Journal of Chromatography, 225 (1981) 219—224
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 937

Note

Quantitative determination of clobazam in the plasma of epileptic patients by gas—liquid chromatography with electron-capture detection

ROBERTO RIVA*, GIOACCHINO TEDESCHI, FIORENZO ALBANI and AGOSTINO BARUZZI

Institute of Neurology, School of Medicine, University of Bologna, Via Foscolo 7, 40123 Bologna (Italy)

(First received January 30th, 1981; revised manuscript received April 10th, 1981)

Clobazam (CLB), with anticonvulsant [1—4] and anxiolytic properties [5—11], was synthesized by Rossi et al. [12] in 1969, and was the first example of a 1,5-benzodiazepine. Clinical investigations have indicated that CLB is effective against various forms of epilepsy [4, 13—15], and for this reason it is now coming into use as an antiepileptic drug. There is no evidence that CLB metabolites have antiepileptic properties.

The pharmacokinetics and the metabolism of the drug have been studied by the radioreceptor technique [16] both in various animal species and in man [17, 18]. The structures of several metabolites of CLB have been clarified recently. The N-desmethylation and the 4′-hydroxylation metabolic pathways were found to be the most important ones in animals and man. The metabolism of the 1,5-benzodiazepines in the hydroxylation pathway is markedly different from that of the more diffuse 1,4-benzodiazepines in the same pathway (4′-oxidation as opposed to 3-oxidation); the metabolism in the dealkylation pathway is similar in both [18].

The concentration of N-desmethylclobazam in plasma has been found to be eight times higher than the concentration of the parent drug after long-term administration [17], whereas no information is available about the concentration of 4′-hydroxyclobazam in plasma. A method for measuring CLB and N-desmethylclobazam in guinea-pig plasma by means of a gas chromatographic (GC) technique has been published [19]; unfortunately it cannot be used to determine the concentration of CLB in the plasma of human subjects who are taking other drugs, because these cause additional interfering peaks in the chromatograms which invalidate the analysis. Another method for the determination of CLB in plasma by a fluorimetric technique [20] has been published

0378-4347/81/0000—0000/$02.50

but it has a low sensitivity (50 ng/ml) and it is possible that co-medications may interfere with the formation of the fluorophore of CLB.

Our purpose was to develop a simple and fast method for determining the concentration of CLB, suitable for routine clinical application and sufficiently specific with regard to the presence of other common co-medications.

EXPERIMENTAL

Reagents and standards

All chemicals were of analytical-reagent grade or better and were checked for chromatographic purity before use. Clobazam [7-chloro-1-methyl-5-phenyl-1H-1,5-benzodiazepine-2,4-(3H,5H)-dione] was kindly supplied by Hoechst (Frankfurt/M., G.F.R.). Methylclonazepam [7-nitro-1-methyl-5-(*o*-chlorophenyl)-3H-1,4-benzodiazepine-2-(1H)-one] was kindly supplied by Roche (Milan, Italy) and was employed as the internal standard. Stock solutions of CLB and methylclonazepam were prepared in methanol to give a concentration of 1 mg/ml of each compound. Plasma standards of 10, 30, 60, 150, 300 and 500 ng/ml of CLB were prepared by adding the required volumes of a standard 30 μg/ml solution of CLB to drug-free pooled plasma. Plasma standards were then divided into 1-ml samples and frozen at −20°C (calibrator samples).

Apparatus

A Sigma 3 gas chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.) equipped with a nickel-63 electron-capture detector (ECD) was used. The glass column (0.5 m × 3 mm I.D.) was packed with GP—2% SP-2510-DA on 100—120-mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.) and was conditioned as indicated by the manufacturer [21]. The GC conditions were: column temperature, 245°C; injector temperature, 300°C; detector temperature, 300°C; carrier gas (nitrogen) flow-rate, 80 ml/min. An LKB 9000 mass spectrometer equipped with a gas chromatograph was used under the following conditions: ionizing energy, 70 eV; ion source temperature, 290°C; accelerating voltage, 3.5 kV; trap current, 60 μA. The sample was introduced by either the direct inlet system (probe temperature 70°C) or the GC procedure using a similar column to that described above.

Extraction procedure

To 1 ml of plasma (calibrator samples or patient samples) were added 100 μl of the dilute internal standard (40 ng/ml in methanol), 0.5 ml of 1 *N* hydrochloric acid and 4 ml of benzene. Test-tubes were mechanically shaken for 10 min, then centrifuged at 1000 *g* for 5 min and 3.5 ml of the organic phase were evaporated to dryness in a vacuum evaporator at 45°C. The residues were dissolved in 100 μl of acetone and 1—2 μl of this solution were injected into the gas chromatograph. Calibration graphs were constructed of the peak-area ratio of CLB to methylclonazepam versus concentration of CLB. For each series of analyses a new calibration graph had to be prepared.

Recovery

Various amounts (0.3, 0.5, 1.5 and 4.0 μg) of CLB were dissolved in 10 ml

of drug-free plasma by adding a given volume of a standard solution of 30 μg/ml CLB in methanol. The plasma was divided into 1-ml samples, which were used for the recovery study. These samples were extracted as described above but without adding the internal standard. The residues were dissolved in 100 μl of acetone containing 4 ng of methylclonazepam. A second series of standards was prepared simultaneously by extracting 1 ml of drug-free plasma and then adding CLB and the marker to the dried extract at the concentrations noted above. The analytical recovery was calculated by comparing the peak-area ratios of the extracted standards to the ratios obtained from the standards to which CLB had been added after extraction. The absolute recovery was calculated by correcting the analytical recovery by a factor representing the ratio between the benzene volume added to and the benzene volume removed from the plasma and evaporated during the extraction procedure.

Linearity

The linearity was calculated by using the results obtained from the calibration graphs (from 10 to 500 ng/ml).

Reproducibility

Four plasma samples of 10 ml each (containing 100 ng, 600 ng, 1.5 μg and 4 μg of CLB) were prepared by adding given volumes of a standard 30 μg/ml solution of CLB in methanol. The plasma was divided into 1-ml samples and was kept frozen at −20°C until taken for analysis. The analyses were performed ten times, about once every twelve days over a period of 4 months using the above method.

Interference from other drugs or substances

To determine the potential usefulness of the procedure, we checked for possible interferences from other antiepileptic drugs (phenobarbital, mephobarbital, primidone, carbamazepine, phenytoin, ethosuximide and valproic acid), some of the most important benzodiazepines (diazepam, 3-hydroxydiazepam, oxazepam, N-desmethyldiazepam, clonazepam, nitrazepam, prazepam, flurazepam, flunitrazepam, lorazepam, medazepam, bromazepam and chlordiazepoxide) and some other drugs and substances [caffeine, gluthetimide, amobarbital, quinidine, theophylline, cholesterol, α-methyl-α-phenylsuccinimide, iminostilbene, 5-(*p*-methylphenyl)hydantoin and 5-(*p*-methylphenyl)-5-phenylhydantoin] by chromatographing physiological samples and pure standards at normal therapeutic or physiological concentrations.

Plasma of patients

Samples of blood from patients receiving CLB orally twice daily (at 8 a.m. and 8 p.m.) were taken in heparinized test-tubes just before the morning dose. The plasma was separated as soon as possible, immediately frozen and stored at −20°C until taken for analysis.

RESULTS AND DISCUSSION

Preliminary experiments indicated that GP—2% SP-2510-DA was the most

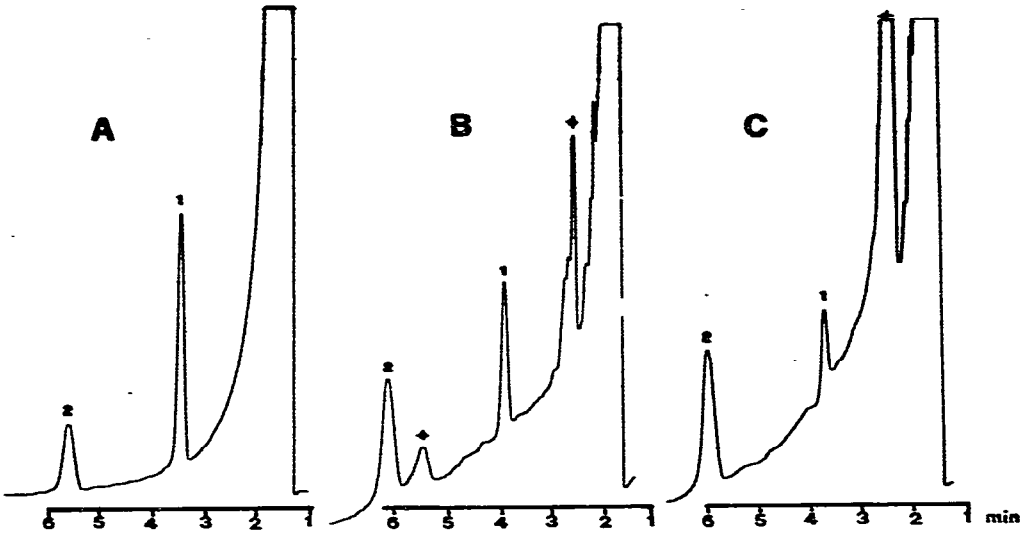

Fig. 1. GC response obtained with (A) extracted calibration sample, (B) extracted plasma of a patient undergoing therapy with clobazam, carbamazepine and phenytoin and (C) extracted plasma of a patient undergoing therapy with clobazam, phenobarbital and valproic acid. Peaks: (1) clobazam; (2) methylclonazepam (internal standard). Peaks marked with an asterisk are unidentified compounds in the extracted plasma of patients undergoing multiple drug therapy.

suitable GC stationary phase for the determination of CLB in the plasma of patients under multiple drug therapy. With other stationary phases commonly used in benzodiazepine analysis, multiple peaks interfering with the CLB peak were evident in chromatograms from the plasma of patients under co-medication. Unfortunately, N-desmethylclobazam (a basic compound) showed indications of irregular adsorption to this stationary phase (which is specific for acidic drugs), so that this metabolite cannot be measured concomitantly. For these reasons, we decided to determine only the concentration of CLB in the plasma of epileptic patients under multiple therapy, leaving aside the problem of its metabolite measurement.

The extracts from drug-free plasma showed no interference from endogenous plasma sustances. Fig. 1 shows a representative chromatogram of an extract from a plasma calibration graph (A) and two chromatograms obtained from epileptic patients taking other antiepileptic drugs (B, C). We did not observe any exogenous interferences in the chromatograms which might have altered the CLB analysis, even when drugs and substances listed above were examined. Fig. 2 shows the mass spectrum of CLB, obtained after the GC procedure. It has a molecular ion at $m/e = 300$, which is also the base peak. By comparing the CLB mass spectrum obtained after the GC inlet with the direct-inlet-system mass spectrum, it could be concluded that CLB leaves the GC column unchanged. Therefore, in our procedure, this substance was being measured in its intact form.

The concentrations of CLB in calibrator samples and their respective readings were seen to be linearly related; the line obtained by calculation with a

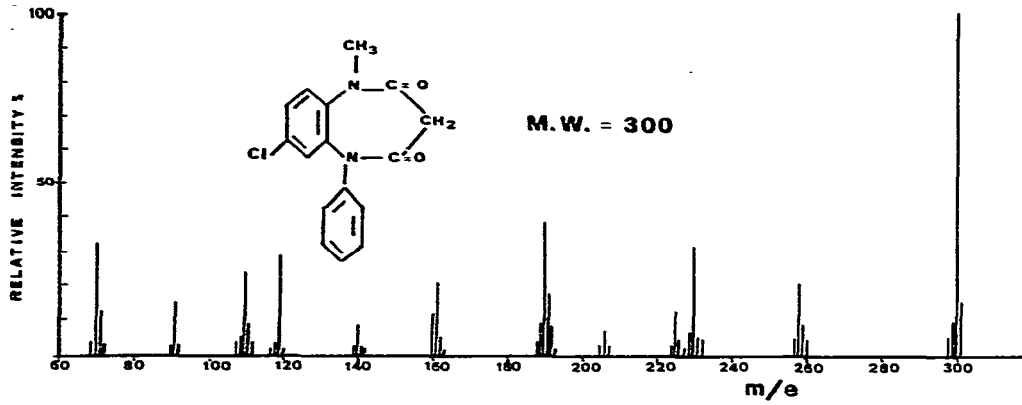

Fig. 2. Normalized electron-impact mass spectrum of clobazam obtained by injecting a plasma extract into an LKB-9000 gas chromatograph—mass spectrometer system.

TABLE I

RECOVERY OF CLOBAZAM FROM HUMAN PLASMA SAMPLES

Amount added (ng/ml)	Amount found* (ng ± S.D.)	Absolute recovery (% ± S.D.)
30	27.2 ± 1.48	90.66 ± 4.93
60	53.7 ± 3.34	89.50 ± 5.57
150	135.2 ± 6.42	90.13 ± 4.28
400	359.3 ± 15.54	89.82 ± 3.88
Mean recovery		90.12 ± 4.83

*Each value is the mean value of four determinations. These values have been corrected as described in the text.

TABLE II

REPRODUCIBILITY OF DETERMINATION OF CLOBAZAM IN HUMAN PLASMA SAMPLES

Amount added (ng/ml)	Amount found* (ng/ml)	S.D.	C.V. (%)
10	9.97	0.45	4.5
60	59.89	2.63	4.4
150	150.30	6.53	4.3
400	398.80	15.96	4.0

*Each value is the mean value of ten determinations.

least-squares linear regression method is $y = 0.0071x + 0.0066$; $r = 0.999$. The detection limit for CLB in plasma using the described procedure is about 5 ng/ml. The mean absolute recovery from four analyses of plasma samples containing four different CLB concentrations was 90.12 ± 4.83% (Table I). The results of the reproducibility study are illustrated in Table II; the mean coefficient of variation was 4.3%.

In a series of 16 patients, aged from 4 to 47 years, with a CLB dosage of 0.3—1.6 mg/kg/day (mean ± S.D. = 0.89 ± 0.38), we found concentrations in plasma of 20—197 ng/ml (mean ± S.D. = 81.0 ± 48.9) regardless of the co-medications. We did not believe it was useful to analyse these data statistically, as there are so many uninvestigated parameters (age, co-medications, etc.). The proposed method is, to the best of our knowledge, the most sensitive available for determining concentrations of CLB in human plasma when multiple drug therapy is used. The procedure is rapid, simple and reproducible. The chromatographic time for twenty analyses is about 2 h (6 min for each analysis), with an additional 40 min for samples preparation.

The drugs tested do not interfere in the analysis of CLB so that kinetic studies of CLB in patients undergoing multiple drug therapy can be performed.

ACKNOWLEDGEMENTS

The authors thank Dr. Maria Patrizia Bertoni and Mr. Luigi Cappellini of the Mario Negri Institute, Milan, Italy, for their kindness in performing the CLB mass spectral analyses and Mr. Francesco Negrini and Mr. Pierino Pollini for their assistance in the preparation of the manuscript.

REFERENCES

1 A.G. Chapman, R.W. Horton and B.S. Meldrum, Epilepsia, 19 (1978) 293.
2 B.S. Meldrum and R.W. Horton, Psychopharmacology, 60 (1979) 277.
3 B.S. Meldrum, A.G. Chapman and R.W. Horton, Brit. J. Clin. Pharmacol., 7 (1979) 50s.
4 H. Gastaut, Concours Med., 100 (1978) 3697.
5 S. Fielding and I. Hoffman, Brit. J. Clin. Pharmacol., 7 (1979) 7s.
6 H.J. Gerhards, Brit. J. Clin. Pharmacol., 7 (1979) 23s.
7 E. Barzaghi, R. Forneux and P. Mantegazza, Arzneim.-Forsch., 23 (1973) 683.
8 H.H. Wieck, L. Blaha and B. Heerklotz, Brit. J. Clin. Pharmacol., 7 (1979) 109s.
9 S.I. Devanthan and S.M. Channabasavanna, Curr. Ther. Res., 21 (1977) 361.
10 P. Moreau, Gaz. Méd. Fr., 83 (1976) 481.
11 D.R. Doongaji, A. Sheth, J.S. Apte, P.D. Lakdawala, C.B. Khare and S.S. Thatte, J. Clin. Pharmacol., 18 (1978) 358.
12 S. Rossi, O. Pirola and R. Maggi, Chim. Ind. (Milan), 51 (1969) 479.
13 H. Gastaut, Nouv. Presse Med., 7 (1978) 2400.
14 H. Gastaut and M.D. Low, Epilepsia, 20 (1979) 437.
15 F. Escobedo, E. Otero, H. Chaparro, T. Flores and R.F. Rubio-Donnadieu, in J.A. Wada and J.K. Penry (Editors), Advances in Epileptology: the Xth Epilepsy International Symposium, Raven Press, New York, 1980, p. 358.
16 P. Hunt, Brit. J. Clin. Pharmacol., 7 (1979) 37s.
17 W. Rupp, M. Badian, O. Christ, P. Hajdù, R.D. Kulkarni, K. Taeuber, M. Vihlein, R. Bender and O. Vanderbeke, Brit. J. Clin. Pharmacol., 7 (1979) 51s.
18 M. Voltz, O. Christ, H.M. Kellner, H. Kuch, H.W. Fehlhaber, D. Gantz, P. Hajdù and F. Cavagna, Brit. J. Clin. Pharmacol., 7 (1979) 41s.
19 S. Caccia, M. Ballabio, G. Guiso and M.G. Zanini, J. Chromatogr., 164 (1979) 100.
20 J.T. Stewart, L.L. Honigberg, A.Y. Isai and P. Hajdù, J. Pharm. Sci., 68 (1979) 494.
21 Supelco Bull. No. 768, Supelco Bellefonte, PA, 1977.