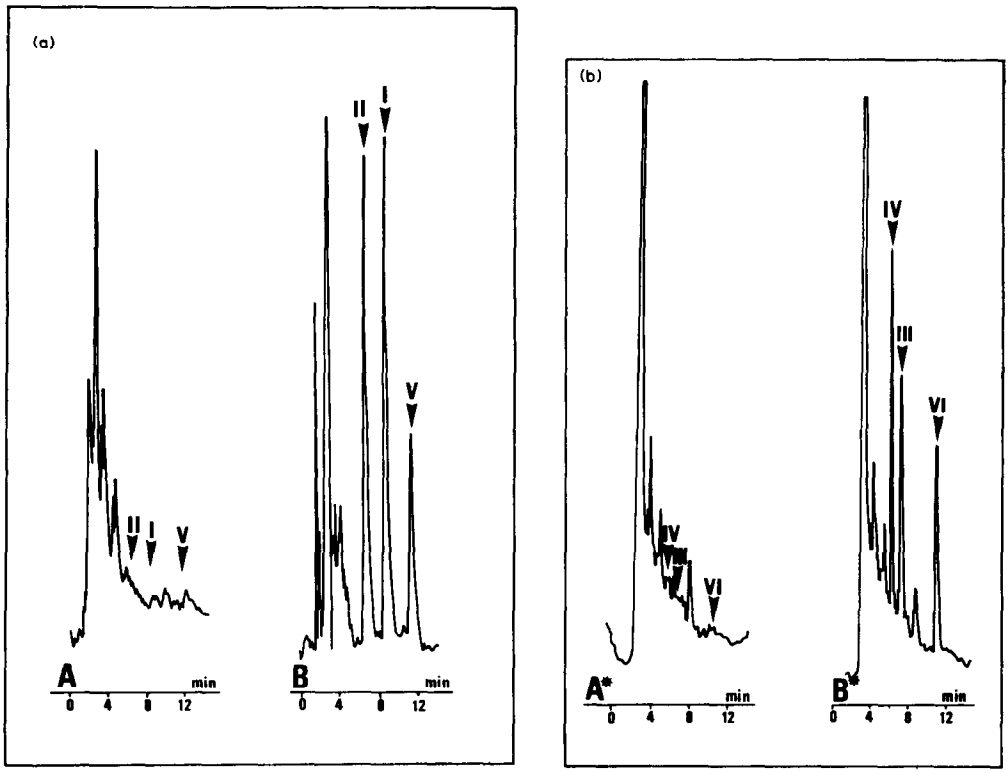

Fig. 2. Chromatograms obtained after extraction at pH 13 of (A) control plasma and (B) control plasma spiked with I, II (200 ng/ml), III and IV (50 ng/ml) with V as an internal standard; and after back-extraction of the aqueous phase at pH 9 of (A*) control plasma and (B*) the concentrated organic phase containing III and IV with VI as an internal standard.

min, respectively, and for III, IV and the internal standard VI 5.8, 6.8 and 10.5 min, respectively (Fig. 2).

Standard solutions and reagents. Standard solutions of I, II, III, IV (Diamant, Puteaux, France), V (diazepam) and VI (nitrazepam) (Hoffmann-La Roche, Basle, Switzerland) were prepared by carefully weighing about 10 mg into a 10-ml volumetric flask and dissolving them in methanol. Serial dilutions in methanol were also made.

Inorganic reagents were prepared in deionized and freshly quartz-glass redistilled water as follows: buffer solution of pH 9 [by 10-fold dilution of Titrisol (Merck, Darmstadt, F.R.G.) concentrated buffer], 1 *M* hydrochloric acid (by 10-fold dilution of Ultrex concentrated hydrochloric acid) and Na_3PO_4 phosphate buffer [by dissolution until saturation of Na_3PO_4 (Fluka, Buchs, Switzerland)]. The solvents acetonitrile (RS grade), diethyl ether (Spectrosol grade) and methanol (Uvasol grade) were supplied by Carlo Erba (Milan, Italy), S.D.S. (Peypin, France) and Merck, respectively. The components of the mobile phase were filtered before degassing through an FG 0.2-μm membrane for acetonitrile and an HA 0.45-μm membrane (Millipore, Bedford, MA, U.S.A.) for water.

Method

Extraction procedure. In a 30-ml cylindro-conical tube a suitable volume of V solution, for internal standardization of I and II analysis, was evaporated to dryness under a stream of pure nitrogen. After addition of 0.5 ml of a plasma or urine sample and 1 ml of Na_3PO_4 buffer solution, yielding a pH of about 12.5, the plasma was extracted twice for 10 min with 5-ml portions of diethyl ether. After centrifugation for 10 min at 2500 *g*, the organic layer was evaporated to dryness and the residue was dissolved in 100 μl of methanol for HPLC determination of I and II. To the aqueous phase was added a suitable volume of VI solution, for internal standardization of III and IV analysis, 1 *M* hydrochloric acid to pH ca. 9 and 1 ml of buffer pH 9. After extraction for 10 min with 5 ml of diethyl ether and centrifugation for 10 min at 2500 *g*, the organic layer was evaporated to dryness, and the residue was dissolved in 100 μl of mobile phase for HPLC determination of III and IV.

Quantification of unknown samples. Data relating to plasma concentrations of I, II, III and IV were obtained from least-squares linear regression curves, established daily from four calibration points using I/V, II/V, III/VI and IV/VI peak-height ratios.

TABLE I

INTRA-ASSAY REPRODUCIBILITY

Compound	Concentration added (ng/ml)	Number of determinations (*n*)	Relative standard deviation* (%)	Accuracy** (%)
I	15	14	6.7	−8.5
	30	10	4.5	−4.7
	50	11	3.0	N.A.***
	100	8	1.7	+2.0
	200	8	1.8	N.A.
	2000	8	2.0	N.A.
II	10	14	9.3	−11.4
	30	9	7.3	N.A.
	50	11	3.3	−3.7
	100	8	1.5	N.A.
	200	8	1.0	N.A.
	2000	8	2.2	N.A.
III	50	12	9.2	−10.0
	100	7	4.1	−2.6
	200	7	2.3	+1.9
	2000	4	4.2	N.A.
IV	50	12	4.2	−6.3
	100	7	3.9	+4.0
	200	7	2.4	N.A.
	2000	4	4.1	−1.7

*The interval of variation for experimentally measured concentrations was estimated with 95% confidence.

**Difference between found and expected concentrations.

***N.A., not available.

RESULTS

The intra-assay reproducibility was checked for plasma concentrations of I and II ranging from 10 to 2000 ng/ml and of III and IV ranging from 50 to 2000 ng/ml. The results reported in Table I were satisfactory in these concentration ranges.

The linearity proved satisfactory for plasma or urine concentrations of I and II from 10 to 2000 ng/ml and for III and IV from 20 to 2000 ng/ml (Table II). In these concentration ranges, the extraction recovery was 92 ± 4% for I and II and 79 ± 6% for III and IV.

The limit of detection was calculated to be about 10 ng/ml for I and II and 15—20 ng/ml for III and IV using a 1.0-ml plasma or urine sample (Fig. 3).

No interference from drugs currently associated with clobazam therapy and compounds I and II was observed under the analytical conditions described, except for nordiazepam, which exhibited a retention time slightly lower than that of I. In contrast, some co-medications might interfere chromatographically with the determination of III and IV, such as carbamazepine and diphenylhydantoin, whereas no interference was observed with primidone, ethosuximide and phenobarbital. These interferences might be partially or totally eliminated by the diethyl ether extraction at pH 13 carried out before the extraction of hydroxy metabolites III and IV from biological fluids.

DISCUSSION

The simultaneous determination of clobazam and its metabolites was

TABLE II

INTRA-DAY LINEARITY

Compound	Biological fluid	Concentrations added* (ng/ml)	Equation of the non-weighted linear regression	Correlation coefficient
I	Plasma	6.6—39.5	$y = 0.02135x - 0.0058$	0.9961
	Plasma	63.1—252.5	$y = 0.01013x + 0.0014$	0.9999
	Plasma	252.5—1893.8	$y = 0.00091x + 0.0046$	0.9999
II	Plasma	10.6—79.5	$y = 0.02178x + 0.0349$	0.9990
	Plasma	57 —228	$y = 0.00467x - 0.0032$	0.9997
	Plasma	228 —1710	$y = 0.00039x + 0.0029$	0.9999
III	Urine	25 —100	$y = 0.00798x + 0.0100$	0.9996
	Plasma	25 —100	$y = 0.00765x - 0.0050$	0.9995
	Plasma	49 —196	$y = 0.00405x + 0.0284$	0.9974
	Plasma	196 —1470	$y = 0.00281x + 0.0905$	0.9989
IV	Urine	16 —64	$y = 0.02259x + 0.0518$	0.9943
	Plasma	16 —64	$y = 0.02526x + 0.0066$	0.9993
	Plasma	64.1—256.5	$y = 0.00042x + 0.0060$	0.9998
	Plasma	256.5—1923.8	$y = 0.00185x + 0.0605$	0.9993

*Each calibration graph was constructed from four or five different points.

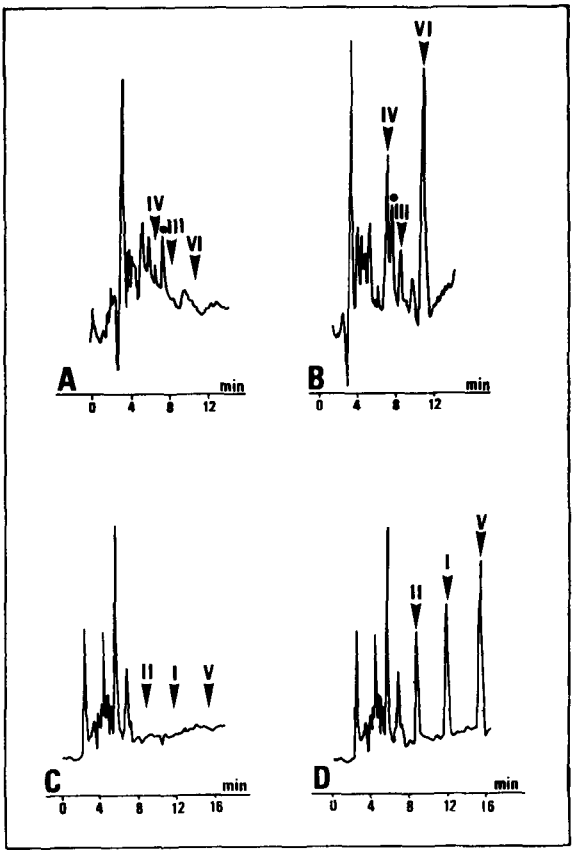

Fig. 3. Chromatograms illustrating the detection limit for plasma quantification of (B) III and IV (20 ng/ml) and (D) I and II (10 ng/ml) with their control plasmas, A and C, respectively.

theoretically possible under the chromatographic conditions described, using a single extraction with diethyl ether at pH 9. However, in this instance, some co-extracted endogenous compounds in plasma or urine interfered chromatographically with low concentrations of III and IV derivatives. Therefore, successive diethyl ether extractions of I and II at pH 13 and of III and IV at pH 9 were carried out. Under such conditions, almost complete extraction of the endogenous compounds interfering with III and IV was achieved by applying two 5-ml diethyl ether extractions at pH 13, whereas the hydroxy metabolites were not extracted. Hence the chromatograms obtained from control plasma or urine after the subsequent extraction at pH 9 were free from impurities at the retention times of III and IV (Figs. 2A* and 3A), allowing a precise and selective determination of these compounds. Such an extraction protocol was complicated and time-consuming, but these inconveniences were reduced by the possibility of automation for HPLC analyses. Moreover, owing to the extraction of VI (internal standard for III and IV) with diethyl ether at pH 13, and the chromatographic interference between VI and II, compound VI must be added to the aqueous phase after extraction at pH 13.

Owing to a lack of selectivity towards some medicaments, our HPLC method

did not allow the hydroxy metabolites in plasma or urine from epileptic patients undergoing polytherapy with such compounds to be quantified. However, as mentioned above, the selectivity was satisfactory for measurements of I and II, except in patients receiving diazepam.

A limit of detection of 15—20 ng/ml for hydroxy derivatives might be insufficient for plasma measurements of hydroxy metabolites after a single administration of a 5—10 mg therapeutic dose of clobazam. However, this sensitivity was adequate for repeated clobazam administration and for the withdrawal phase after discontinuation of clobazam therapy.

Despite these problems, the proposed HPLC procedure represents the only available method allowing the determination of clobazam and its three main

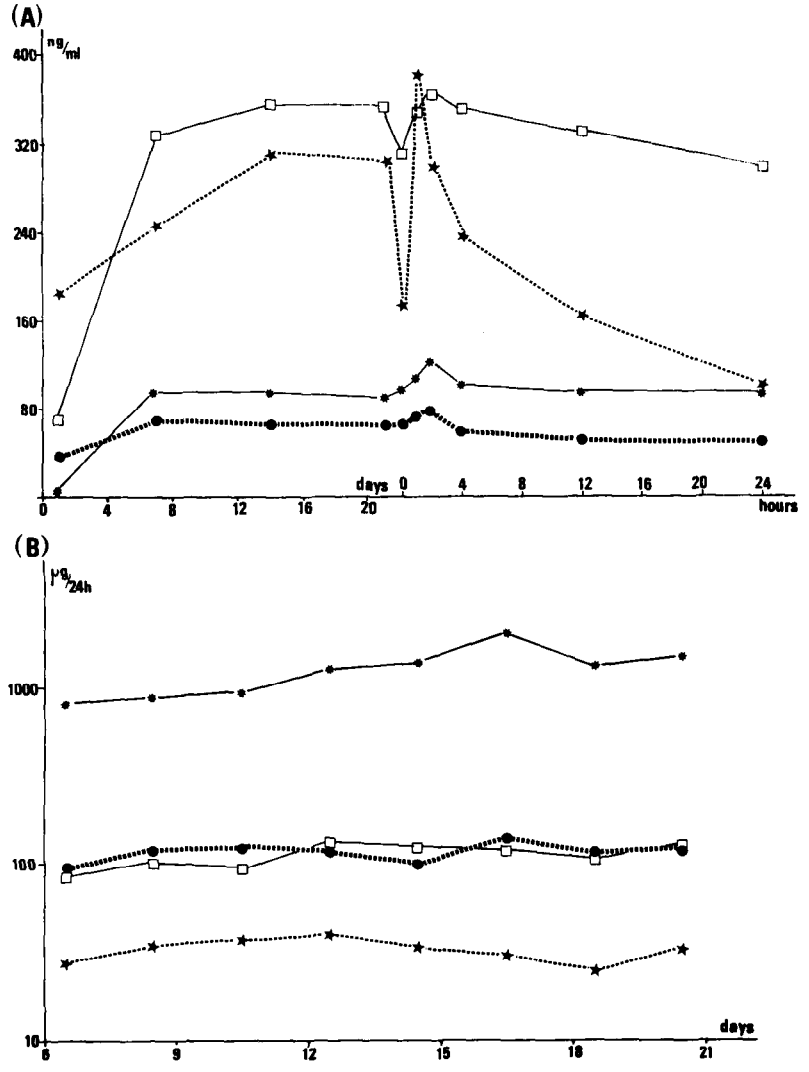

Fig. 4. Plasma concentration time courses (A) and daily urine excretion (B) of I (★), II (□), III (●) and IV (∗) after oral administration of 10 mg clobazam at 12-h intervals in a representative healthy volunteer (J.-L. T.).

metabolites in the same plasma or urine sample, and with the same chromatographic conditions (column, mobile phase, detector, etc.). Moreover, other techniques, especially gas—liquid chromatography, are difficult to operate; derivatization of the hydroxyclobazam metabolites is required, but quantitative efficiency of the derivatization reaction cannot be achieved, whatever the reagent, solvent, temperature and duration chosen [32].

Validation of HPLC measurements of I and II: correlation with a GLC method

I and II were analysed in parallel in several tens of plasma samples using our HPLC method and a previous GLC method [15]. The plasma samples were obtained from different epileptic patients receiving chronic administration of 20—50 mg of clobazam in association with some drugs such as carbamazepine, phenytoin, diphenylhydantoin, ethosuximide, primidone, clonazepam and/or phenobarbital. Correlations between the two techniques were generally satisfactory, with regression curves $y = 0.965x + 6.04$, $n = 37$, correlation coefficient $r = 0.992$, for compound I and $y = 1.022x - 2.70$, $n = 37$, $r = 0.985$, for compound II.

Applications

As an example, the proposed HPLC method was applied to the determination of clobazam and its three metabolites in plasma and urine from a healthy volunteer who had received repeated administrations of 10 mg of clobazam every 12 h during 21 days. Blood samples were taken before the first administration, 2 h after the morning administration on days 1, 7, 14 and 21 and, for the last (44th) administration, after 0, 1, 2, 4, 12 and 24 h; 24-h urine samples were taken on days 1, 6, 8, 10, . . ., 20. The time courses of plasma concentration (A) and urine excretion (B) of I, II, III and IV are shown in Fig. 4. An extensive pharmacokinetic treatment of the results will be the subject of a further publication.

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