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Note

Simple and sensitive method for monitoring clonazepam in human plasma and urine by high-performance liquid chromatography

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Clonazepam, 7-nitro-5-(2-chlorophenyl)-1,3-dihydro[2*H*]1,4-benzodiazepin-2-one, a member of the benzodiazepine series of drugs, has anticonvulsant properties [1]. The pharmacokinetics of clonazepam and its metabolites, 7-aminoclonazepam and 7-acetamidoclonazepam has been reported previously [2–4]. Owing to a relatively low therapeutic index, the measurement of drug levels is important in maintaining a therapeutic concentration without side-effects.

Most methods for the determination of clonazepam in serum employ gas chromatography with either electron-capture or nitrogen–phosphorus detection [5–7]. Other methods are limited by the need of hydrolysis or derivatization [8,9], lack of sensitivity [10], cost of mass spectrometry [11], use of

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multiple extractions in the analysis [12,13] or use of toxic extraction solvents [14–17].

This paper describes a simple, sensitive and rapid method for the determination of clonazepam in human plasma and urine using reversed-phase high-performance liquid chromatography (HPLC) with desmethylflunitrazepam as internal standard (I.S.).

EXPERIMENTAL

Materials

Methanol and acetonitrile were HPLC grade (Merck, Darmstadt, F.R.G.). All other chemicals and solvents used were analytical grade and provided by Merck and Prolabo (Paris, France). The enzyme β-D-glucuronidase was obtained from Sigma (St. Louis, MO, U.S.A.).

Clonazepam (CZ, free base) and desmethylflunitrazepam (Nor-FNZ, free base) were purchased from Roche (Basel, Switzerland). The structures are shown in Fig. 1.

Stock solutions of pure CZ and Nor-FNZ were prepared in methanol at 20 mg/l, stored at 4°C and found to be stable for several months. The working solution of Nor-FNZ contained 2.5 mg/l.

The clonazepam standard concentrations were 5, 10, 30, 50, 100, 300 and 500 μg/l and were obtained by appropriate dilution just before use of clonazepam stock solution in drug-free plasma.

Chromatographic conditions

The HPLC system consisted of a pump (Waters 510) and an automatic sample injection module (Waters Wisp 710 B), which were coupled to a programmable multi-wavelength detector (Waters 490) operated at 242 nm. The sensitivity was 0.01 a.u.f.s. Quantifications were carried out by measuring the peak-area ratio with a computing integrator and chromatography control station (Waters 840). The separation was performed on a Waters reversed-phase Nova Pak C_{18} column (5 μm particle size, 150 mm × 4.6 mm I.D.).

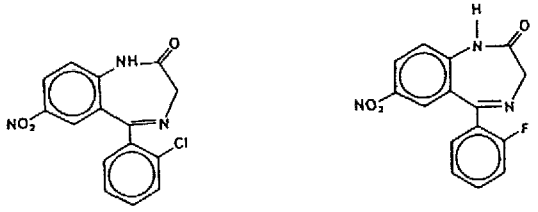

Fig. 1. Structures of clonazepam (CZ) and the internal standard desmethylflunitrazepam (Nor-FNZ).

The mobile phase was acetonitrile–methanol–6 m*M* phosphate buffer (30:10:60, v/v) prepared with stock solutions (94 ml of 0.2 *M* NaH_2PO_4 added to 6 ml of 0.2 *M* $Na_2HPO_4 \cdot 7H_2O$). This mobile phase was adjusted to pH 5.7 with 0.1 *M* HCl, filtered and degassed through 0.45-μm Durapore filters (GVW P047).

The flow-rate was set at 1.3 ml/min, giving a pressure of 158.6 bar; the column was washed with methanol and water at the end of each session.

Preparation of biological samples

Urine samples were prepared by adjusting the pH to 5.0 with 2 *M* hydrochloric acid, then hydrolysing at 37°C for 5 h with 20 μl of a solution of $2 \cdot 10^4$ U/ml β-D-glucuronidase.

For extraction, 15 μl of desmethylflunitrazepam solution (2.5 mg/l), 50 μl of 0.5 *M* sodium hydroxide and 5 ml of diethyl ether were added to 0.5-ml aliquots of plasma or urine in glass centrifuge tubes. After agitation and centrifugation, the organic phase was removed and evaporated to dryness at 45°C in a Speed Vac concentrator (Savant Instruments, Farmingdale, NY, U.S.A.) connected to a vacuum pump (Yamato Scientific, Tokyo, Japan). The residue was dissolved in 100 μl of methanol, and 25 μl were injected into the chromatographic system.

RESULTS AND DISCUSSION

Fig. 2 shows a typical chromatogram obtained after extraction of a patient's plasma. The retention times and capacity factors (k') were 3.65 min and 2.72 for the I.S. and 4.43 min and 3.52 for clonazepam, respectively.

An eight-point standard curve was constructed by extracting plasma samples spiked with CZ (at 5, 10, 30, 50, 100, 200, 300 and 500 μg/l) in order to evaluate the linearity of the method. The curve was linear within the range of concentrations tested. The regression equation and linear regression coefficient were $y = 0.005x + 0.054$ and $r = 0.996$, respectively (y = peak area of clonazepam/peak area of the I.S. and x = concentration of clonazepam in μg/l).

The accuracy of the method (Table I) was also determined. Mean errors (six samples for each concentration) ranged from −3.3% to 5.0%. The R.S.D. was never higher than 10%. The limit of detectability, determinated by decreasing the concentration of clonazepam, was found to be 3 μg/l at a signal-to-noise ratio of 3. This limit is adequate for forensic and clinical analysis.

Methylclonazepam, obtained by methylation of clonazepam with diazomethane, was previously used as the I.S. [18] but in our conditions its retention time was too long. Therefore we chose desmethylflunitrazepam since it is not currently used in antiepileptic therapy and has a structural analogy with clonazepam.

The absolute recovery of clonazepam with different extraction solvents was determined by comparing representative peak areas of extracted plasma sam-

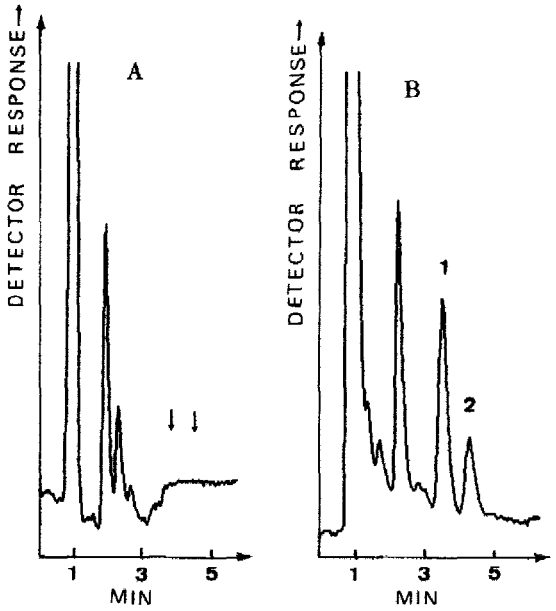

Fig. 2. (A) Typical chromatogram of a control plasma. There are no interferences at the retention times of clonazepam and the internal standard (arrows). (B) Chromatogram of an extract of a test plasma sample obtained from a child under clonazepam treatment (concentration, 19 μg/l). Peaks: 1 = desmethylflunitrazepam; 2 = clonazepam.

TABLE I

ACCURACY OF THE DETERMINATION OF CLONAZEPAM IN PLASMA

Concentration added (μg/l)	Concentration found (mean ± S.D., $n=6$) (μg/l)	R.S.D. (%)	Error (%)
20	20.60 ± 1.82	9.1	3.0
40	41.83 ± 3.09	7.7	4.6
60	58.00 ± 5.31	8.8	−3.3
100	105.00 ± 7.71	7.7	5.0
140	136.50 ± 13.95	9.9	−2.5

ples at a concentration of 0.2 mg/l, with the peak areas of the methanolic standards at the same concentration. Diethyl ether was chosen as the extraction solvent since it give the best recovery (89.3%), is easily evaporated and produced emulsion-free extracts (Table II).

The within-run precision was evaluated by analysing eight samples of a drug-free plasma spiked with clonazepam at the therapeutic concentration of 40 μg/

TABLE II

RECOVERY OF CLONAZEPAM FROM DIFFERENT EXTRACTION SOLVENTS

Solvent	Mean recovery ($n=4$) (%)
Hexane–isoamyl alcohol (98:2, v/v)	4.6
Heptane–isoamyl alcohol (98:2, v/v)	9.2
Chloroform	92.7
Dichloromethane	85.0
Diethyl ether	89.3
Ethyl acetate	82.8
Ethyl acetate–dichloromethane–2-propanol (44 44:12, v/v)	86.8
Diethyl ether–chloroform–1-butanol (32:32 36, v/v)	75.0
Ethyl acetate–chloroform–1-butanol (44:44 12, v/v)	90.2
Ethyl acetate–dichloromethane–1-butanol (44:44:12, v/v)	66.0

TABLE III

ANALYSIS OF CLONAZEPAM AFTER SINGLE-STEP EXTRACTION USING 200 µl OF SERUM

Concentration of drug (µg/l)	Mean recovery ($n=4$) (%)	Coefficient of variation (%)
10	74.4	9.1
25	89.1	6.7
50	92.4	3.0
80	88.4	5.0
100	89 8	3.9
150	89 4	3.1

l. Analyses were performed every day for two weeks. The coefficients of variation were 6.2% for within-run precision and 7.4% for day-to-day precision.

For pediatric samples, a minimum of 200 µl of serum or human urine after hydrolysis can be analysed without significant loss of precision (Table III).

The selectivity of the Nova Pak C_{18} column enables other benzodiazepines commonly used as concomitant therapy or in case of intoxication, to be resolved. Several other benzodiazepines were assayed by the proposed method; retention data are given in Table IV. Clearly, only oxazepam and nitrazepam interfere with the determination of clonazepam. These interferences would necessitate alternative methods for toxicological screening.

TABLE IV

RETENTION TIMES AND CAPACITY FACTORS OF DRUGS TESTED FOR INTERFERENCE WITH CLONAZEPAM

Drug	Retention time (min)	Capacity factor (k')
Barbital	1.37	0.40
7-Acetamidoclonazepam	1.45	0.48
7-Aminoclonazepam	1.55	0.58
Aprobarbital	2.0	1.04
Butabarbital	2.03	1.07
Hexobarbital	2.40	1.45
Heptabarbital	2.73	1.79
Desmethylflunitrazepam	3.65	2.73
Clonazepam	4.33	3.42
Oxazepam	4.43	3.52
Nitrazepam	4.56	3.65
Estazolam	4.90	4.0
Desmethylclobazam	4.93	4.03
Lorazepam	5.11	4.21
Flunitrazepam	6.23	5.36
Alprazolam	6.25	5.38
Triazolam	6.60	5.73
Decarboxyloflazepate	6.72	5.86
Chlordiazepoxide	7.73	6.89
Clobazam	7.82	6.98
Desmethyldiazepam	7.92	7 08
Bromazepam	8.10	7.27
Desmethylprazepam	8.17	7.34
Medazepam	8.20	7.37
Ethylloflazepate	9.70	8.90
Diazepam	13.23	12.50
Acepromazine	N.E.[a]	–
Aceprometazine	N.E.	–
Clothiazepam	N.E.	–
Loprazolam methane sulfonate	N.E.	–
Prazepam	N.E.	–

[a]N.E., not eluted within 15 min.

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