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QUANTITATION OF CLONAZEPAM AND ITS 7-AMINO AND 7-ACETAMIDO METABOLITES IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method is reported for the determination of clonazepam and its metabolites 7-amino- and 7-acetamidoclonazepam. Extraction from buffered plasma is carried out at pH 9.5 with hexane—ethyl acetate (7:3) for clonazepam and with chloroform for the metabolites. Flunitrazepam, 7-aminodemethylflunitrazepam and 7-acetamidoflunitrazepam are used as the internal standards for clonazepam and its 7-amino and 7-acetamido metabolites, respectively. To prevent decomposition of 7-amino-clonazepam a high concentration of 7-aminomethylclonazepam is added to the plasma. Chromatography is carried out on a reversed-phase column with detection at 254 nm for clonazepam and 240 nm for the metabolites. Using the method it was possible to determine 5 ng/ml clonazepam, 7-aminoclonazepam and 7-acetamidoclonazepam in plasma with coefficients of variation of 9.5%, 5.9% and 8.9%, respectively.

This method can be used to measure clonazepam in plasma from patients treated with other antiepileptics. It may also be utilized for in vitro studies on the metabolism of clonazepam in subcellular fractions from the liver.

INTRODUCTION

Clonazepam is a relatively new anticonvulsant drug, preferentially used in the treatment of childhood epileptic disorders such as minor motor and petit mal epilepsia which are refractory to treatment with other drugs [1, 2]. Several analytical methods have been developed in order to improve the selectivity, the sensitivity and the simplicity of clonazepam assays. The most commonly used methods employ gas—liquid and high-performance liquid chromatography

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(HPLC) [3—8]. Analytical methods for 7-aminoclonazepam have employed gas—liquid chromatographic [9] and mass spectrometric techniques [10].

In the present paper we describe a selective and sensitive HPLC method for the determination of clonazepam and its metabolites, 7-amino- and 7-acetamidoclonazepam in plasma samples from patients and in incubates with subcellular fractions from the liver.

Our method combines the advantages that only one extraction step is required and no derivatization or acid hydrolysis [3] is necessary. With ultraviolet (UV) detection of the compounds linearity over a wide concentration range is achieved.

MATERIAL AND METHODS

Apparatus

The liquid chromatograph consisted of a Constametric III pump and a Spectromonitor III variable-wavelength detector (both from Laboratory Data Control, Riviera Beach, FL, U.S.A.) operating at 254 nm (clonazepam) and 240 nm (the metabolites). A Rheodyne Model 7125 was used as injector (Berkeley, CA, U.S.A.) with a 20-μl sample loop. For the absorbance spectra a Beckman 34 spectrophotometer with scanner was employed.

Chemicals and materials

Clonazepam (CLZ), flunitrazepam (FLZ, internal standard), 7-aminoclonazepam (7-ACLZ), 7-aminodemethylflunitrazepam (7-ADFLZ, internal standard), 7-aminomethylclonazepam (7-AMCLZ, protector, see Results and discussion), 7-acetamidoclonazepam (7-AACLZ), 7-acetamidoflunitrazepam (7-AAFLZ, internal standard) were products of Hoffmann-La Roche (Basel, Switzerland). The drugs tested for interference were obtained from the respective manufacturer. Glycine (p.a.), sodium hydroxide, sodium acetate (p.a.), methanol (p.a.), hexane (p.a.), ethyl acetate, acetonitrile (LiChrosolv) and chloroform (p.a.) were purchased from Merck (Darmstadt, F.R.G.). All glass tubes were silanized, washed, and rinsed twice from detergent with distilled water.

The stock solutions were 1 mg/ml of the respective compound in methanol. All working solutions were prepared by dilution with methanol and they contained 1 μg/ml except for the solution of 7-AMCLZ and 7-AAFLZ which contained 100 and 2 μg/ml, respectively. A 2 *M* glycine—sodium hydroxide buffer, pH 9.5, was prepared.

Chromatographic conditions

The column (150 × 4.6 mm I.D.) was prepacked with Supelcosil LC-18 (5-μm octadecylsilane, Supelco, Bellefonte, PA, U.S.A.). The mobile phase for CLZ was a mixture of acetonitrile—0.1 *M* sodium acetate (35:65), pH 7.7, and for the metabolites a mixture of acetonitrile—0.02 *M* sodium acetate (18:82), pH 7.4. The mobile phases were degassed by filtration. Column equilibrium was achieved within 30 min at a flow-rate of 3 ml/min. Distilled water was used to measure the retention time for an unretained solute.

Analytical procedure

CLZ and the metabolites were analysed separately. To 1 ml of plasma were added 1 ml of 2 *M* glycine—sodium hydroxide buffer, pH 9.5, 50 μl of the internal standard and 50 μl of the protector when metabolites were analysed. When CLZ was analysed, the mixture was extracted for 45 min with 7 ml of hexane—ethyl acetate (7:3). When the metabolites were analysed extraction was performed with 7 ml of chloroform for 45 min. The samples were centrifuged for 5 min and the organic phase was transferred to new tubes and evaporated to dryness. The residue was reconstituted in 40 μl of the respective mobile phase (see chromatographic conditions). Thirty microlitres of the sample were injected into the column. An additional extraction step was needed when analysing 7-ACLZ in plasma from patients treated with carbamazepine. After the 45 min extraction the water phase was discarded and 1 ml of 2 *M* glycine—sodium hydroxide buffer, pH 9.5 was added to the chloroform phase, which was extracted for 5 min.

Standards were prepared in duplicate by the addition of known amounts of CLZ (10—100 ng/ml), 7-ACLZ (10—100 ng/ml) and 7-AACLZ (10—100 ng/ml) to drug-free plasma. Quantitations were performed from a standard curve of the ratio of the peak heights of the sample and the internal standard versus the plasma concentration, obtained by analysing the above-mentioned standards.

Test of stability

7-ACLZ and 7-AACLZ were tested for stability in chloroform and ethyl acetate at low concentrations (1.4, 7.1 and 14.3 ng/ml, equal to 10, 50 and 100 ng in 7 ml of the organic solvent) for 1 h at room temperature.

10, 50 and 100 μl of 1 μg/ml 7-ACLZ and 7-AACLZ in methanol were added to empty tubes (used as reference standards) and to tubes with 7 ml of chloroform with and without protector, and with 7 ml of ethyl acetate, respectively. The organic solutions were stored at room temperature for 1 h before evaporation to dryness. The small amount of methanol in the reference standards was added and evaporated to dryness immediately before injection. The residue from each tube was dissolved in 40 μl of mobile phase and 30 μl were injected into the column. The peak heights were measured for each organic solution and concentration and compared with the peak heights of the reference standards (see Fig. 4 and Results and discussion).

RESULTS AND DISCUSSION

The absorbance spectra for 7-ACLZ and 7-AACLZ dissolved in the mobile phase are shown in Fig. 1. In order to monitor both metabolites at the same wavelength we chose 240 nm as the optimum wavelength. The absorbance spectrum for CLZ (not shown) revealed that 254 nm was the optimum wavelength.

Fig. 2 and 3 show the chromatograms of CLZ (Fig. 2) and its metabolites (Fig. 3) in spiked plasma, blank plasma and plasma from treated patients. FLZ was chosen as internal standard because of its similar chemical structure to CLZ. Even though flunitrazepam (Rohypnol) is used as a hypnotic agent, this

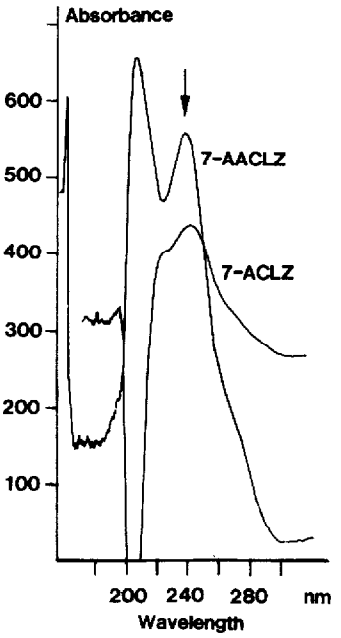

Fig. 1. Absorbance spectrum of 7-aminomethylclonazepam (1.0 μg 7-ACLZ per ml mobile phase) and 7-acetamidoclonazepam (1.0 μg 7-AACLZ per ml mobile phase). Conditions: mobile phase, acetonitrile—0.02 *M* sodium acetate (18:82); scan speed, 50 nm/min; chart speed, 25 mm/min.

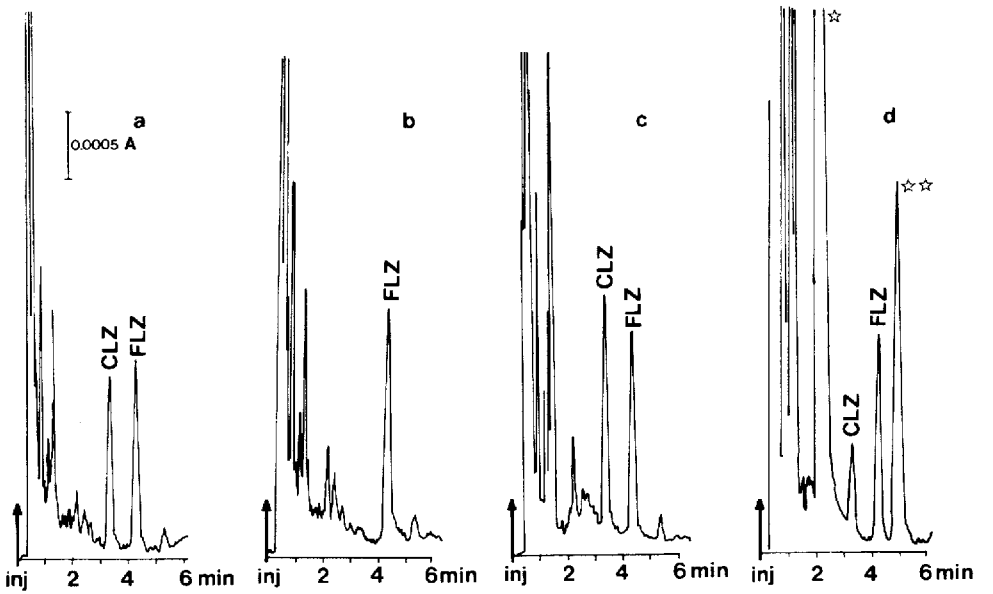

Fig. 2. Chromatograms of clonazepam. Column: Supelcosil LC 18 (5 μm, 150 × 4.6 mm I.D.). Mobile phase: acetonitrile—0.1 *M* sodium acetate (35:65). Flow-rate: 3 ml/min. Detector: Spectromonitor III (Laboratory Data Control) at 254 nm. Peaks: CLZ = clonazepam, FLZ = flunitrazepam (internal standard), ☆ = carbamazepine and phenytoin, ☆☆ = unidentified peak. The figure shows chromatograms from plasma of: (a) a sample spiked with 30 ng/ml CLZ; (b) a blank sample with FLZ; (c) a sample from a patient treated with CLZ (the found CLZ concentration was 46 ng/ml); (d) a sample from a patient treated with CLZ, carbamazepine and phenytoin (the found CLZ concentration was 15 ng/ml).

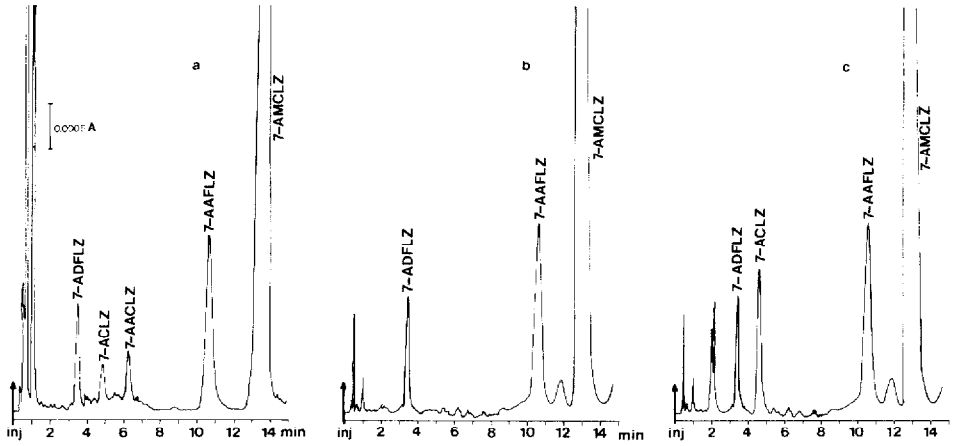

Fig. 3. Chromatograms of clonazepam metabolites. Chromatographic conditions as in Fig. 2 except for the mobile phase: acetonitrile—0.02 *M* sodium acetate (18:82). Peaks: 7-ADFLZ = 7-aminodemethylflunitrazepam (internal standard), 7-ACLZ = 7-amino-clonazepam, 7-AACLZ = 7-acetamidoclonazepam, 7-AAFLZ = 7-acetamidoflunitrazepam (internal standard), 7-AMCLZ = 7-aminomethylclonazepam. The figure shows chromatograms from plasma of: (a) a sample spiked with 40 ng/ml 7-ACLZ and 7-AACLZ; (b) a blank sample with internal standards; (c) a sample from a patient treated with CLZ. The chromatogram shows a peak of 7-ACLZ (110 ng/ml), but no detectable peak of 7-AACLZ.

drug is seldom employed in epileptic patients with clonazepam treatment since this form of epilepsy occurs mainly in children.

Standard curves for CLZ, 7-ACLZ and 7-AACLZ were generated by addition of these compounds to drug-free plasma. The regression equations and the coefficients of correlation in the 10—100 ng/ml range were: for CLZ, $Y = 0.031X + 0.030$, $r = 0.998$; for 7-ACLZ, $Y = 0.012X - 0.009$, $r = 0.995$; and for 7-AACLZ, $Y = 0.0074X + 0.0015$, $r = 0.998$.

Precision

Samples spiked with CLZ, 7-ACLZ and 7-AACLZ at concentrations shown in Table I were analysed within one analysis ($n = 10$) and for CLZ in addition between analyses in duplicate ($n = 10$). The estimated mean concentration and the coefficient of variation (C.V.) are also given in Table I. As seen in Table I, the C.V. did not exceed 11% for any of the compounds at either concentration. Using the method it was possible to determine 5 ng/ml of CLZ, 7-ACLZ and 7-AACLZ in plasma with C.V. of 9.5%, 5.9% and 8.9%, respectively.

Stability

CLZ stability in plasma and in methanol solution was tested and found to be stable over several months. In hexane and ethyl acetate, CLZ was stable for at least two days. These solvents were considered for extraction of CLZ. Chloroform gave too much baseline noise when used for CLZ extraction from plasma and therefore hexane—ethyl acetate (7:3) was chosen.

We found that 7-AACLZ was stable when stored in plasma or methanol for at least one month (—20°C) or in chloroform for at least one week (—20°C, +4°C, +25°C).

TABLE I

PRECISION AND COEFFICIENTS OF VARIATION (C.V., %) FOR ANALYSIS OF PLASMA SAMPLES ($n = 10$) SPIKED WITH CLONAZEPAM (CLZ), 7-AMINOCLONAZEPAM (7-ACLZ) AND 7-ACETAMIDOCLONAZEPAM (7-AACLZ)

Compound	Concentration (ng/ml)		C.V. (%)
	Added	Estimated mean	
CLZ within analysis	15.6	15.8	7.2
	31.1	28.1	5.5
CLZ between analyses	15.6	16.8	9.1
	31.1	28.7	10.6
7-ACLZ within analysis	10	8.3	7.5
	50	49.1	4.1
7-AACLZ within analysis	10	10.1	10.4
	50	51.0	8.5

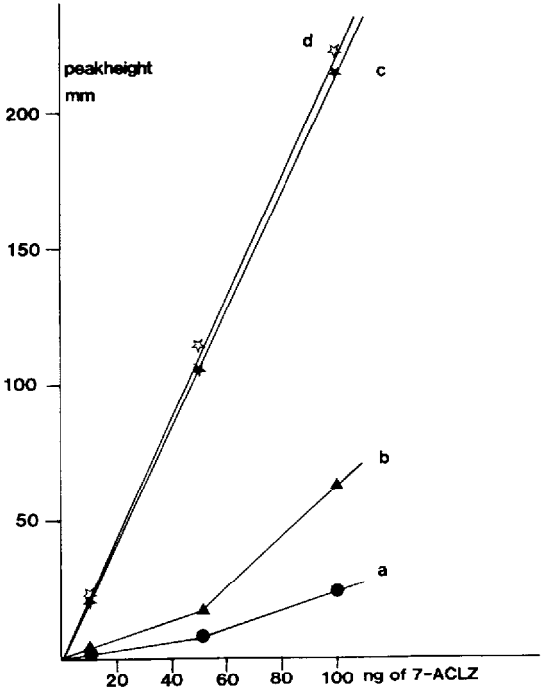

Fig. 4. Decompositon of 7-aminoclonazepam (7-ACLZ) in ethyl acetate and chloroform and the effect of 7-aminomethylclonazepam (7-AMCLZ, 5 μg) in preventing decomposition of 7-ACLZ in 7 ml of chloroform. Procedure according to test of stability: 10, 50 and 100 ng of 7-ACLZ stored for 1 h in (a) 7 ml of ethyl acetate, (b) 7 ml of chloroform, and (c) 7 ml of chloroform with 5 μg of protector (7-AMCLZ), compared with (d) 10, 50, 100 ng reference standards.

In contrast, 7-ACLZ was found to decompose with time when stored in chloroform, ethyl acetate and methanol at low concentrations. It was stable, however, for at least one month when stored in plasma.

At a concentration of 1 μg/ml 7-ACLZ in methanol there was a 10—20% decomposition of the compound when stored at −20°C over 48 h. During extraction of 7-ACLZ from patient plasma to chloroform, concentrations as low as 1 ng/ml in chloroform may occur. Decomposition of 7-ACLZ in chloroform and ethyl acetate at concentrations of 1—20 ng/ml is 80% and 90%, respectively, when stored for 1 h at room temperature (Fig. 4). Despite the decomposition of 7-ACLZ in chloroform this solvent was chosen for extraction of the metabolites instead of ethyl acetate since primary and secondary amines react with esters. 7-AMCLZ, a compound chemically very similar to 7-ACLZ, was tested as a protector and was found to be effective in preventing decomposition of 7-ACLZ (Fig. 4).

Recovery

The recoveries of CLZ and its metabolites were determined by comparison of peak heights of standards without extraction with standards extracted from plasma according to the analytical procedure. The recoveries of CLZ and its metabolites were 90% to 95% when extracting for 45 min.

Interferences

Concomitant treatment with other drugs, particularly antiepileptics, gives rise to a potential risk of interference since clonazepam is present at low concentrations. To test this possibility various drugs were dissolved in the mobile phase and injected into the chromatograph. The retention behaviour of these drugs is listed in Table II. None of the drugs listed in the table interfered with CLZ.

When plasma from patients treated with some of these drugs, notably phenobarbital, carbamazepine, phenytoin and nitrazepam, were processed according to the analytical method, no peaks interfering with CLZ or FLZ were detected.

TABLE II

CAPACITY FACTORS (k') OF VARIOUS COMMONLY USED DRUGS

Column: Supelcosil LC-18 (5 μm, 150 × 4.6 mm I.D.). Mobile phase: acetonitrile—0.1 *M* sodium acetate (35:65). Flow-rate: 3.0 ml/min. Unretained solute: distilled water.

Drug	k'	Drug	k'
Caffein	0.30	Nitrazepam	6.08
Theobromine	0.30	Lorazepam	6.45
Theophylline	0.30	Chlordiazepoxide	6.55
Ethosuximide	0.90	Clonazepam	7.25
Phenobarbital	1.35	Flunitrazepam	9.43
Carbamazepine	4.18	Nordiazepam	11.25
Phenytoin	4.35	Diazepam	19.55
Oxazepam	5.85	Valproic acid	Not detected

However, plasma from carbamazepine-treated patients contained a peak interfering with 7-ACLZ. This peak was identical to the carbamazepine-10,11-dihydrodiol metabolite. This problem can be circumvented by introducing an additional extraction step (see Analytical procedure).

Clinical application

Our HPLC method for CLZ has been used for routine analyses of patient plasma over a period of one year. The dose versus plasma concentration relationship observed is similar to that reported previously [7]. Although the value of clonazepam metabolite monitoring is doubtful we have measured 7-ACLZ and 7-AACLZ in seven patients. The preliminary data indicate that the levels of 7-ACLZ are in the same range as those of CLZ. 7-AACLZ was not measurable in these patient samples. The method can not be applied to plasma from patients treated with FLZ since this drug is used as internal standard. This is the only limitation that we are aware of so far. However, if the metabolites 7-ACLZ and 7-AACLZ are to be analysed, concomitant treatment with carbamazepine (and possibly other antiepileptic drugs) may give interfering peaks.

Conclusion

We have developed an HPLC system for the determination of both CLZ and its metabolites (7-ACLZ and 7-AACLZ) based on a single solvent extraction, reversed-phase column system and UV detection. To obtain good stability for 7-ACLZ it was necessary to add 7-AMCLZ as a protector in high concentration to the extraction solvent (Fig. 4). The selectivity of the column system enables other antiepileptic drugs and benzodiazepines to be well resolved and quantitated (Table II). The method can also be used without modification for the assay of CLZ and its metabolites in studies of CLZ metabolism in vitro in subcellular preparations of, for example, liver.

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