Journal of Chromatography, 116 (1976) 445–450

CHROM. 8684

A SIMPLE AND SENSITIVE GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF CLONAZEPAM IN HUMAN PLASMA

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(Received July 28th, 1975)

SUMMARY

A simple gas chromatographic method for the determination of clonazepam in human plasma has been developed. After solvent extraction, the compound is measured by an electron capture detector on an OV-17 column. The electron-capture response is linear for 5–120 ng/ml of plasma. There is no interference from other commonly used anti-epileptic drugs or endogenous substrates. Preliminary data from routine monitoring of epileptic patients shows a 10-fold variation in their clonazepam plasma levels.

INTRODUCTION

Knowledge of the pharmacokinetic profiles of various compounds and the routine monitoring of drug plasma levels have been very useful in the management of anti-epileptic therapy[1–4]. The pharmacokinetics of clonazepam, a newly developed anticonvulsant agent, has been characterized in volunteers[5,6]. However, there is little kinetic information on epileptic patients receiving constant treatment with the drug[7,8]. In addition it has been shown for other compounds that extrapolation of kinetic data from volunteers to patients might not be always correct[9,10]. One of the factors which has partially limited our knowledge of the kinetics of this interesting compound in patients has been the lack of suitable, simple, analytical procedures for the kinetic evaluation and routine determination of clonazepam plasma levels. In the present paper we describe a gas–liquid chromatographic procedure which, with its specificity, rapidity and simplicity and the small amount of plasma required, has a real advantage over the procedures presently available[11,12].

EXPERIMENTAL

Standard and reagents

Clonazepam [5-(chlorophenyl)-1,3-dihydro-(2H)-7-nitro-1,4-benzodiazepin-2(1H)-one], 7-aminoclonazepam and 7-acetamidoclonazepam were kindly supplied by Prodotti Roche, Milan, Italy. 2-Aminobenzyl-5-chlorobenzophenone (ABCB)

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(Fluka, Buchs, Switzerland) was employed as internal marker. The following reagents were obtained from Carlo Erba, Milan, Italy: acetone (R.P.), diethyl ether (R.P.) and disodium hydrogen phosphate (R.P.). Diethyl ether was distilled before use. A solution of the phosphate was made up in double-distilled water.

Apparatus

A Carlo Erba Fractovap G-1 chromatograph equipped with a ^{63}Ni electron-capture detector (ECD) was used. The column (glass, 1 m × 4 mm I.D.) was packed with 3% OV 17 on Chromosorb Q (100–120 mesh) (Applied Science Labs., State College, Pa., U.S.A.), and conditioned for 1 h at 250° (nitrogen flow-rate, 40 ml/min), for 4 h at 340° (no nitrogen) and for 24 h at 275° (nitrogen flow-rate, 40 ml/min). The operating conditions were: column temperature, 280°; injection-port temperature, 290°; detector temperature, 300°; carrier-gas (nitrogen) flow-rate, 60 ml/min. The ECD was used with a pulse current at an excitation voltage of 50 V and a pulse interval of 30 msec.

For mass spectrometry (MS), a Finnigan 3100 mass spectrometer combined with a Model 9500 gas chromatograph and a Model 6000 computer were used. The conditions were: electronic energy, 70 eV; electron beam current, 500 μA; temperature of ion source, 100°. The column (glass, 2 m × 2 mm I.D.) was packed with 3% OV 17 on Gas-Chrom Q (100–120 mesh), and operated at 280° with a helium flow-rate of 30 ml/min.

Determination of calibration graphs

Aliquot portions (5–80 ng) of a solution of clonazepam in acetone (2 ng/ml) were brought to dryness in triplicate under a gentle stream of nitrogen on a water-bath at 30°. The dry residue was re-dissolved in 50 μl of a solution of ABCB in acetone (5 μg/ml). 1 μl of this solution was injected on to the gas chromatograph. The calibration graph, constructed by plotting the ratio of the peak area of clonazepam to that of the internal marker (ABCB) against clonazepam concentration, was linear from 100 pg to 1.6 ng (injected amounts) with an absolute sensitivity of 50 pg. Reproducibility was excellent with a 1.5–2% variation for samples run in triplicate and on different days. Similar calibration graphs were constructed on adding known amounts of clonazepam to 0.2–1 ml of plasma and processing them as described below.

Extraxtion procedure

To 0.5 ml of plasma (in 10-ml glass-stoppered test-tubes), containing different amounts of clonazepam (5–120 ng), were added 1 ml of 0.5 *M* Na_2HPO_4 (previously adjusted to pH 9 with 1 *N* NaOH), 250 ng of ABCB (50 μl of a solution in acetone) as internal marker and 5 ml of freshly distilled diethylether. The tubes were gently shaken in a horizontal position for 30 min and then centrifuged at 4° for 10 min. 4 ml of the ether phase were transferred to a second tapered test-tube and brought to dryness under a gentle stream of nitrogen in a water-bath at 40°. The dry residue was then redissolved in 50 μl of acetone and 1–2 μl of this solution was injected as before on to the gas chromatograph. In the case of samples from plasma of patients, aliquot portions of 0.2–1 ml were used and an internal calibration graph, involving the addition of various amounts (5–120 ng/ml) of clonazepam to fresh plasma, was always contructed along side the determination of the unknown samples.

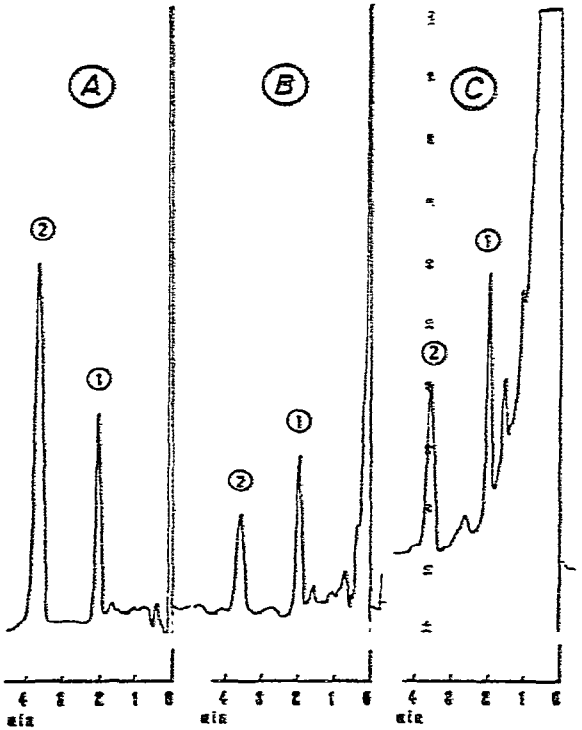

Fig. 1. Gas chromatograms of ABCB (1) and clonazepam (2) obtained before (A) (external standard) and after the extraction procedure (B) (internal standard). (C) shows the results for the extracted plasma of a patient receiving clonazepam, carbamazepine and phenobarbital in combination. For conditions, see text.

RESULTS AND DISCUSSION

Typical chromatograms obtained with external and internal standards and with plasma extract from patients are shown in Fig. 1. Clonazepam has a retention time of 3.5 min and ABCB of 2 min. The identity of the peaks was checked by gas–liquid

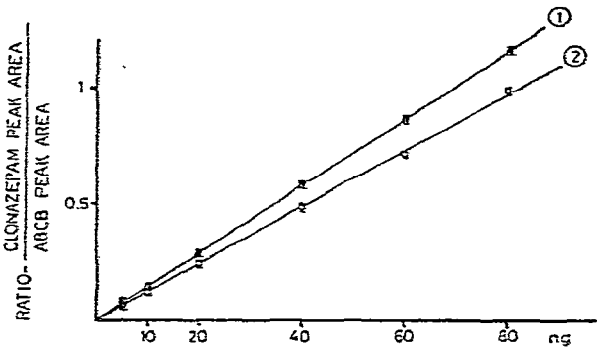

Fig. 2. Calibration graphs for clonazepam with external (1) and internal ABCB standard (2). For conditions, see text.

TABLE I

RECOVERY OF CLONAZEPAM FROM HUMAN PLASMA SAMPLES

Amount added (ng)	*Amount found* (ng ± standard error)*	*Recovery (% ± standard error)*
20	16.66 ± 0.88	83.3 ± 4.40
40	32.80 ± 0.44	81.6 ± 0.88
60	50.33 ± 1.40	83.6 ± 2.30
80	67.70 ± 0.66	82.0 ± 2.00
	Mean	82.6 ± 1.18

* Each value is the mean of three determinations.

chromatography–mass spectrometry (GLC–MS) and the analysis confirmed that the peaks were intact clonazepam and ABCB. The use of ABCB as internal marker appears to be very satisfactory. The calibration graph for extracted clonazepam, obtained on plotting the ratio of the peak area of clonazepam to that of the internal marker against known amounts of clonazepam, is shown in Fig. 2. The linearity of the method ranges from 5 to 80 ng/ml of plasma. The minimum detectable amount with the described procedures is 4 ng/ml of plasma.

The recovery from human plasma was constant in the range examined with a mean of 82.6 ± 1.1% (Table I). No interference from endogenous substances was noted. Other anticonvulsant drugs such as diphenylhydantoin, carbamazepine, phenobarbital, ethosuximide and di-*n*-propyl acetate, added to the internal standard

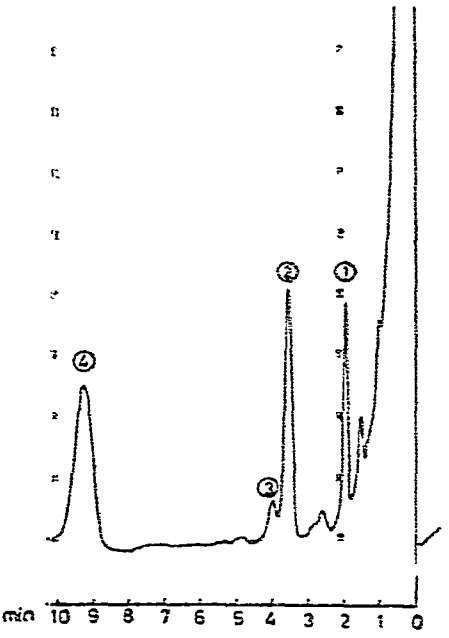

Fig. 3. Gas chromatogram of ABCB (1), clonazepam (2), 7-aminoclonazepam (3) and 7-acetamidoclonazepam (4). Equal amounts (100 ng) of the four compounds were added to plasma. For conditions, see text.

TABLE II

RELATION BETWEEN THE PLASMA LEVELS AND THE DAILY DOSE OF CLONAZEPAM IN EPILEPTIC PATIENTS RECEIVING CONSTANT TREATMENT WITH THE DRUG FOR AT LEAST 4 WEEKS

Plasma samples were taken before the morning dose, *i.e.*, 11–12 h after the evening dose.

Daily dose (mg/kg) *	*Plasma level (ng/ml)* **	*Range of variation*
0.06	22, 70, 90	4 fold
0.08	15, 40, 48, 60, 65, 125	8 fold
0.10	5, 8, 28, 32, 34, 40, 50	10 fold
0.13	25, 30, 32, 54, 60	2.5 fold
0.16	15, 25, 60, 120, 132	9 fold
0.23	34, 42, 105	3 fold

* Administered in two or three separate doses.
** Each figure represents a patient.

before the extraction procedure, did not interfere, as expected from their physicochemical properties. Similar findings were obtained with plasma from epileptic patients receiving the above drugs in combination with clonazepam. There was also no interference in cases in which diazepam or other benzodiazepines were administered simultaneously. Diazepam, its metabolites, and other benzodiazepine derivatives, have a shorter retention time than ABCB at the operating conditions we employed. Possible interference from clonazepam metabolites was also evaluated: 7-aminoclonazepam and 7-acetamidoclonazepam gave peaks with a retention time of 4 and 9.5 min, respectively. 7-Aminoclonazepam was very poorly extracted under the conditions used as shown in Fig. 3. No interference is expected from these two major metabolites of clonazepam.

A skilled technician can run through the procedure described for 15–20 plasma samples plus an internal calibration graph of four points in duplicate in a time of 3.5–4 h. The method has been applied to the routine determination of clonazepam in plasma of epileptic patients receiving constant oral treatment with the drug, in combination with other drugs, for at least 3 weeks. Preliminary data indicate that there is no relation between the daily dose (mg/kg) and the plasma levels (ng/ml) obtained

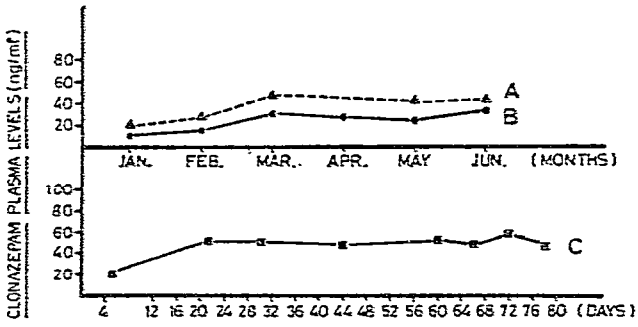

Fig. 4. Clonazepam plasma levels in three epileptic patients over a prolonged period of observation (80–180 days). The administered daily doses were 0.10 mg/kg for patient A and 0.12 mg/kg for patients B and C. Plasma levels refer to samples withdrawn before the morning dose.

before the morning dose. As reported in Table II, there was a *ca.* 3–10-fold variation in the plasma levels from patient to patient for a given dose. On the other hand, for the same patient, in cases following constant therapy, clonazepam plasma levels appeared to be relatively stable if samples were taken at fixed intervals of time after ingestion of the drug (Fig. 4). Fluctuations of *ca.* 20–40% were observed between the pre-dose morning values and the values obtained 4–6 h after drug intake.

The lack of a relation between plasma levels and the daily dose over a period of constant drug treatment stresses the necessity of routine measurement of clonazepam plasma concentrations in epileptic patients.

CONCLUSIONS

We have developed an analytical method for the determination of clonazepam based on a single solvent extraction followed by detection with GLC using an ECD. At variance with previously described procedures, no hydrolysis is necessary, and this improves greatly the specificity of the analysis. The rapidity and simplicity of the method allows the determination of 20 samples in 4 h, and this makes it particularly useful for routine analysis. The sensitivity (4 ng/ml) and the specificity of the method are compatible with pharmacokinetic studies. The small amount of plasma (0.2 ml) required makes the procedure suitable for the determination of clonazepam in infants and children using capillary blood samples.

ACKNOWLEDGEMENTS

We wish to thank Drs. Rovei and Frigerio for their kindness in performing the GLC–MS analysis, Dr. Avanzini (Istituto Neurologico C. Besta, Milan), Dr. Canger (Epilepsy Center, Guardia II, University of Milan), Dr. Baruzzi (Department of Neurology, University of Bologna) and Dr. Zagnoni (Department of Neurology, University of Genova) for supplying plasma from patients and Prodotti Roche, Milan, for kindly supplying samples of 7-aminoclonazepam and 7-acetamidoclonazepam.

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