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A RAPID GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF DIAZEPAM AND METABOLITES IN BODY FLUIDS

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SUMMARY

A rapid method is described for the extraction of diazepam and its metabolites from plasma and urine. The procedure is applicable to subsequent analysis by electron capture gas chromatography, and has been used for the analysis of clinical samples. The detection limit for diazepam is about 0.01 μg/ml, using a 2-ml sample. Quantification of lower levels of benzodiazepines requires a sample clean-up procedure, and the method is not suitable for this purpose.

INTRODUCTION

Gas chromatography (GC) is now widely used for the analysis of diazepam and its metabolites in biological fluids. As these compounds are often present in low concentrations, electron capture detectors (ECD) are most frequently used for their quantification.

Earlier methods, dependent on chromatography of the benzophenones following hydrolysis of the parent compounds[1,2], have now been generally superseded by techniques which separate the intact benzodiazepines[3–7]. Various procedures have been reported for extracting the compounds, the most thorough of which involves back extraction from acid to remove lipids and other interfering compounds. The technique is lengthy and somewhat tedious, and though necessary in the analysis of low levels of benzodiazepines, can be shortened when dealing with relatively high concentrations of these compounds[4–6]. These more rapid methods have usually been applied to the assay of diazepam and its metabolites during chronic administration or following overdosage. All of these procedures involve some manipulation of the extracting solvent, and it seemed appropriate to develop a method, using ECD, which would involve only a very simple extraction procedure, and as far as possible avoid clean-up and solvent transfer steps. One approach to this problem which seemed attractive was that used by Ramsey and Campbell[8] in their method for amphetamines, which makes use of a small volume of chloroform as the extracting solvent. In the present work, halogenated solvents could not be

used, being incompatible with the ECD. Ethyl benzoate was selected as a suitable solvent, being dense, having low water solubility, and giving rise to a relatively small ECD response.

EXPERIMENTAL

Apparatus

The entire extraction procedure is carried out in a 10-ml finely tapered centrifuge tube. All glassware should be silanised with a 2% v/v solution of hexamethyldisilazane in petroleum ether (b.p. 60–80 °) prior to use.

Reagents

Benzodiazepines were obtained from the following sources: diazepam, N-desmethyldiazepam and 3-hydroxydiazepam from Hoffman-La Roche (Basle, Switzerland), oxazepam from Wyeth (Maidenhead, Great Britain) and prazepam from Warner-Lambert (Eastleigh, Great Britain).

β-Glucuronidase was purchased from Sigma (London, Great Britain). All other reagents were obtained from BDH (Poole, Great Britain) and were of analytical reagent grade when available.

Reagent-grade ethyl benzoate was found to give an acceptably low ECD response, and was used without further clean-up.

Buffer solutions were prepared by dissolving 10 g of sodium acetate in doubly distilled water (100 ml). The pH of this solution was then adjusted to the required value by addition of glacial acetic acid.

Internal standard. A stock solution of 0.1 mg/ml of prazepam in ethyl benzoate was prepared. This was diluted to a working strength of 4 μg/ml as required.

Standard solutions. 0.1 mg/ml stock solutions of diazepam, N-desmethyldiazepam, 3-hydroxydiazepam, and oxazepam were made up in methanol and stored under nitrogen.

Gas chromatography

A Pye 104 gas chromatograph (Pye-Unicam, Cambridge, Great Britain), equipped with a ^{63}Ni ECD, was used. The separation was carried out on a 1 ft × 4 mm I.D. silanised glass column, packed with 3% OV-225 on 60–80 mesh Gas-Chrom Q (Field, Richmond, Surrey, Great Britain) and maintained at 235 °, with a carrier gas flow-rate (nitrogen) of 100 ml/min.

The detector was operated at 320 ° with a purge gas flow-rate of 40 ml/min. The detector pulse width was 150 μsec. Under these conditions retention times were: oxazepam, 0.8 min; diazepam, 1.4 min; prazepam, 2.4 min; N-desmethyldiazepam (Ro 5-2180), 3.5 min; 3-hydroxydiazepam (Ro 5-5345), 7.9 min.

Method

Urine samples. Two millilitres urine are pipetted into a 10-ml stoppered glass centrifuge tube and buffered to pH 5.3 with 2 ml acetate solution. 10 μl β-glucuronidase is added, and the mixture is incubated for 2 h at 37 °. This incubation step is necessary for the hydrolysis of benzodiazepine glucuronide and sulphate conjugates. Extraction is carried out by vortex-mixing for 3 min with 200 μl of the internal standard solution. The mixture is then centrifuged at 1500 *g* for 5

min. It is generally found that the extracting solvent forms a layer in the bottom of the centrifuge tube which is unsuitable for chromatographic analysis due to the presence of solid material in the organic phase. A clear layer can be obtained by gently stirring the organic layer with a fine wire or syringe needle. This causes conglomeration of the solid matter which can then be easily pushed aside. A sample of the organic phase is then drawn into a micro-syringe for injection into the chromatograph.

Plasma samples. The overall procedure with plasma is similar to that for urine, except that the incubation stage is omitted. Vortex-mixing of plasma samples results in the formation of emulsions which are broken by freezing to −78° in dry ice–acetone, and then melting, prior to centrifugation. Emulsion formation can be reduced to some extent by carrying out the extractions on a mixing-wheel, the centrifuge tubes being gently rotated for 15 min. Even with this less vigorous extraction method, the freezing-step is usually required.

RESULTS

Fig. 1 shows calibration curves derived from the extraction of spiked urine samples. It can be seen that linear graphs are obtained from the extraction of oxazepam, Ro 5-2180, and Ro 5-5345. The curve which was obtained for diazepam is due to the use of the ECD outside the linear response range for this compound.

The reproducibility, and hence the accuracy, of this method has been studied by multiple determinations of two solutions of diazepam and its metabolites, in drug-free urine. The results of this investigation are given in Table I. The limits of detection, based upon the extraction procedure outlined above, are given in Table II.

The present method has been used for the assay of plasma samples from psychiatric patients who had been taking oral diazepam for a number of weeks, and in the analysis of blood from persons who had taken an overdose of the drug. A typical chromatogram is shown in Fig. 2, from which it can be seen that the

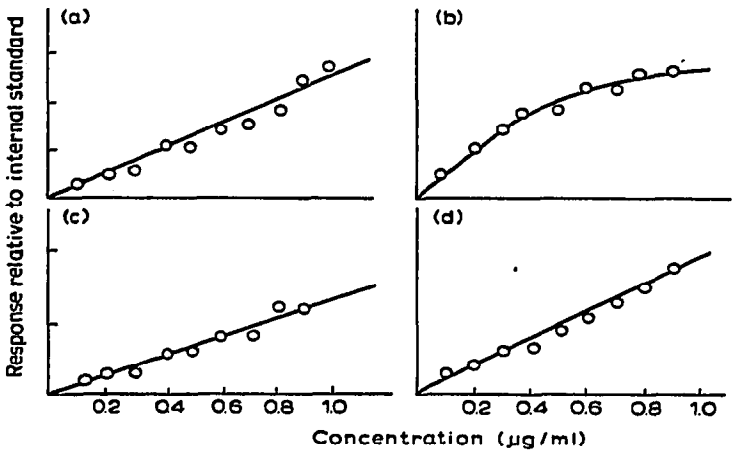

Fig. 1. Calibration curves for diazepam and metabolites following extraction from spiked urine samples. (a) Oxazepam; (b) diazepam; (c) N-desmethyldiazepam; (d) 3-hydroxydiazepam.

solvent response is small and no major peaks are produced by coextracted endogenous constituents.

TABLE I
REPRODUCIBILITY OF EXTRACTION FROM SPIKED URINE SAMPLES

Compound	*Concentration*			
	0.2 μg/ml		*0.8 μg/ml*	
	No. of extractions	*Standard deviation (%)*	*No. of extractions*	*Standard deviation (%)*
Diazepam	7	4.9	5	6.7
Ro 5-2180	6	6.0	5	5.4
Ro 5-5345	7	8.3	5	13.0
Oxazepam	6	8.3	5	11.5
Mean	6.5	6.9	5	9.6

TABLE II
DETECTION LIMITS USING ETHYL BENZOATE EXTRACTION

Compound	*Detection limit (μg/ml)*
Diazepam	0.01
Ro 5-2180	0.03
Ro 5-5345	0.10
Oxazepam	0.04

DISCUSSION

A method has been presanted by which the levels of diazepam and its metabolites in urine, blood, end plasma can be determined rapidly and with acceptable accuracy. Achievement of 100% recoveries has not been the prime goal of this work. Provided that the extraction is reproducible, low recoveries can often be justified by the time-saving involved. It is possible, by adjustment of the pH of the aqueous phase before extraction, to increase the accuracy and detection limits of this procedure. This is considered unnecessary in the present method, the main merit of which is the speed with which it can be performed.

The extraction procedure is applicable to the analysis of diazepam and its metabolites in clinical samples. It is therefore to be expected that the method may also be applicable to the extraction of other benzodiazepines from body fluids. With minor modifications, the method should permit rapid extraction of many other compounds prior to analysis by GC using an ECD. The method is not considered suitable for detection of very low levels of benzodiazepines and quantita-

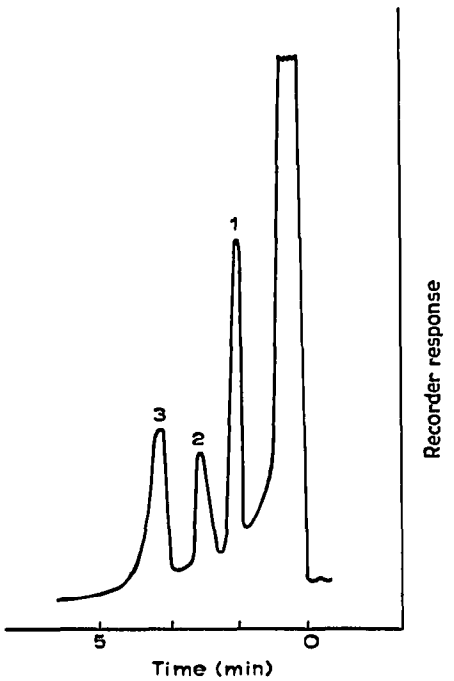

Fig. 2. Typical chromatogram from an extract of a plasma sample. Plasma concentrations: diazepam, 0.16 μg/ml; N-desmethyldiazepam, 0.20 μg/ml. 1 = Diazepam; 2 = prazepam; 3 = Ro 5-2180.

tion of these compounds at levels below those indicated in Table II requires the use of a clean-up procedure[3].

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