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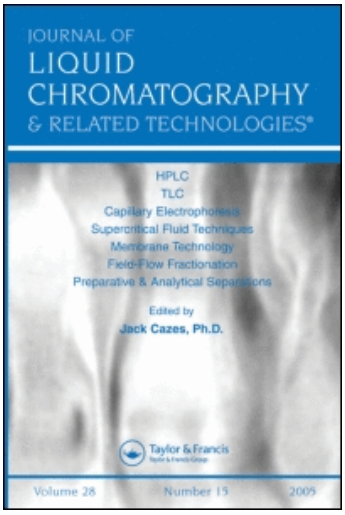

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Simultaneous Determination of Diazepam and its metabolites in Plasma by High-Performance Liquid Chromatography

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SIMULTANEOUS DETERMINATION OF DIAZEPAM AND ITS METABOLITES IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A reversed phase high-performance liquid chromatographic method (HPLC) for the simultaneous determination of diazepam and its three active metabolites, nordazepam, oxazepam and temazepam, in plasma was proposed. The compounds were isolated by solid-phase extraction. The chromatographic mobile phase was metanol-water (55:45, v/v) at a flow rate of 1 mL/min. UV detection was performed concurrently at 240 and 254 nm.

INTRODUCTION

Since the introduction of chlordiazepoxide in 1960 and diazepam a year later into psychiatric drug therapy and General Medicine, the benzodiazepines (BZD)

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have become the most widely employed psychoactive drugs (1-4). The reasonable tolerance to them, easy administration by diverse routes, low cost and low toxicity have led to a steady increase in their abuse, accidents with children and use by the drug-dependent population.

Diazepam and its metabolites are usually assayed by chromatography. Gas-Liquid Chromatography (GLC),via different detection systems, column packings and/or extraction procedures, is a versatile technique in the analytical-toxicological investigation of these drugs (5-8). High-Performance Liquid Chromatography (HPLC) also offers the advantages of easy sample handling and volatile derivatives are not required for analysis. Separation and assay of BZD's and their hydrolysis products have been performed using various HPLC techniques (9-13). UV Spectrophotometry is the most widely used detection method for these compounds.

This paper describes the application of reversed-phase HPLC for the simultaneous determination of diazepam and its three active metabolites in plasma which was pre-treated by a solid-phase extraction process in RP-18 columns.

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MATERIALS

Drug Standards:

Diazepam and Nordazepam were supplied by Roche S.A. (Madrid, Spain), Temazepam by Europharma (Madrid, Spain), Oxazepam by Alonga S.A.(Madrid,Spain) and Carbamazepine, used as internal standard, by Geigy (Barcelona, Spain). The concentration of BZD and carbamazepine standard solutions were 1 mg/mL of methanol. The working solutions (10 μg/mL) were prepared by adding O.1 mL of standard solution to 10 mL of mobile phase.

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Reagents:

Methanol was HPLC grade and obtained from Merck. Sodium carbonate was reagent grade and also obtained from Merck. Milli Q distilled water.

Apparatus:

Waters HPLC system comprising manual inyector, 125 x 4 mm stainless steel analysis column packed with 5 μm Lichrospher 100 RP-18 (Merck), Waters 501 pump, Waters 490 programmable multiwavelength UV detector, Professional 350 Digital Computer (for recording) and Interface Module.

As mobile phase, Methanol-Milli Q water mixture (55:45, v/v) was pumped isocratically at 1 mL/min and a pressure of 2,000-2,200 psi. UV detection was carried out at 240 and 254 nm simultaneously.

METHOD

Calibration procedure

BZD's isolated: The BZD working solutions were diluted appropriately to give three series of solutions of concentrations 0.1, 0.2, 0.5, 1.0, 1.5, 2.0 and 2.5 µg/mL of mobile phase (therapeutic range), to which carbamazepine was added as internal standard (1 µg/mL). Samples (25 µL) of each solution were injected into the liquid chromatograph. Mean calibration curves were plotted as the peak-area ratio (BZD/carbamazepine) against BZD concentration.

BZD's in mixture: In the same way three series of solutions containing a mixture of all four BZD's studied were prepared. Samples (25 µL) were injected into the liquid chromatograph and mean calibration curves were obtained as above.

Extraction procedure

Samples of drug-free plasma were mixed with appropriate aliquots of the BZD working solutions to give solutions containing either diazepam or one of its three metabolites of concentration 1 µg/mL. Solid-phase extraction of the BZD's was performed using an Adsorbex SPU-Sample Preparation Unit and a 100 mg RP-18 column (Merck). The column was activated by washing with 2 x 1 mL of methanol followed by 2 x 1 mL of water. 100 µL of

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0.1 M Na CO_3 was then added. A BZD-spiked plasma sample (0.5 mL) was applied to the column, washed with 2 x 1 mL of water and then dried by passing methanol (50 µL) through it. The BZD's were eluted by successive addition of 200 and 100 uL of methanol and evaporated to dryness under a stream of Nitrogen. The dried extract was dissolved in 250 µL of internal standard solution (1 µg/mL of mobile phase). Finally, a sample (25 µL) was injected into the chromatograph. The sample would contain 0.050 µg of BZD if extraction were 100%.

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The appropriate mean calibration curve was used to calculate the amount of BZD extracted and hence, by comparison with the theoretical amount , the recovery.

For simultaneous assay of diazepam and its metabolites, solutions in drug-free plasma were prepared containing 1 µg/mL and 2 µg/mL of each BZD and subjected to the extraction procedure previously described. At the first case redissolution of the dried extract and sample injection were carried out as for the BZD's isolated. At the second case the dried extract was redissolved in 400 µL of the carbamazepine solution (1 µg/mL) and a sample (25 µL) was injected into the chromatograph. If extraction were complete the sample would contain 0.0625 µg of each BZD.

Recoveries were calculated as already described, using the calibration curves plotted for the four compounds studied dissolved together in mobile phase.

The reproducibility of the method was studied by making replicate measurements in four plasma samples of each BZD obtained by the extraction technique described (BZD's isolated , 1 µg/mL; BZD's in mixture, 1 and 2 µg/mL). Coefficients of variation were calculated.

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RESULTS AND DISCUSSION

The HPLC system described gave a good separation of diazepam and its metabolites with retention times of 13.30 min (diazepam), 10.53 min (nordazepam), 7.92 min (temazepam) and 6.59 min (oxazepam). Carbamazepine was chosen as the internal standard because it gave a good response at the working wavelengths and did not interfere in the analysis of the compounds (4.65 min retention time).

The regression data obtained for the four drugs, studied individually and simultaneously, at the two wavelengths (240 and 254 nm) are shown in Table 1. In the range studied (0.1-2.5 µg/mL) linear correlation was always good between the peak-area ratios

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TABLE 1. Regression Equations of the BZD studied (Range of Concentrations: 0.1-2.5 μg/mL)

COMPOUND	WAVELENGTH (nm)	REGRESSION EQUATION	CORRELATION COEFFICIENT
D(isolated)	240	Y = 1.834 X + 0.0135	0.998
	254	Y = 2.060 X - 0.0672	0.999
D(in mixture)	240	Y = 1.081 X + 0.0106	0.998
	254	Y = 2.041 X + 0.0593	0.999
N(isolated)	240	Y = 1.546 X - 0.0331	0.999
	254	Y = 2.074 X + 0.0096	0.999
N(in mixture)	240	Y = 1.451 X + 0.0612	0.994
	254	Y = 1.961 X + 0.0619	0.999
T(isolated)	240	Y = 1.184 X - 0.0026	0.998
	254	Y = 1.529 X - 0.0041	0.999
T(in mixture)	240	Y = 1.195 X - 0.0149	0.999
	254	Y = 1.540 X + 0.0254	0.999
O(isolated)	240	Y = 0.407 X + 0.0019	0.999
	254	Y = 0.577 X - 0.0047	0.997
O(in mixture)	240	Y = 0.399 X - 0.0111	0.998
	254	Y = 0.568 X + 0.0261	0.998

D.Diazepam; N.Nordazepam; T.Temazepam; O.Oxazepam.

(BZD/carbamazepine) and the concentrations of each compound.

Responses to the BZD's individually and simultaneously, for the same concentrations, correlated strongly as shown by the correlation coefficients and the analysis of variance of the regression (Table 2).

The results obtained on a blank plasma extract showed the practical absence on interfering substances. Four replicate extractions were performed on each of the four individual BZD-spiked plasma solutions (1 μg/mL). The mean recoveries obtained were acceptable in the case of diazepam, nordazepam and temazepam and lower in the case of oxazepam (Table 3). Lastly, extraction was performed on four solutions of plasma spiked (2 μg/mL) with all four BZD's, obtaining individual chromatograms (Figure 1). The calculated mean recoveries had similar values to those achieved for BZD's individually (Table 3).

Reproducibility of the method was acceptable, as shown by mean coefficients of variation less than 3.5%.

Good linearity was obtained at both working wavelenghts; nevertheless, 240 nm was considered as the best of the two since the sensitivity of the method was

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TABLE 2. Correlation between Responses Obtained for the Single BZD and in Mixture (Range of Concentrations: 0.1-2.5 μg/mL)

COMPOUND	WAVELENGTH (nm)	REGRESSION EQUATION	CORRELATION COEFFICIENT	F	α
Diazepam	240	Y = 0.981 X - 0.0232	0.999	3404.79	0.000
	254	Y = 0.985 X - 0.0040	0.998	1624.32	0.000
Nordazepam	240	Y = 0.975 X + 0.0148	0.999	7659.73	0.000
	254	Y = 0.959 X + 0.0543	0.999	9721.52	0.000
Temazepam	240	Y = 1.008 X - 0.0091	0.999	3785.33	0.000
	254	Y = 1.020 X + 0.0297	0.999	73,349.42	0.000
Oxazepam	240	Y = 0.975 X - 0.0129	0.999	3875.78	0.000
	254	Y = 0.943 X + 0.0196	0.999	5765.57	0.000

Y. Ratio of BZD/Carbamazepine areas (in mixture)

X. Ratio of BZD/Carbamazepine areas (singly)

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TABLE 3. Extraction Recoveries of the BZD from Plasma

COMPOUND	CONCENTRATION (μg/mL)	RECOVERIES (%) 1	2	3	4	$\bar{X}$	COEFFICIENT VARIATION(%)
D(isolated)	1	88.4	86.8	87.6	86.2	87.2	1.09
D(in mixture)	1	87.3	89.7	90.0	87.9	88.7	1.50
	2	86.8	90.1	85.3	87.5	87.4	2.29
N(isolated)	1	78.2	79.6	79.0	80.1	79.2	1.03
N(in mixture)	1	82.8	80.5	81.2	84.0	82.1	1.92
	2	82.0	80.7	83.2	85.3	82.8	2.36
T(isolated)	1	76.0	76.9	75.6	75.3	76.0	0.92
T(in mixture)	1	80.2	80.5	76.9	77.8	78.9	2.25
	2	76.0	75.8	78.2	79.1	77.3	2.11
O(isolated)	1	55.9	57.1	55.2	56.0	56.0	1.41
O(in mixture)	1	55.3	58.2	57.6	54.8	56.5	2.97
	2	59.9	57.3	56.0	55.6	57.2	3.39

D.Diazepam; N.Nordazepam; T.Temazepam; O.Oxazepam

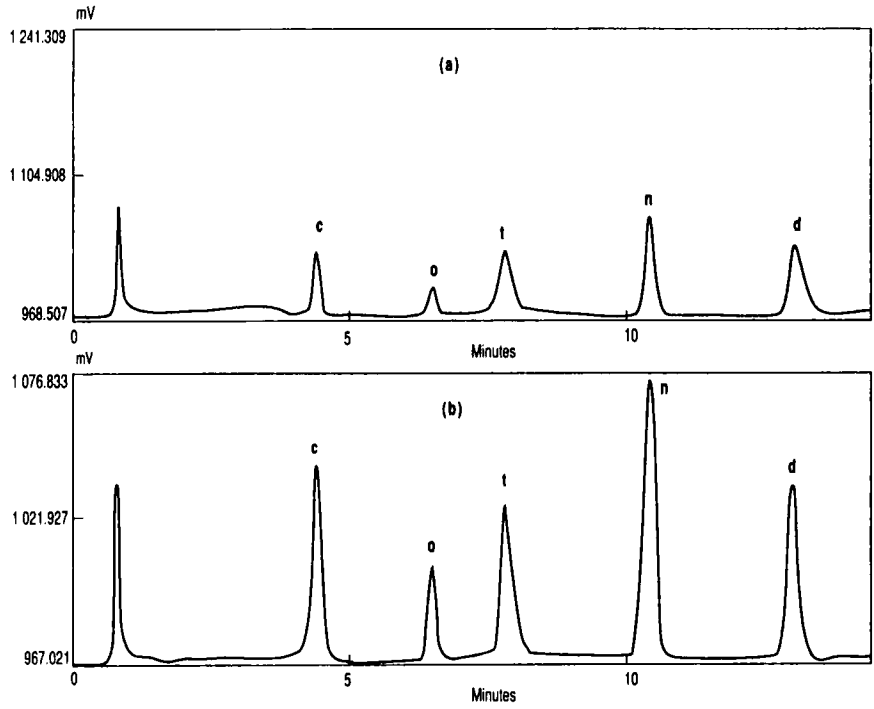

Figure 1: Chromatogram of the BDZ mixture, after extraction of plasma. λ = 240 nm. (a) 1 µg/mL (b) 2 µg/mL
* c: carbamazepine o: oxazepam t: temazepam
n: nordazepam d: diazepam

improved, showing as a greater response in the chromatogram.

In conclusion, the reversed-phase HPLC method proposed is sensitive, rapid, easily reproducible and can be applied to plasma, which makes it a good choice for monitoring patients being treated with BZD's or in the case of diazepine poisoning.

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REFERENCES

1. Rumak,B.H. and Lovejoy,Jr.F.H. Clinical Toxicology In "Casarett and Doull's Toxicology. The basic science of poisons", Klaasen,C.D.; Amdur,M.O.; Doull,J.(Eds.), Macmillan Publ. Co., New York, 1986, p. 887.

2. Proudfoot,A., Intoxicaciones agudas: Diagnóstico y Tratamiento, Ed.Doyma, Barcelona, 1985, p. 75.

3. Ellenhorn, M.J. and Barceloux, D.G., Medical Toxicology. Diagnosis and Treatment of Human Poisoning, Elsevier Science Publ. Co., Inc., New York, 1988, p. 580.

4. Gottschalk,L.A. and Cravey,R.H., Toxicological and pathological studies on psychoactive drug-involved deaths, Biomed. Publ., California, 1980.

5. Karnes,H.T. and Farthing,D., Improved method for the determination of oxazepam in plasma using capillary gas chromatography and nitrogen-phosphorus detection, LC-GC, 5 (11), 978-79 (1987).

6. Drovet-Coassolo, C.; Aubert, C.; Coassolo,P. and Cano,J.P., Capillary gas chromatography-mass spectrometric method for the identification and quantification of some benzodiazepines and their unconjugated metabolites in plasma, J.Chromatogr., 487 (2), 295-311 (1989).

7. Ferguson,J.L. and Couri,D., Electron-capture gas chromatography of benzodiazepines and metabolites, J.Anal.Toxicol., 1 (4), 171-74 (1977).

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8. McAllister,C.B. , Use of internal standards in the estimation of diazepam in plasma, J. Chromatogr., 151 (1), 62-66 (1978).

9. Japp ,M.; Garthwaite, K.; Geeson, A.V. and Osselton,M.D., Collection of analytical data for benzodiazepines and benzophenones, J.Chromatogr., 439, 317-39 (1988).

10. Sohr,C.J. and Buechel,A.T., Separation of parent benzodiazepines and their major metabolites by reverse-phase ion-pair chromatography, J.Anal. Toxicol., 6,286-89 (1982).

11. Lau, C.E.; Dolan, S. and Tang,M., Microsample determination of diazepam and its three metabolites in serum by reversed-phase High-Performance Liquid Chromatography, J.Chromatogr., 416 (1), 212-18 (1987).

12. Klockwoski, P.M. and Levy, G., Simultaneous determination of diazepam and its active metabolites in rat serum, brain and cerebrospinal fluid by HPLC, J.Chromatogr., 422, 334-39 (1987).

13. Bogusz, M. and Aderjan, R., Corrected retention indices in HPLC: Their use for the identification of acidic and neutral drugs, J.Anal.Toxicol., 12, 67-72 (1988).

14. Nordholm,L. and Mengel,H., Determination of an oxadiazole-substituted 1,4-Benzodiazepine in plasma by HPLC with ultraviolet detection and by a radioreceptor assay , J.Chromatogr., 494, 257-65 (1989).

15. StPierre, M.V and Pang ,K.S., Determination of diazepam by High-Performance Liquid Chromatography and Thin Layer Chromatography, J.Chromatogr., 421 (2), 291-307 (1987).

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