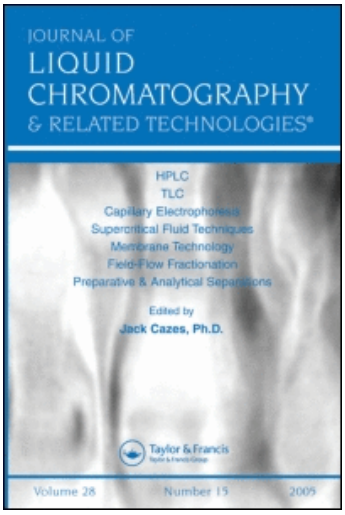

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QUANTITATION OF BENZODIAZEPINE HYDROLYSIS PRODUCTS IN URINE USING SOLID-PHASE EXTRACTION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Solid-phase extraction and high performance liquid chromatography (HPLC) were used to indirectly (via products of acid hydrolysis) confirm the presence of benzodiazepine metabolites in the urine of patients who had received overdoses of these compounds. The use of solid-phase extraction method for quantitation of benzodiazepine hydrolysis products in urine offers numerous advantages in comparison to extraction with chloroform.The chromatograms of urine extracts were free of interferences.The recoveries of the benzodiazepine hydrolysis products and the internal standard were greater than 96%, which makes this method highly reliable for quantitative analytical purposes.

INTRODUCTION

Benzodiazepines are widely used because of their hypnotic, tranquilizing and anticonvulsant properties

and, therefore, considerable interest exists in their detection and determination in body fluids. Numerous methods for the chromatographic analysis of benzodiazepines have already been described; in a handbook by Schutz (1), a book by Piemonte et al (2) and a review by Sioufi (3). Separation of diazepam and oxazepam metabolites in saliva using thin layer chromatography (TLC) was reported by Reszka and Tyfczyńska (4). Application of TLC in the analysis of benzodiazepines and their metabolites in urine have also been described (5). A database for the choice of optimal qualitative and quantitative eluent composition for the toxicological analysis of drugs was given by Matyska and Soczewiński (6). Recently a series of benzodiazepines encountered in forensic samples were separated using isocratic reversed-phase liquid chromatography (7).

In this paper, we report the application of solid-phase extraction for the isolation of benzodiazepine hydrolysis products from urine and determination of these compounds by HPLC.

EXPERIMENTAL

Materials; Table 1 shows list of compounds investigated. Benzodiazepines were obtained from Polfa (Poznań and Tarchomin-Poland). The **MACB, ACB** and **ANB** were kindly donated by Hoffmann-La Roche (Switzerland).

The solvents were of HPLC grade and were obtained from Merck (E.Merck,FRG);all other chemicals were of analytical grade.

Instrumentation; The experiments were carried out using a liquid chromatograph Type 302 (Institute of Physical

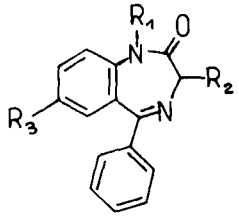
TABLE 1

List of Compounds Investigated

No	Name of compound	Abbreviation	Structure	Substituents
1	Diazepam	DIA	1	R_1 = CH_3 , R_2 = H, R_3 = Cl
2	Oxazepam	OXA	1	R_1 = H, R_2 = OH, R_3 = Cl
3	Temazepam	TEM	1	R_1 = CH_3 , R_2 = OH, R_3 = Cl
4	Clonazepam	CLO	1	R_1 = H, R_2 = H, R_3 = NO_2
5	Chlordiazepoxide	CHL	2	
6	Benzophenone	B	3	R_4 = R_5 = H
7	2-Methylamino-5-chlorobenzophenone	**MACB**	3	R_4 = $NHCl_3$, R_5 = Cl
8	2-Amino-5-chlorobenzophenone	**ACB**	3	R_4 = NH_2 , R_5 = Cl
9	2-Amino-5-nitrobenzophenone	**ANB**	3	R_4 = NH_2 , R_5 = NO_2

STRUCTURES:

1



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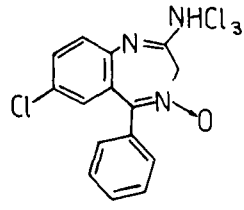

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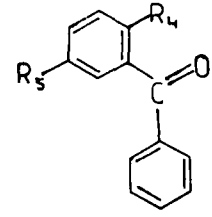

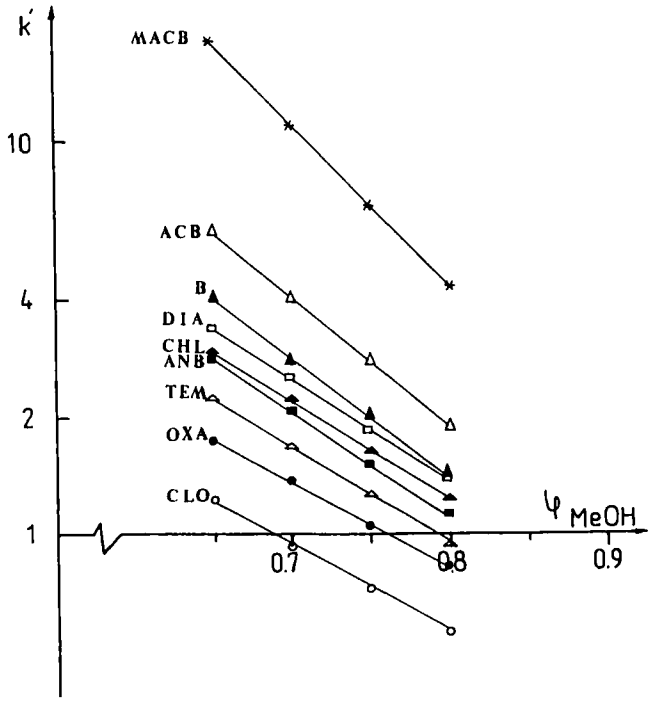

FIGURE 1. Plots of k'(logarithmic scale) vs. volume fraction φ of the modifier (methanol) in potassium phosphate buffer (pH 7.15).For identification of solutes see Table 1.

Chemistry of the Polish Academy of Sciences,Poland) equipped with an UV detector (254nm).A 250 x 4 mm i.d. stainless-steel column was packed with 10 μm LiChrosorb RP-18 (E.Merck).The mobile phase was prepared by mixing methanol and aqueous potassium phosphate buffer (pH 7.15). Before use, the mobile phase was filtered and degassed.

Methods; (a).Hydrolysis.

Samples of urine (20 mL) from patients who had taken benzodiazepines in overdose amounts were first

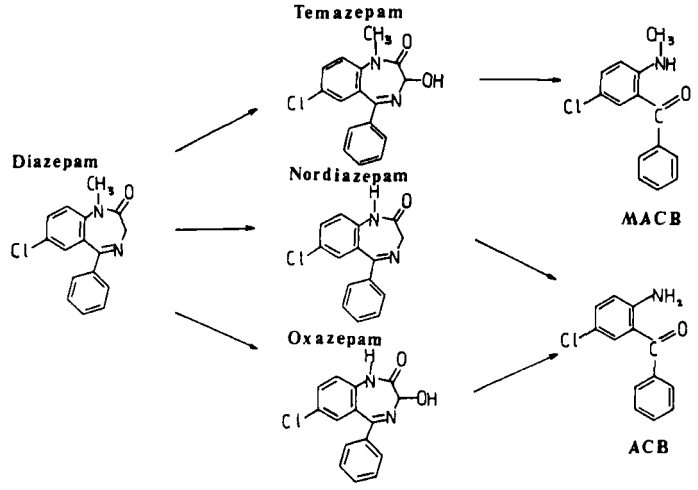

FIGURE 2.Metabolism of diazepam and its hydrolysis products.

hydrolyzed by hydrochloric acid 6 mol/L (1:1) to benzophenones (60 min.at 100 ^{o}C). A reference sample of urine (20 ml) from a healthy man was also hydrolyzed.Each sample was divided into two parts: one for extraction with chloroform and the second for solid-phase extraction.

(b).*Liquid-liquid extraction.*

For the extraction of **MACB** and **ACB** (benzodiazepine hydrolysis products - see Table 1) after hydrolysis 5 mL of ammonium buffer (pH 10.1) was added to 10 mL of sample and the extraction was carried out with chloroform (20 mL). The extract was evaporated under nitrogen flow at 30 ^{o}C to dryness and the residue disolved in 0.2 mL of methanol.

(c).*Solid-phase extraction.*

Extraction of samples of urine (10 mL) after hydrolysis, as well as an artificially prepared mixture

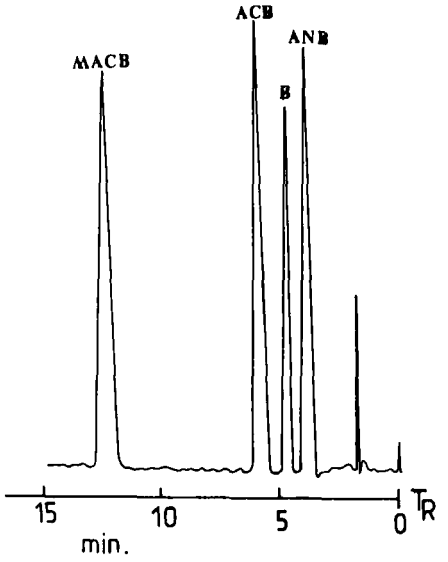

FIGURE 3.Chromatogram of benzodiazepine hydrolysis products and internal standard (benzophenone). Mobile phase: Methanol + potassium phosphate buffer (pH 7.15), 75:25 v/v.Flow rate:1.2 ml/min.For notation of solutes see Table 1.

of benzodiazepine hydrolysis products (**MACB**,**ACB** and **ANB** - 10 mg in 10 mL methanol), was carried out.The columns for solid-phase extraction were packed with octadecylsilanized silica (ZOCh,Lublin,Poland)and were conditioned with 5 mL methanol and 5 mL water.Care must be taken not to let the column run dry.The prepared sample was slowly passed through the column.The column was than air-dried under vacuum for 10 min. A collection tube was placed under the column, which was eluted with 1 mL of methanol.

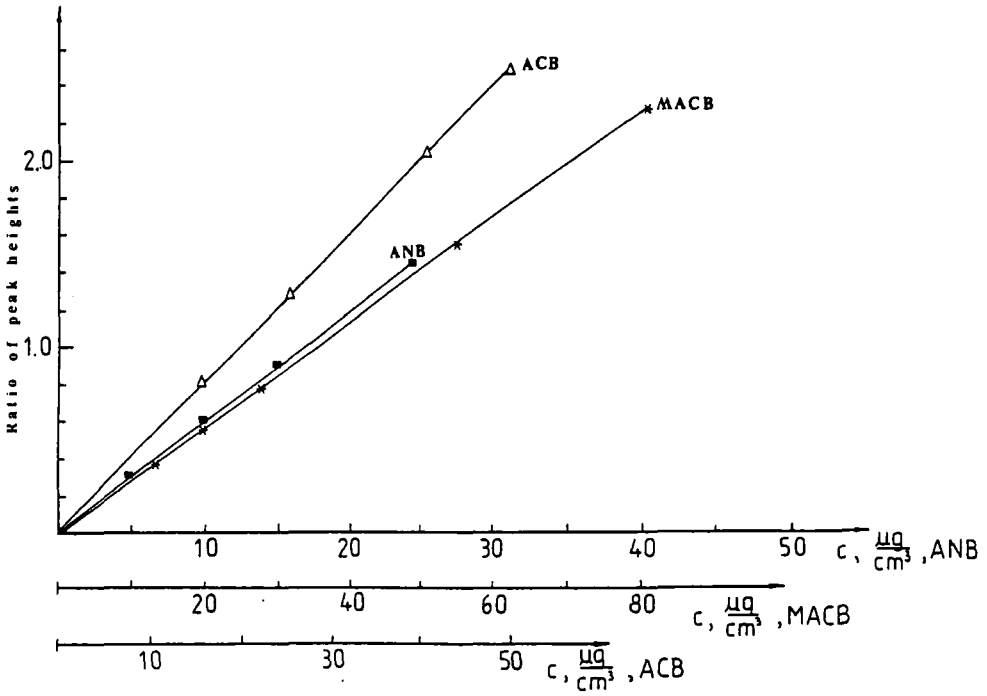

FIGURE 4.Calibration curves for **MACB**,**ACB** and **ANB** (see Table 1) plotted vs. ratio of peak heights.

RESULTS AND DISCUSSION

The isocratic normal-phase separation of the benzodiazepines and their hydrolysis products is shown in Figure 1. Under the conditions of the separation, a wide range of capacity factors for these compounds were obtained.Based on these results, for routine determinations the mobile phase consisting of 75 % v/v methanol in aqueous potassium phosphate

TABLE 2

Recovery of Standards

No	Name of Standard	Water Recovery(%)	Urine Recovery(%)
1	MACB	99.8	97.1
2	ACB	99.2	96.5
3	ANB	99.1	96.3
4	B	99.6	96.9

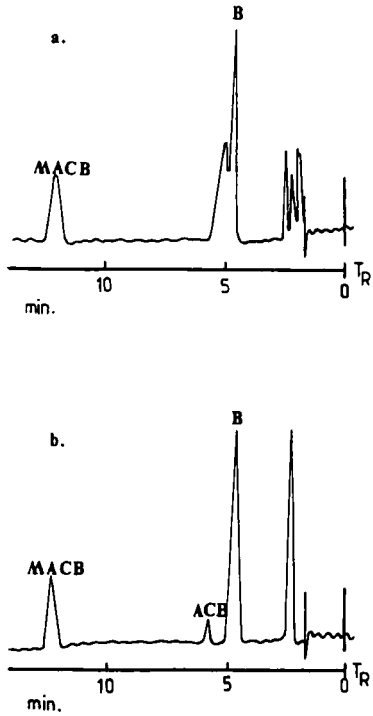

FIGURE 5.(a).Chromatogram after extraction of urine with chloroform.
(b).Chromatogram after solid-phase extraction of urine. Mobile phase for (a) and (b): Methanol + potassium phosphate buffer (pH 7.15), 75:25 v/v.Flow rate: 1.2 ml/min. For notation of solutes see Table 1.

buffer (pH 7.15) was chosen. Figure 2 shows metabolism of diazepam and its hydrolysis products.

The chromatogram obtained for diazepam hydrolysis products and the internal standard is shown in Figure 3

Quantitation of benzodiazepine hydrolysis products from urine using the above procedure was carried out with an internal standard using the ratio of peak heights.Figure 4 shows typical calibration curves for **MACB**,**ACB** and **ANB** plotted vs.the ratio of peak heights.For determination of different levels of these compounds in urine , the curves are linear over a rather wide range of concentrations. The detection limits were for: **ACB**-5.1 ng, **MACB**-10.1 ng, **ANB**-5.3 ng.

Table 2 shows the almost complete recoveries of standards from urine and water after solid-phase extraction.

The separation results when using solid-phase extraction and extraction with chloroform in analysis of urine are given in Figure 5. The chromatogram of urine after solid-phase extraction is free of interferences. Additionally, both peaks of the hydrolysis products of diazepam (**MACB** and **ACB**) were obtained ,whereas with chloroform extraction,only the **MACB** peak is present.

The described procedure has already been utilized for emergency toxicological analyses . The sensitivity and the reproducibility of the results make this method highly reliable for toxicological analysis and drug monitoring.

ACKNOWLEDGEMENTS

Thanks are expressed to the PHARMACEUTICAL COMPANY "**HOFFMANN - LA ROCHE**" in Switzerland for samples of **MACB**, **ACB** and **ANB**.

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