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Note

Quantitative gas chromatographic determination of diazepam and its major metabolite in human serum

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The benzodiazepines have, as mentioned in a recent review[1], become increasingly important in clinical practice, and as a result are being misused to a greater extent than in the past. Methods devised for the determination of these drugs should therefore be suitable for both therapeutic and toxic levels, but although a number of methods for the determination of benzodiazepines have been described[2–6], most of them suffer from the disadvantage that they are linear only over a limited range of concentrations and are therefore suitable for the determination only of therapeutic levels. In most of the methods, no internal standard is used and a standard graph must be prepared for every series of determinations. This technique is also liable to errors as daily changes in gas chromatographic conditions, extraction efficiency, etc., are not compensated for. When an internal standard is used, it is important that it should be possible to add it at the start of the extraction procedure. In this way, one ensures that any losses of the drug to be determined are automatically compensated for by an equivalent loss of internal standard. As our department is interested in the determination of drugs at both therapeutic and toxic levels, a method was designed that is sensitive enough to be used to determine diazepam in therapeutic concentrations and is linear over a range of concentrations that also makes it suitable for toxicological application.

MATERIALS AND METHODS

Apparatus

A Varian Aerograph Model 1400 single-column gas chromatograph with a flame ionization detector was used.

Peak areas were determined by means of a Hewlett-Packard Model 3373B integrator. The column used for determinations was a 2-m stainless-steel column packed with 3% SE-30 on Chromosorb W. The column was conditioned at 250° for 24 h after silylation with Silyl 8.

Operating conditions

The column was operated isothermally at 180°, with the injection port and detector at 270°. Nitrogen was used as the carrier gas. The flow-rates of hydrogen and air were optimised so as to obtain maximum peak heights.

Reagents and materials

The following were used: phosphate buffer containing 13.61 g of potassium dihydrogen orthophosphate per 100 ml of water, the pH being adjusted to 6.9 ± 0.1 with 1 *M* dipotassium hydrogen orthophosphate solution; 6 *N* hydrochloric acid; 6 *N* sodium hydroxide solution; bromothymol blue, 0.1 % in methanol; diethyl ether, reagent grade, re-distilled; carbon tetrachloride, analytical-reagent grade; methylamino-5-chlorobenzophenone (MACB), obtained by hydrolyzing diazepam with 6 *N* hydrochloric acid; amino-5-chlorobenzophenone (ACB), obtained by hydrolyzing chlordiazepoxide with 6 *N* hydrochloric acid and purifying the product by column chromatography on silica gel; the internal standard, *o*-aminobenzophenone (*o*-AB), synthesized as described by Scheifele and De Tar[7].

Extraction procedure

To 3 ml of serum in a 30-ml glass-stoppered centrifuge tube were added 5 μg of internal standard in 50 μl of methanol and 6 ml of phosphate buffer. The mixture was extracted three times with 7 ml of diethyl ether by shaking for 2 min on a vortex mixer. The organic phase was separated from the aqueous phase by centrifuging and the ether layers were pooled.

The combined ether layer was extracted with 3 ml of 6 *N* hydrochloric acid by shaking for 2 min on a vortex mixer followed by centrifuging. The ether layer was discarded and the hydrochloric acid extract thoroughly cleaned by washing three times with 10-ml portions of diethyl ether.

The purified hydrochloric acid extract was placed in a glycerol bath at 40° in order to remove the ether. The temperature was then increased to 100° and the centrifuge tubes were stoppered, and kept at this temperature for 1 h.

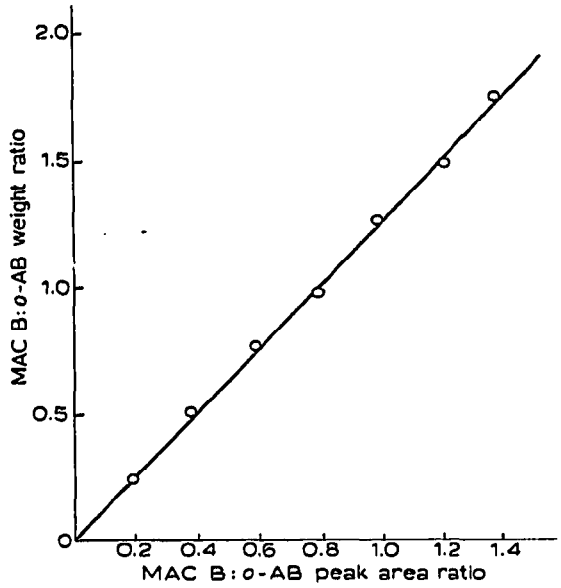

Fig. 1. MACB: *o*-AB weight ratio *versus* peak area ratio.

TABLE I

RECOVERY OF DIAZEPAM FROM SERUM

Amount added (μg per 100 ml)	*Amount recovered (μg per 100 ml)*	*Mean ± S.D. (μg per 100 ml)*
10	10.79, 10.44, 10.44, 10.09	10.44 ± 0.08
33	33.64, 32.45, 34.57, 34.57	33.82 ± 1.0
100	99.76, 103.24, 98.30, 98.30	99.98 ± 2.3

After cooling, the reaction mixture was transferred to a conical centrifuge tube. Two drops of a bromothymol blue indicator solution were added and, while maintained at *ca.* 10° in an ice-bath, the solution was neutralized with 6 *N* sodium hydroxide solution till the colour of the indicator changed from red to blue.

To the neutral mixture, 100 μl of carbon tetrachloride were added and the benzophenones extracted by shaking for 3 min on a vortex mixer and centrifuging to separate the layers. A 5-μl volume of this extract was injected into the gas chromatograph.

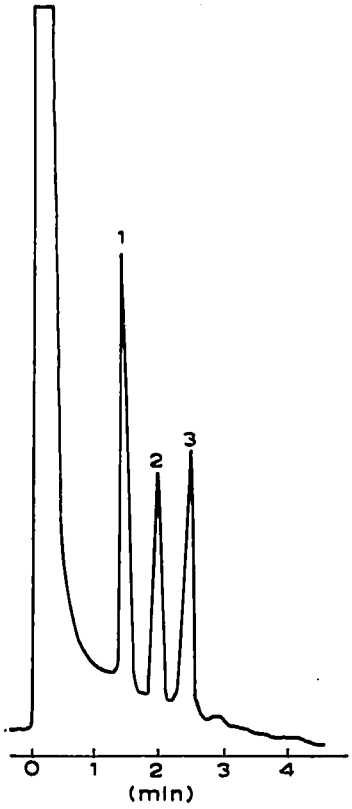

Fig. 2. Chromatogram of an extract of a patient's serum.

Standardization

Standardization of the method was carried out by taking 3-ml portions of normal serum and adding to it the internal standard and concentrations of diazepam between 10 and 400 μg per 100 ml. These serum fractions were then extracted as described above, analyzed and a graph was constructed of MACB: *o*-AB weight ratio *versus* MACB: *o*-AB peak area ratio (Fig. 1). A summary of the results for the recovery of diazepam obtained with serum in the range between 10 and 100 μg per 100 ml added to the serum is presented in Table I.

RESULTS AND DISCUSSION

The method for the determination of diazepam and its major metabolite, N-demethyldiazepam, is based on the technique described by De Silva *et al.*[2]. Benzodiazepines are extracted and hydrolyzed to their corresponding benzophenones (MACB from diazepam and ACB from the metabolite). These benzophenones are then analyzed by gas chromatography.

We found that the extraction time of 10 min described by De Silva *et al.*[2] could be reduced to 2 min without any loss in either recovery or reproducibility.

The internal standard *o*-aminobenzophenone was chosen because it closely resembles the hydrolysis products MACB and ACB in structure and because it appears at a convenient position in the chromatogram (Fig. 2).

Standard serum curves were constructed by plotting the MACB: *o*-AB peak area ratio *versus* MACB: *o*-AB weight ratio for standard samples that were run simultaneously with the unknown samples. The peak area ratio of the unknown sample was then used in order to determine the weight of MACB which, in turn, was converted into weight of diazepam by multiplying by 1.16.

Quantitation of the metabolite was achieved by preparing a series of solutions containing MACB and ACB in a known weight ratio. These solutions were then analyzed gas chromatographically and the detector response ratio for the various weight ratios was determined. A graph was then constructed of MACB: ACB weight ratio

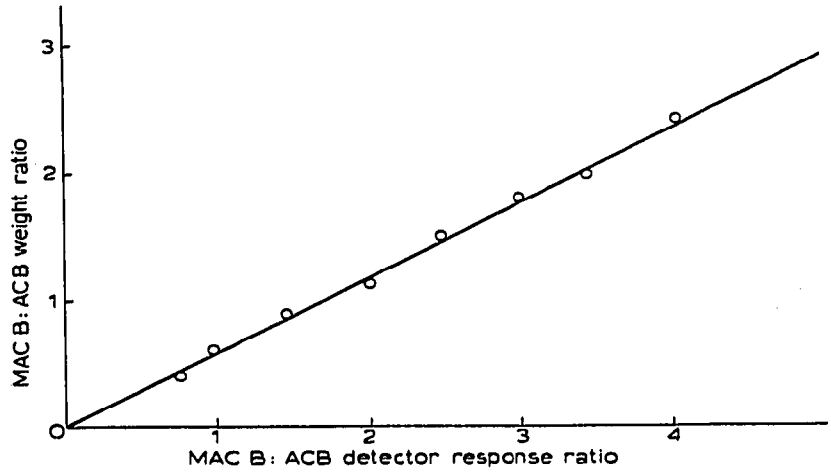

Fig. 3. MACB: ACB detector response ratio *versus* weight ratio.

versus MACB : ACB detector response ratio (Fig. 3). The concentration of the metabolite in an unknown sample was determined by relating the peak area ratios from the gas chromatograph to the weight ratio. As the weight of MACB was already known, the weight of ACB could be determined and converted into weight of N-demethyldiazepam by multiplying by 1.50.

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