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SCREENING PROCEDURE FOR BENZODIAZEPINES IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING A RAPID-SCANNING MULTICHANNEL DETECTOR

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SUMMARY

A sensitive and specific determination of benzodiazepines in biological fluids is described, based on reversed-phase high-performance liquid chromatography. The compounds are extracted with a C_2 AASP cartridge within 5 min. The recovery ranges from 92 to 104% and is independent of the concentration. Using a suitable gradient elution, complete separation of nineteen benzodiazepines is achieved in 50 min with detection limits of less than 3 ng/ml in urine and 5 ng/ml in other biological fluids. Using a rapid-scanning multichannel detector, the identities of the benzodiazepines can be confirmed. Prazepam is employed as internal standard. The precision and accuracy of the described method are suitable for monitoring benzodiazepine levels in clinical studies and an example is given.

INTRODUCTION

Benzodiazepines (BZD) are for the most part antianxiety agents used in the management of many psychiatric disturbances [1]. Some of them, such as clonazepam and diazepam, have anticonvulsant properties and are therefore used in the treatment of epilepsy [2, 3]. Others, such as flunitrazepam, nitrazepam and triazolam, are hypnotic agents [4]. Numerous clinical studies [5, 6] have adequately demonstrated the importance of total plasma concentrations of benzodiazepines in relation to their efficacy [5, 6] and toxicity [7, 8]. Consequently, knowledge of the BZD plasma levels is helpful in therapeutic drug monitoring and in the control of overdosed patients.

Several methods for BZD analysis in biological samples have been described in the literature, employing such diverse techniques as thin-layer chromatography (TLC) [9–11], electron-capture [12, 13] or flame ionization [14, 15] gas chromatography (GC) and high-performance liquid chromatography (HPLC)

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[16–18]. TLC is a commonly used technique for the detection of drugs, but the detection systems lack sensitivity and selectivity [19]. GC procedures require the conversion of BZD into benzophenones [14, 20], or derivatization [21], or the use of capillary column chromatography [22, 23]. Since chlordiazepoxide and its metabolites are thermally unstable and since the conversion into benzophenones does not allow the differentiation of all the BZD, HPLC seems to be a method well suited to the determination of BZD in biological samples. This is why numerous methods using HPLC have been published. However, to the best of our knowledge, few of these methods include most of the drugs routinely analysed in a clinical laboratory in a simultaneous programme of determination.

This report describes an HPLC assay for the BZD in biological samples in the reversed-phase mode using a suitable concentration gradient, a rapid-scanning multichannel UV detector and an automated HPLC sample preparation–injection system.

EXPERIMENTAL

Chemicals

HPLC-grade acetonitrile, hexane and methanol were obtained from E. Merck (Darmstadt, F.R.G.). Phosphate buffer used for elution was prepared by dissolving 0.53 g of disodium hydrogen phosphate hexahydrate and 8.93 g of potassium dihydrogen phosphate in a final volume of 1 l of tri-distilled water. The pH was brought up to 5.40 with phosphoric acid.

The extracting solvent was acetonitrile–0.1 *M* dipotassium hydrogen phosphate (10:90), pH 9.

Apparatus

A Varian 5000 high-performance liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) was used. Samples were prepared and introduced by means of an Advanced Automated Sample Processor (AASP) system from varian. The AASP integrates solid-phase extraction using bonded-silica sorbents with automated, on-line syringeless injection of extracted samples into the liquid chromatograph. Bonded-silica sorbent cassettes used for sample purification and concentration were C_2 (20×2 mm I.D.) AASP cartridges (Varian). The separations were performed on a 300×4.6 mm I.D. μBondapak 5-μm reversed-phase column (Waters). The detection was carried out with a photodiode array spectrophotometric detector, LKB Model 2040, connected to an IBM PC microcomputer and a Cannon graphic plotter. The program used for calculating the results was the Wavescan program (LKB).

Chromatographic parameters

The mobile phase flow-rate was set to 0.7 ml/min. The gradient elution components were acetonitrile and phosphate buffer pH 5.40; the gradient programme is given in Table I.

TABLE I

GRADIENT PROGRAMME

Step	Acetonitrile (vol. %)	Phosphate buffer pH 5.40 (vol. %)	Time (min)
1	38	62	0
2	38	62	15
3	70	30	22
4	70	30	40
5	38	62	45

Standards

All pure drugs used were kindly donated by Roche (Neuilly, France), Cassenne (Paris, France), Upjohn (Kalamazoo, MI, U.S.A.), Roussel (Paris, France), Clin Midy (Paris, France) and Warner-Lambert Labs. (Morris Plains, NJ, U.S.A.). Stock solutions were prepared by weighing 10 mg of each compound into a 100-ml volumetric flask and dissolving it in methanol. Working solutions containing 10 μg/ml were obtained by dilution of the stock solution in distilled water. Prazepam was used as an internal standard (I.S.) and was prepared in the same way.

Extraction procedure

Volumes of 50 μl of biological sample, 500 μl of I.S. and 300 μl of extraction buffer (pH 9) were passed through the C_2 AASP cartridge. A wash was then performed with 1 ml of extraction buffer, and the cartridge was loaded into AASP LC Module for on-line elution and analysis.

Calibration curves

Calibration curves were obtained by plotting the ratio of the maximum UV absorbance of each BZD to that of the I.S. as a function of the amount of BZD added to the human sample control. The concentration of BZD in the unknown samples was calculated by interpolation from the standard curve.

RESULTS

Chromatography

Fig. 1 shows a chromatogram from a spiked plasma (1 μg/ml for each drug).

Recovery

To test the efficiency and reproducibility of the extraction procedure, absolute recoveries were performed from plasma in quadruplicate (Table II). The recovery ranged from 92 to 104% and was independent of the concentration.

Precision

Table III shows the precision of the method. The only drugs with a relatively poor day-to-day precision [coefficient of variation (C.V.) 12.0 and 14.2%] are bromazepam and oxazepam because of their poor stability. To compensate for

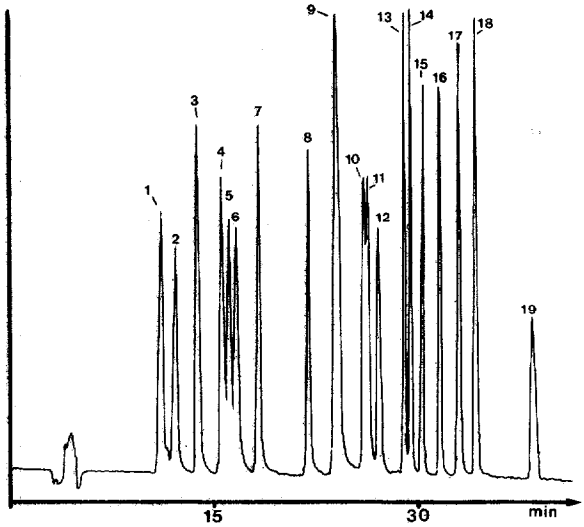

Fig. 1. Chromatogram of a plasma extract containing 1 μg/ml of each drug. Peaks: 1 = bromazepam; 2 = oxazepam; 3 = estazolam; 4 = nitrazepam; 5 = chlordiazepoxide; 6 = triazolam; 7 = clonazepam; 8 = chlorazepate; 9 = desmethyldiazepam; 10 = tofizopam; 11 = clobazam; 12 = flunitrazepam; 13 = diazepam; 14 = loflazepate; 15 = desalkylprazepam; 16 = clotiazepam; 17 = tetrazepam; 18 = prazepam; 19 = medazepam.

their high C.V. we ran biological samples with bromazepam or oxazepam twice and used the mean value as a result, when the two results were inside our within-day C.V.

Linearity

A linear response to the UV detector was obtained for up to 20 mg/l.

Detection limit

The detection limit was dependent on the quality and the age of the column used. Based on a signal-to-noise ratio of 3:1, and with new, high-quality columns,

TABLE II

RECOVERIES OF THE DRUG FROM PLASMA ($n = 4$)

Compound	Concentration added (mg/l)	Recovery (%)	C.V. (%)
Diazepam	1.0	98.5	2.3
Desmethyldiazepam	0.5	101.6	1.9
Oxazepam	1.0	97.2	3.4
Chlordiazepoxide	1.0	103.8	2.1
Triazolam	0.1	97.9	2.2
Flunitrazepam	0.1	101.2	2.8
Clonazepam	0.1	96.4	1.9
Prazepam	1.0	98.6	3.2
Clobazam	1.0	92.3	2.3
Estazolam	0.1	99.4	2.2

TABLE III

PRECISION OF THE METHOD

$n = 17$ in every case.

Compound	Concentration added (mg/l)	Mean concentration found (mg/l)	C.V. (%)
Day-to-day			
Diazepam	1.0	0.97	5.0
Desmethyldiazepam	0.5	0.54	4.4
Chlordiazepoxide	1.0	1.08	2.4
Flunitrazepam	0.1	0.12	6.7
Triazolam	0.1	0.09	7.8
Bromazepam	0.5	0.40	12.0
Oxazepam	1.0	0.92	14.2
Prazepam	1.0	0.98	7.2
Estazolam	0.1	0.10	3.1
Within-day			
Diazepam	1.0	1.08	3.8
Desmethyldiazepam	0.5	0.57	4.6
Chlordiazepoxide	1.0	0.92	2.1
Flunitrazepam	0.1	0.12	5.2
Triazolam	0.1	0.10	4.3
Bromazepam	0.5	0.42	7.1
Oxazepam	1.0	0.94	6.5
Prazepam	1.0	1.04	5.7
Estazolam	0.1	0.11	1.9

this limit was ca. 3 ng/ml in urine and 5 ng/ml in other biological fluids.

Selectivity

Retention times and maximum absorption levels of 21 BZD or metabolites and other compounds which are also extracted by this method are shown in Table IV. Even though retention times were nearly the same for some of them (bromazepam/demethylchlordiazepoxide, nitrazepam/chlordiazepoxide and tofisopam/clobazam), these drugs could still be easily identified by their different UV spectra.

DISCUSSION

With the assay procedure described, most of the commonly used BZD and their major therapeutically active metabolites can be measured in biological fluids. Several papers have described rapid methods that involve isocratic elution [24–26], but their purpose was to measure one or only a few compounds. Indeed, according to these methods, it is not possible to have weakly polar drugs, such as diazepam or medazepam, and very polar drugs, such as bromazepam or oxazepam, on the same chromatogram. The concentration gradient reported here makes it possible not only to group all the compounds on a single chromatogram, but also to have well resolved and separated chromatographic peaks. This resolution was

TABLE IV

RETENTION TIMES AND MAXIMUM UV ABSORPTION LEVELS FOR SOME COMPOUNDS EXTRACTED BY THE METHOD

Compound	Retention time (min)	Maximum UV absorption (nm)
Bromazepam	12.25 + 0.08	233
Demethylchlordiazepoxide	12.60 + 0.07	258
Oxazepam	13.14 + 0.10	228
Estazolam	14.32 + 0.09	227
Alprazolam	16.25 + 0.09	221
Nitrazepam	16.54 + 0.14	216, 257
Chlordiazepoxide	17.16 + 0.04	261
Triazolam	17.52 + 0.11	221
Clonazepam	19.17 + 0.08	215, 308
Chlorazepate	23.15 + 0.18	222
Desmethyldiazepam	25.17 + 0.10	227
Tofisopam	27.13 + 0.12	234, 307
Clobazam	27.29 + 0.08	230
Flunitrazepam	28.05 + 0.16	218, 250
Diazepam	29.25 + 0.08	240
Loflazepate	30.35 + 0.07	229
Desalkylprazepam	31.30 + 0.12	226
Clotiazepam	32.37 + 0.09	210, 241
Tetrazepam	34.05 + 0.19	226, 254
Prazepam	35.29 + 0.05	226
Medazepam	40.01 + 0.09	229

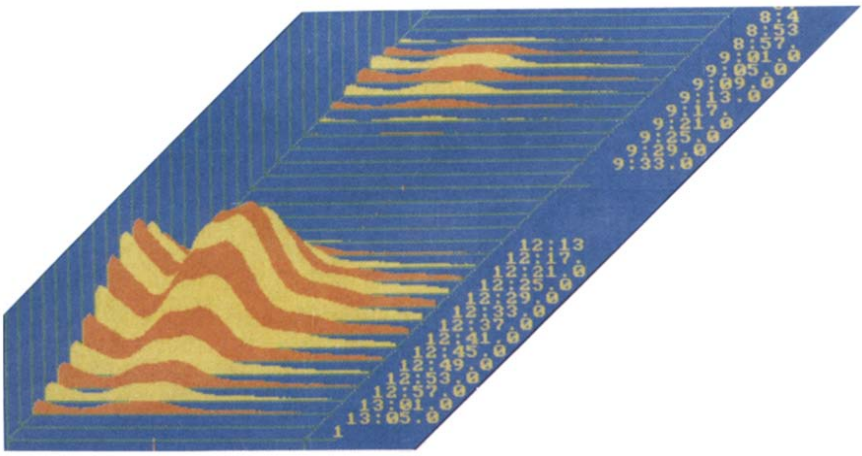

Fig. 2. Three-dimensional (A, λ, t) chromatogram presenting sequential spectra captured at 4.0-s intervals during elution. Plasma extract from a chlordiazepoxide-treated patient containing chlordiazepoxide (2.46 μg/ml) and demethylchlordiazepoxide (0.23 μg/ml).

particularly good for the most strongly retained BZD; e.g., the numbers of theoretical plates calculated for diazepam and prazepam were, respectively, 175 000 and 162 000.

A good reproducibility of retention times was made possible by the use of a high buffering capacity in the mobile phase, with a standard deviation (S.D.) ranging from 0.04 to 0.19.

Since prazepam is rapidly metabolized in the stomach, it is not present in the blood or in the urine in its unchanged form. Moreover, it is well separated on the chromatogram, so we selected it as the internal standard for this assay.

Liquid–liquid extraction is used by the majority of clinical laboratories, but it is time-consuming and does not offer good recoveries. Liquid–solid extraction is becoming increasingly preferred [24, 27,28] because of its ease of use, low sample elution volume and the good recoveries. However, traditional liquid–solid procedures do not eliminate the evaporation step of the elution solvent, which is often a cause of loss of the compound. The use of an automated HPLC sample preparation–injection system appears to be the best solution since it injects the isolated sample into the liquid chromatograph. In the development of our present assay method, various bonded-silica phases were evaluated. C_8 and C_{18} bonded silicas led to a slight tailing on the chromatographic peaks. This phenomenon was not observed with C_2 bonded-silica phases.

The method is highly selective within the BZD group. Since patients treated with BZD frequently receive other psycho-active drugs, the clinical usefulness of any method for monitoring BZD will largely be determined by the degree of freedom from interference by these drugs. In our assay, barbiturates, caffeine (often present in human biological samples) and theophylline were discarded by the extraction step since they are not retained on the C_2 cartridge at pH 9. Other drugs, such as tricyclic antidepressants or phenothiazines, could be present on the chromatogram, but the differentiation is possible either by retention times or by UV spectral studies, which are very dependent on compound type. Coeluting compounds may not be detectable by UV analysis because of a vast difference in molar absorptivity or because of similar UV spectra. Therefore, we have not found any compound eluting at the same time as one of the BZD studied. A major limitation of conventional UV–visible detectors is that only one wavelength resolution element is registered at a time. To a certain degree this disadvantage can be overcome by the use of a scanning spectrophotometer in the stop-flow mode. More convenient, however, are the rapid-scanning spectrophotometric detectors based on a linear photodiode array, since spectral data in digital form can be readily stored in computer memory and manipulated by a number of digital techniques, including presentation of chromatograms at a number of observation wavelengths and spectral deconvolution of overlapping chromatographic peaks. Although an apparatus of this sort is more expensive than a conventional UV detector, it can be very helpful in characterizing the drugs in question with greater specificity.

The extraction procedure can be performed in 5 min and chromatography takes ca. 45 min.

The described method has been successfully used in our laboratory for moni-

toring clinical and forensic cases, as well as in fundamental research [29]. As an illustration, a chromatogram obtained by analysing plasma from a patient undergoing anxiolytic therapy is shown in Fig. 2. The plasma was collected 2 h after oral administration of 10 mg of chlordiazepoxide. The concentrations of chlordiazepoxide and of its main metabolite, desmethylchlordiazepoxide, were 2.46 and 0.23 μg/ml, respectively.

REFERENCES

1 R.I. Shader and D.J. Greenblatt, Am. J. Psychiatry, 134 (1977) 652.
2 J.W. Lance and M. Antony, Arch. Neurol., 34 (1977) 14.
3 L.O. Randall, G.A. Heise, W. Schallek, R.E. Bagdon and W.B. Abrams, Curr. Ther. Res., 3 (1961) 405.
4 E. Stepanski, F. Zorick, M. Kaffeman, J. Sicklesteel and T. Roth, Nouv. Presse Med., 11 (1982) 2987.
5 P.L. Morselli, P. Deniker, C. Radonco-Thomas and A. Villeneuve (Editors), Neuropsychopharmacology, Pergamon, Oxford, 1978, pp. 877–888.
6 E. Deisenhammer and R. Sommer, Wien Klin. Wochenschr., 9 (1979) 571.
7 R.C. Kelly, R.M. Anthony, L. Krent, W.L. Thompson and I. Sunshine, Clin. Toxicol., 14 (1979) 445.
8 D.N. Bailey, Clin. Toxicol., 22 (1984) 433.
9 A.T. Howarth and G. Clegg, Clin. Chem., 24 (1978) 804.
10 J. Straughan, W.F. Cathcart-Rake, D.W. Shoeman and D.L. Azarnoff, J. Chromatogr., 146 (1978) 473.
11 T. Inoue and T. Niwaguchi, J. Chromatogr., 339 (1985) 163.
12 R.G. Lister, D.R. Abernethy, D.J. Greenblatt and S.A. File, J. Chromatogr, 277 (1983) 201.
13 J.E. Wallace, H.A. Shwertener and E.L. Shimek, Clin. Chem., 25 (1976) 1296.
14 C. Violon and A. Vercruysse, J. Chromatogr, 189 (1980) 94.
15 C. Violon, L. Pessemier and A. Vercruysse, J. Chromatogr., 236 (1982) 157.
16 S. Bouquet, P. Aucouturier, A.M. Brisson, P. Courtois and J.B. Fourtillan, J. Liq. Chromatogr., 6 (1983) 301.
17 G.L. Lensmeyer, C. Rajani and M.A. Evenson, Clin. Chem,. 28 (1982) 2274.
18 J.L. Tomasini, H. Bun, P. Coassolo, C. Aubert and J.P. Cano, J. Chromatogr., 343 (1985) 369.
19 R.N. Gupta, J. Chromatogr., 340 (1985) 139.
20 A.K. Dhar and H. Kutt, J. Chromatogr., 222 (1981) 203.
21 M.S. Greaves, Clin. Chem., 20 (1974) 141.
22 P. Coassolo, C. Aubert and J.-P. Cano, J. Chromatogr., 274 (1983) 161.
23 P. Coassolo, C. Aubert and J.-P. Cano, J. Chromatogr., 338 (1985) 347.
24 P. Heizmann, R. Geschke and K. Zinapold, J. Chromatogr., 310 (1984) 129.
25 R.J. Perchalski and B.J. Wilder, Anal. Chem., 50 (1978) 554.
26 H.L. Komiskey, A. Rahman, W.P. Weisenburger and W. Silvius, J. Anal. Toxicol., 9 (1985) 131.
27 P.M. Kabra and E.U. Nzekwe, J. Chromatogr., 341 (1985) 383.
28 A.P. Pakuts, R.H. Downie and T.I. Matula, J. Liq. Chromatogr., 6 (1983) 2557.
29 M. Barrière, A. Piriou, C. Tallineau, P. Mura and O. Pourrat, Toxicol. Lett., 19 (1983) 287.