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A method for gas chromatographic determination of benzodiazepines

It has been recently reported by RUELIUS *et al.*[1] and by SCHWARTZ *et al.*[2], that diazepam in man and dog undergoes N-demethylation and C_3-hydroxylation. The metabolites produced were characterized by SCHWARTZ *et al.*[3] by combining thin-layer chromatography with high-resolution mass-spectrometry.

A gas chromatographic method for the determination of diazepam and its demethylated metabolites, involving an acidic hydrolysis to chlorobenzophenone derivatives, was described by DE SILVA *et al.*[4]. This method, although later modified (DE SILVA *et al.*[5]), permits a separation between diazepam and its N-demethylated metabolites, but does not allow differentiation of the C_3-hydroxylated metabolites.

An improved gas chromatographic procedure is described here which employs a liquid phase of 3 % OV_1. This gives an excellent resolution and allows a quantitative estimation of the individual intact benzodiazepine derivatives. The present method has been successfully applied to the determination of diazepam, N-demethyl-diazepam, N-methyl-oxazepam, oxazepam and nitrazepam.

Experimental

Reagents. All reagents must be of reagent grade purity. All inorganic reagents were made up in triple distilled water.

1 M KH_2PO_4 buffer: pH 7.

Diethyl ether: Analytical reagent grade ether, containing not more than 0.00005 % peroxide, must be used from a bottle opened no more than 1 day previously.

Acetonitrile: Reagent grade pure for spectrophotometric use.

Procedure

In a glass-stoppered centrifuge tube place 1 ml of the sample containing benzodiazepine derivatives, 2 ml of buffer and 4 ml of H_2O. Add 10 ml of ether, seal the stopper with water and shake on a reciprocating shaker for 10 min. Centrifuge at 0° for 5 min and transfer the ether phase into a glass tube. Re-extract the water phase with another 10 ml of ether and combine the ether extracts. Place the tube containing the ether extracts in a hot water bath (45°) to evaporate to dryness and then dry the residue in a vacuum desiccator for 15 min. Dissolve the residue in a suitable amount of acetonitrile and ensure uniform distribution and solution of the material by tapping and stirring the tube for 60 sec.

A suitable aliquot, from 1 to 3 μl, is chromatographed and the different peaks are identified by their retention times.

Gas chromatography. The gas chromatograph used was Model G.V. dual column (Carlo Erba, Milan) equipped with a hydrogen flame ionization detector. In several experiments an electron capture detector was used to improve the sensitivity of the method.

The stationary phase was OV_1 3 % on Gas Chrom Q (60–80 mesh) packed into a 2-m glass column (int. diam. 2 mm, ext. diam. 4 mm). The flow rate of carrier gas (nitrogen) was 22 ml/min and the column temperature was 245°.

Quantitative analysis. For identification and calculations the internal standard

technique was used. 2-N-Benzylamino-5-chloro-benzophenone was chosen as an internal standard because of its suitable retention time. The area of the peaks was calculated by measuring, in convenient units, the height and width of the peak at half height.

Benzodiazepines can be quantitated by gas chromatography when the relative peak area is used as an index of concentration, since a linear relationship exists between relative peak area and drug concentration in the range of 0.1 to 2 μg.

A hundred-fold increase in sensitivity was obtained by using the electron capture detector.

Preliminary studies of the partition coefficient of benzodiazepines tested between water and ether have shown that these drugs are substantially quantitatively extracted from aqueous media with a 2-fold volume of this organic solvent. A typical gas chromatogram showing the separation of a mixture containing five benzodiazepine derivatives and the internal standard is illustrated in Fig. 1.

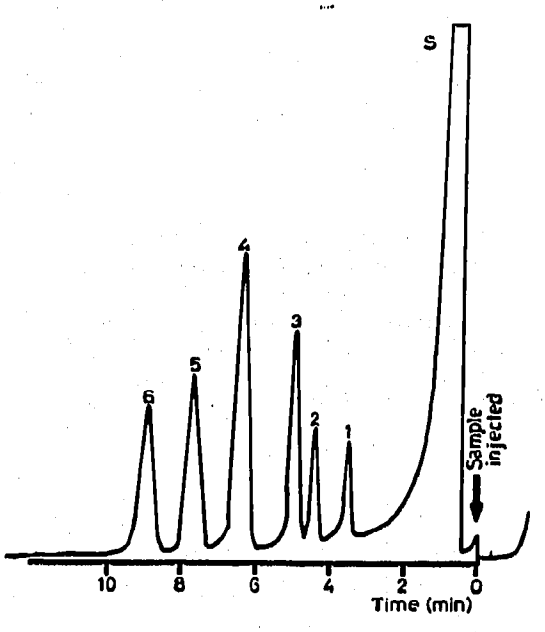

Fig. 1. Separation of a mixture of five benzodiazepines. 1 = Oxazepam; 2 = diazepam; 3 = N-demethyl-diazepam; 4 = N-methyl-oxazepam; 5 = nitrazepam; 6 = internal standard; S = solvent. Experimental conditions as described in the text.

TABLE I

RECOVERY STUDIES

Drug	% *Recovery* ± *S.E.*	
	From water	*From rat blood*
Diazepam	92 ± 3	90 ± 3
N-Demethyl-diazepam	100 ± 4	100 ± 3
N-Methyl-oxazepam	93 ± 1	65 ± 2
Oxazepam	91 ± 1	63 ± 2
Nitrazepam	96 ± 2	87 ± 3

The recovery from water and from rat blood is reported in Table I. The lower recovery from blood suggests a possible protein-binding of the hydroxylated derivatives.

Preliminary studies indicate that blanks from extracts of rat blood or tissue do not show peaks that interfere seriously with a biological application of the gas chromatographic procedure.

The method described in this paper has the advantage over the previous methods of being simple and rapid. It also permits measurements of diazepam and its metabolites in the same sample.

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An accurate siphon for measuring fractions during column chromatography

A number of commercial fraction collectors employ a counterbalanced arm, which causes the turntable to rotate as it rises and falls. The arm is operated by the filling and emptying of a siphon attached to one end. This is a convenient system but the uniformity of the fractions produced depends entirely on the consistency of the siphon. The only form of siphon available commercially, as far as I know, is of type A1 (Fig. 1). Devices of this sort were described by NEDERBRAGT[1] and LIGON[2] but were first studied as an aid to column chromatography by BOVÉ[3]. Modifications intended for special purposes have since been reported[4,5] but BOVÉ's basic design has remained