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Note

Rapid and specific high-performance liquid chromatographic determination of clonazepam in plasma

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Clonazepam (CNZ) is a relatively new anticonvulsant drug, preferentially used in the treatment of childhood epileptic disorders. Because of the narrow therapeutic range (20–50 ng/ml)[1], it is necessary to monitor clonazepam in plasma. The most commonly used methods are gas chromatography[2–7] and high-performance liquid chromatography (HPLC)[8–11].

We describe a rapid, simple, accurate and sensitive HPLC method for the determination of clonazepam in plasma.

MATERIALS AND METHODS

Apparatus

The analysis was performed on a 2B liquid chromatograph (Perkin-Elmer, Monza-Milan, Italy) equipped with a Perkin-Elmer LC 85 variable-wavelength detector. A Rheodyne Model 7125 (Supelchem, Milan, Italy) injector with a 20-μl sample loop was used.

Reagents and standards

Clonazepam (CNZ) and flunitrazepam (FNZ) were from Hoffman-La Roche (Basle, Switzerland), methanol (Lichrosolv), acetonitrile (Lichrosolv), diethyl ether (Uvasolv), ammonia solution (25%, w/v; p.a.) and sodium sulphate (anhydrous, p.a.) from Merck-Bracco (Milan, Italy). The stock solutions of CNZ and FNZ contained 1 mg/ml methanol. The working solution of FNZ (internal standard) contained 0.5 μg/ml methanol. The standard curve was constructed by dilution of CNZ stock solution in drug-free plasma. The standards were stable for at least 3 months at 4°C.

Chromatographic conditions

A C_{18} high-speed column, 3 μm, 30 mm × 4.6 mm (Perkin-Elmer), was used. The mobile phase was a 25% (v/v) solution of acetonitrile in water, degassed by vacuum filtration. The flow-rate was 2.5 ml/min. The detector was set at 306 nm and the sensitivity was 0.01 a.u.f.s.

Analytical procedure

To 1 ml of plasma in a 100 mm × 12 mm screw-cap glass tube were added 30 μl of working internal standard and 50 μl of 1 *M* ammonium hydroxide, and the

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contents were briefly vortex-mixed. Then 8 ml of diethyl ether were added and, after through mixing for 2 min, the aqueous phase (bottom) was discarded. The organic phase was dried with 0.5 g of anhydrous sodium sulphate, transferred to a conical glass tube and evaporated to dryness in a water-bath at 55°C after addition of a few grains of sodium chloride to prevent bumping. The dry residue was redisolved in 25 μl of methanol, and 20 μl of the solution were injected into the liquid chromatograph.

RESULTS

Fig. 1 shows chromatograms of a plasma from a patient treated with clonazepam (flunitrazepam as internal standard) (A) and of a blank sample (B).

The calibration plot was linear for concentrations between 5.0 and 250 ng/ml. The regression equation and linear regression coefficient were $y = 0.0044x - 0.013$ and $r = 0.9998$, respectively. The sensitivity limit of detection was 3 ng/ml.

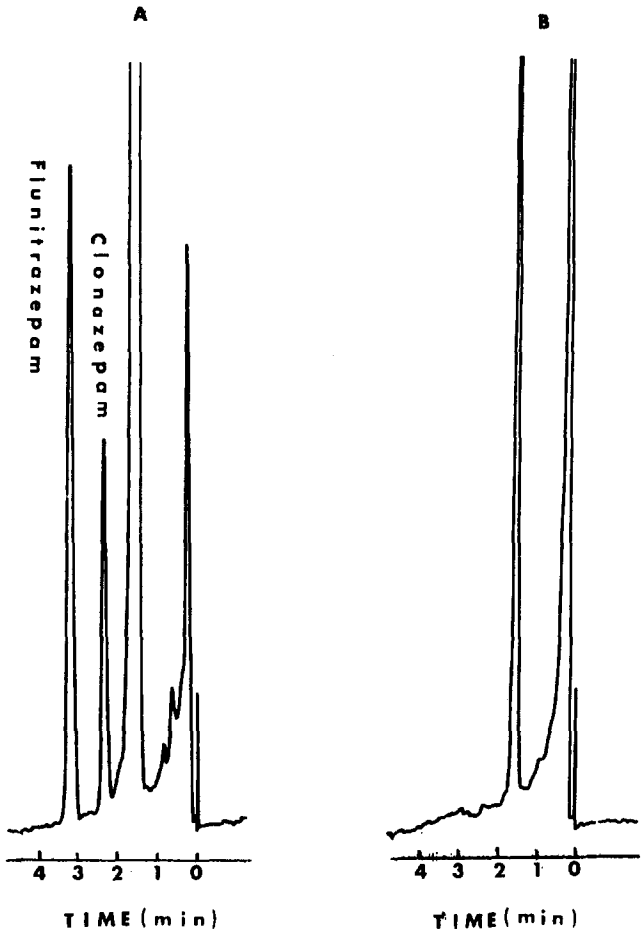

Fig. 1. Chromatograms of plasma extract from a patient treated with clonazepam (flunitrazepam as internal standard) (A) and of a blank sample (B).

TABLE I

PRECISION OF THE ANALYSIS OF PLASMA SAMPLES SPIKED WITH CLONAZEPAM

n = 15.

Amount of CZP in plasma (ng/ml)	*Within assay*		*Between assay*	
	Quantity found (ng/ml)	*C.V. (%)*	*Quantity found (ng/ml)*	*C.V. (%)*
23.5	22.2	3.3	22.9	4.3
47.0	44.1	3.8	46.0	4.6
125.0	124.4	4.3	126.1	3.2

The within-analysis and day-to-day precisions were estasblished by analysing drug-free samples, spiked with three different concentrations of clonazepam (23.5, 47.0 and 125.0 ng/ml). The coefficients of variation (C.V.) are listed in Table I.

The interferences from endogenous compounds, observed at 245 nm, were eliminated by choosing a detection wavelength of 306 nm, corresponding to another absorbance maximum of clonazepam. The potential interference from drugs used as concomitant therapy was examined. The retention times of the most common benzodiazepines, antiepileptics and methylxanthines in this system are listed in Table II.

DISCUSSION

Diethyl ether was chosen as the extraction solvent because it gave a very good recovery of clonazepam and is easily evaporated (Table III). With the high-speed C_{18} column the analysis time was very short (a chromatogram took only 4 min). The selectivity of this column enables other drugs commonly used as concomitant therapy to be well separated.

TABLE II

RETENTION TIMES OF THE MOST COMMON BENZODIAZEPINES, ANTIEPILEPTICS AND METHYLXANTHINES

Only lorazepam and desmethylclobazam may interfere with clonazepam analysis.

Drug	*Retention time (min)*	*Drug*	*Retention time (min)*
Clobazam	4.5	Caffein	1.0
Clonazepam	2.4	Teobromine	0.2
Desmethylclobazam	2.3	Teophylline	0.2
Diazepam	7.2	Carbamazepine	0.3
Desmethyldiazepam	4.1	Diphenylhidantoin	0.2
Flunitrazepam	3.4	Carbamazepine 10,11-epoxide	0.2
Flurazepam	Not eluted	Esobarbital	0.2
Lorazepam	2.5	Phenobarbital	0.1
Oxazepam	2.1	Ethosuximide	0.2
Medazepam	2.0	Primidone	0.2
Nitrazepam	2.1		

TABLE III

RECOVERY OF CLONAZEPAM AND FLUNITRAZEPAM FROM DIFFERENT EXTRACTION SOLVENTS

Solvent	*Recoveries (%)*	
	CZP	*FZP*
Diethyl ether	94.0	104.5
Dichloromethane	88.0	79.5
Chloroform	96.0	87.5
Hexane–ethyl acetate (7:3)	84.5	84.0

Aqueous acetonitrile as mobile phase gave the same results as acetonitrile–phosphate buffer (pH 4.8 or 7.8) mixtures but less problems.

The total analysis time for ten samples was between 20 and 25 min.

We chose flunitrazepam as internal standard because this drug is not used in antiepileptic therapy. The only limitation of our method is the concomitant therapy with clobazam, because its metabolite, desmethylclobazam, interferes with clonazepam. Interference by lorazepam is not a problem, because this drug is not used in therapy.

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