Journal of Chromatography, 378 (1986) 492—497
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3102

Note

Simple and sensitive method for the determination of clobazam, clonazepam and nitrazepam in human serum by high-performance liquid chromatography

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(First received October 2nd, 1985; revised manuscript received January 27th, 1986)

Benzodiazepines, which are frequently prescribed for stress-related symptoms, are clinically monitored to evaluate compliance with a therapeutic regimen and to detect abuse of these drugs [1].

We chose a high-performance liquid chromatographic (HPLC) method to monitor the three benzodiazepines that are routinely administered in our hospital, namely, clobazam, clonazepam and nitrazepam. These drugs are never administered simultaneously, but it is very useful to have at our disposal a unique analytical method whereby only one extraction and only one eluent is used, so that we can assay all samples containing any one of these benzodiazepines. Furthermore, this method can be useful for screening when drug abuse is suspected.

Although other investigators have separated these, benzodiazepines or a similar combination of such drugs [2—7], we believe this to be the first report on the separation of clobazam, clonazepam and nitrazepam. Moreover, we have tried to simplify the extraction procedure and to improve the sensitivity in comparison with other methods previously published.

EXPERIMENTAL

Materials

Nitrazepam, clonazepam and flunitrazepam (the internal standard) were obtained from Roche (Milan, Italy) and clobazam from Hoechst (L'Aquila, Italy). Sodium borate and ethyl acetate were purchased from Carlo Erba (Milan, Italy) and all other chemicals were from Merck (Darmstadt, F.R.G.).

0378-4347/86/$03.50

Apparatus and chromatographic conditions

We used a Perkin-Elmer S-2 liquid chromatograph equipped with a Rheodyne 7105 injection valve and a Perkin-Elmer LC 75 UV spectrophotometric detector set at 220 nm and 0.080 a.u.f.s. Separation was achieved using a reversed-phase C_8, 150 × 4.6 mm I.D. analytical column, 5 μm particle size (Supelco, Bellefonte, PA, U.S.A.), preceded by a 2-cm Pelliguard LC-8 guard column with 40-μm packing (Supelco).

The eluent was a mixture of acetonitrile—1.75 m*M* hydrochloric acid—50 m*M* sodium acetate (36:10:54). The flow-rate was set at 1.5 ml/min and the column temperature was ambient.

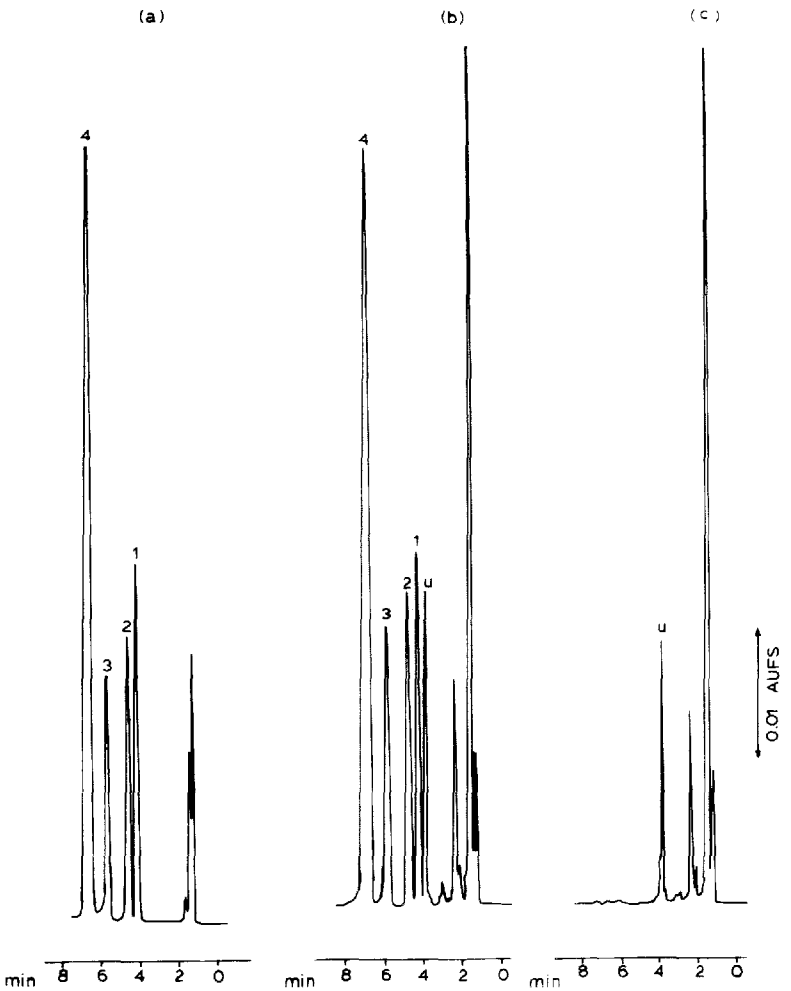

Fig. 1. (a) Aqueous standard mixture of benzodiazepines nitrazepam (1), clonazepam (2), flunitrazepam (3) and clobazam (4). (b) Chromatogram of an extracted human serum pool spiked with 100 ng/ml nitrazepam, clonazepam and flunitrazepam (internal standard), and 200 ng/ml clobazam. (c) Representative chromatogram of the same serum pool before spiking. u = Unknown peak. See text for chromatographic conditions.

Sample preparation

A 1-ml volume of a saturated sodium tetraborate solution was added to 1 ml of serum sample or spiked serum pool (100 ng/ml for each drug, including internal standard). Two aliquots of a 7.5-ml mixture of *n*-hexane–ethyl acetate (9:1) were added, and the mixture was vortexed for 5 min and centrifuged at 1000 *g* each time. The combined organic extracts were evaporated to dryness and the residue was redissolved in 120 μl of eluent. A 100-μl volume of each sample was injected.

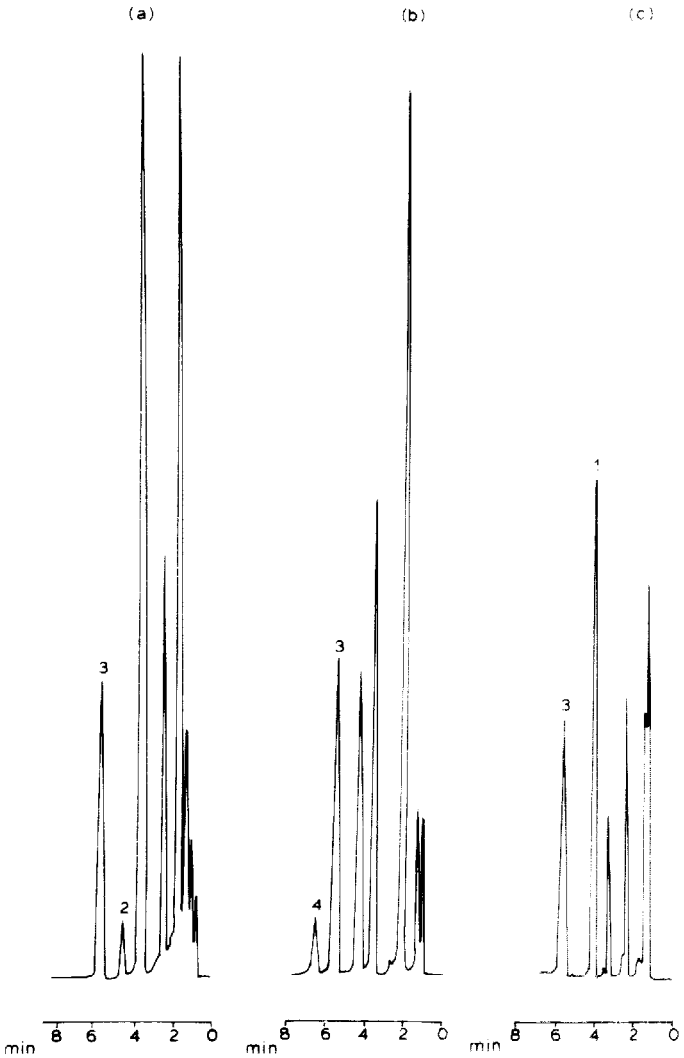

Fig. 2. Representative chromatograms of patients receiving (a) 0.1 mg/kg/day of clonazepam (2), 65 mg/kg/day of valproic acid and 25 mg/kg/day of ethosuximide; (b) 0.1 mg/kg/day of clobazam (4) and 6.0 mg/kg/day of phenobarbital; (c) 1.0 mg/kg/day of nitrazepam (1). Peak 3 = flunitrazepam (internal standard).

RESULTS AND DISCUSSION

Fig. 1 shows the chromatogram obtained from a mixture of standards and from an extracted human serum pool before and after addition of known amounts of the three benzodiazepines and internal standard. Capacity factors for nitrazepam, clonazepam, flunitrazepam and clobazam were 4.55, 5.38, 6.95 and 8.50, respectively.

Fig. 2 shows representative chromatograms for patients receiving each of the three benzodiazepines together with other drugs.

Recovery (i.e. extraction efficiency) was more than 97% for nitrazepam and clonazepam and nearly 100% for clobazam. Linearity was verified up to 250 ng/ml for nitrazepam and clonazepam and up to 500 ng/ml for clobazam (see

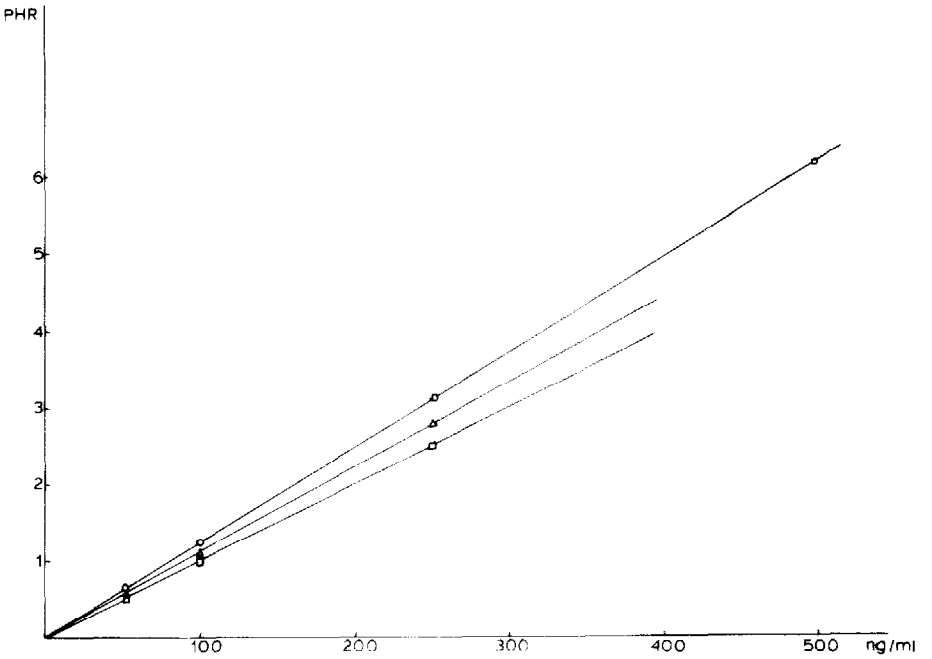

Fig. 3. Peak-height ratio (PHR) versus sample concentration (ng/ml) for clobazam (○), clonazepam (□) and nitrazepam (△) in a spiked serum pool.

TABLE I

EVALUATION OF LINEARITY OF THE METHOD BY SPIKING A SERUM POOL WITH SCALAR AMOUNTS OF BENZODIAZEPINES

Regression equations and correlation coefficients (r) between peak-height ratios, i.e. drug/internal standard (y), and concentration (x, ng/ml) of the three considered benzodiazepines. See also Fig. 3.

Drug	Regression equation	r
Clobazam	$y = 0.0473 + 0.0123x$	0.9995
Clonazepam	$y = 0.0080 + 0.0100x$	0.9998
Nitrazepam	$y = -0.0012 + 0.0109x$	0.9998

TABLE II

PRECISION OF THE METHOD FOR THE THREE DESCRIBED BENZODIAZEPINES

Data refer to a spiked serum pool at 100 ng/ml. For clonazepam, whose serum therapeutic range is below 100 ng/ml, and clobazam, whose therapeutic range is uncertain, the 50 ng/ml level is also reported. Peak-height ratios (PHR) are described.

Drug	Level (ng/ml)	*n*	PHR (mean ± S.D.)	Coefficient of variation on PHR (%)
Within-day precision				
Clonazepam	50	10	0.51 ± 0.01	2.15
Clobazam	50	10	0.65 ± 0.02	2.45
Clonazepam	100	12	1.02 ± 0.02	1.79
Clobazam	100	12	1.28 ± 0.05	3.54
Nitrazepam	100	12	1.14 ± 0.03	2.66
Day-to-day precision				
Clonazepam	50	10	0.51 ± 0.02	4.69
Clobazam	50	10	0.62 ± 0.02	2.92
Clonazepam	100	10	1.01 ± 0.03	2.57
Clobazam	100	10	1.25 ± 0.06	4.74
Nitrazepam	100	10	1.10 ± 0.04	3.63

TABLE III

EVALUATION OF THE ACCURACY OF THE METHOD AT THE TWO LEVELS DESCRIBED IN TABLE II

Drug	Level (ng/ml)	Added (ng/ml)	Found (mean ± S.D., $n = 10$) (ng/ml)
Clonazepam	50	50	50.20 ± 2.39
Clobazam	50	50	46.56 ± 1.47
Nitrazepam	100	100	101.03 ± 3.67
Clonazepam	100	100	100.20 ± 2.60
Clobazam	100	100	97.78 ± 4.82

Fig. 3 and Table I). Precision and accuracy of the method are shown in Tables II and III, respectively.

Choice of wavelength was suggested by the remarkable increase of sensitivity with respect to other wavelengths found in the literature. The lower limits of detection (at a signal-to-noise ratio of 2) were ca. 1 ng/ml for nitrazepam and clonazepam and nearly 0.5 ng/ml for clobazam, which suggests that lower amounts of sample can be employed.

We chose a liquid extraction method because it is simple and quick, and much less expensive than a solid extraction method [6, 8].

We found that the most common antiepileptic and antiasthmatic drugs (i.e. carbamazepine, diphenylhydantoin, ethosuximide, phenobarbital, primidone, valproic acid, theophylline and caffeine) do not interfere with this assay.

Other benzodiazepines may be assayed with this method, e.g. diazepam, but this will be the object of further investigations.

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