Journal of Chromatography, 122 (1976) 471–477

CHROM. 9070

DETERMINATION OF PHENOBARBITAL IN HUMAN SALIVA BY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

A gas chromatographic method for the determination of phenobarbital in saliva has been developed. Phenobarbital is converted into its bispentafluorobenzyl derivative by extractive alkylation at pH 9 with 0.1 *M* tetrabutylammonium ion as extracting agent and 0.1 *M* pentafluorobenzyl bromide as alkylating reagent in methylene chloride. A reaction time of 20 min is required.

Quantitation is effected by electron-capture detection in a gas chromatograph equipped with a pre-column venting system for removal of methylene chloride and pentafluorobenzyl bromide. This procedure allows the direct introduction of the reaction mixture into the gas chromatograph.

A 60-ng amount of phenobarbital in 100 μl of human saliva can be determined with a precision of 1.9% (S.D.) and a recovery of 93%.

INTRODUCTION

Good gas chromatographic (GC) properties can be conferred upon barbiturates by alkylation. Several alkylation procedures have been proposed, *e.g.*, flash methylation with trimethylanilinium hydroxide[1] and alkylation with pentafluorobenzyl (PFB) bromide in the presence of triethylamine[2]. Extractive alkylation has also been used for the derivatization of barbiturates[3]. The barbiturate is extracted in anionic form as an ion pair with a quaternary ammonium ion into an organic phase that contains the alkylating reagent, methyl iodide.

This principle has been used in this investigation in the alkylation of phenobarbital with PFB bromide. The PFB group is an excellent electrophore that gives a high electron-capture detector (ECD) response[4–11]. Extractive alkylation with PFB bromide has been used in the determination of pentazocine[4] and theophylline[5] in serum at the nanograms per millilitre level.

The organic phase from the extractive alkylation will contain, in addition to the derivative, an excess of PFB bromide, which will disturb the quantitation. Different methods for separating the excess of reagent from the derivative have been used, such as evaporation[8,9], extraction into an aqueous phase[4,5], separation on a silica gel

column[10] and coupling of the reagent to an aminophenol to give a product that can be extracted with water[11].

In this investigation, a pre-column venting system has been used for removal of the excess of reagent. Pre-columns of different constructions have been used in GC to permit the injection of large sample volumes[12,13], direct introduction of the reaction mixture[14] and group separation of sample components[15–20]. In this study, a simple venting system of a new design has been made. It gives a constant flow of carrier gas during the chromatographic process, which is of fundamental importance in electron-capture gas chromatography.

This paper describes the determination of phenobarbital in human saliva by extractive alkylation with PFB bromide, removal of excess of reagent by the pre-column venting system and electron-capture detection.

EXPERIMENTAL

Apparatus

Gas chromatography. A Varian Model 1400 gas chromatograph with a flame ionization detector was used, equipped with a glass column (150 × 0.2 cm I.D.), packed with 3% OV-17 on Gas-Chrom Q (80–100 mesh). The column temperature was 240° and the flow-rate of nitrogen carrier gas was 30 ml/min.

A Varian Model 1400 gas chromatograph equipped with a ^{63}Ni ECD was used and modified as shown in Fig. 1. The glass pre-column (11.5 × 0.3 cm I.D.) and the glass analytical column (140 × 0.2 cm I.D.) were filled with the same packing as above. The flow-rate of nitrogen carrier gas was 20 ml/min. The temperature of the pre-column (injector block) was 280°, that of the analytical column 270° and that of the detector 300°.

Mass spectrometry. The derivatives were identified by mass spectrometry (MS) in an LKB 9000 instrument after a GC separation on a glass column (210 × 0.4 cm

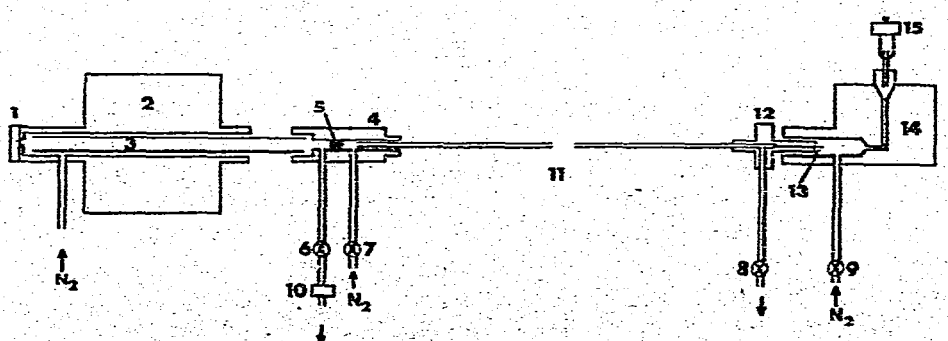

Fig. 1. Layout of apparatus. 1 = Septum; 2 = injector block; 3 = pre-column (1/4 in. × 3 mm I.D., 11.5 cm long); 4 = stainless-steel reducing union (1/4 in. to 1/8 in.); 5 = porous metal plug; 6, 7, 8, 9 = toggle valves (Whitey OGS 1A); 10 = needle valve (Nupro B-2SA); 11 = analytical column (140 × 0.2 cm I.D.); 12 = stainless-steel reducer (1/8 in.)[21]; 13 = stainless-steel restrictor (0.009 in. I.D. × 15 mm); 14 = detector base; 15 = ^{63}Ni ECD cell. Toggle valves 6 and 7 are connected to 4 by stainless-steel tubing which is soldered to 4. Normally, valves 8 and 9 are closed. They are opened when the gas chromatograph is not in use in order to protect the ECD. Toggle valve 8 is also used to measure the carrier gas flow-rate.

I.D.) filled with 6% SE-30 on Chromosorb W (80–100 mesh). The ionization energy was 70 eV.

Reagents and chemicals

PFB bromide was obtained from Pierce (Rockford, Ill., U.S.A.). Tetrabutylammonium hydrogen sulphate was kindly supplied by AB Hässle (Mölndal, Sweden). Phenobarbital and heptabarbital were of pharmacopoeial grade. Methylene chloride and *n*-heptane (Uvasol) were supplied by Merck (Darmstadt, G.F.R.).

Derivatization

To 1 ml of 0.1 *M* tetrabutylammonium hydrogen sulphate in phosphate buffer (pH 9) was added 1 ml of methylene chloride containing 1 mg of phenobarbital (or heptabarbital) and 1 mg of octacosane (internal standard). PFB bromide was added and the tube shaken for a certain time (see Fig. 4). The reaction was stopped with 1 ml of 1 *M* sulphuric acid and the mixture was shaken for 2 min before centrifugation. A 1-μl volume of the organic phase was injected into the gas chromatograph equipped with a flame ionization detector.

Determination of the electron-capture response

PFB derivatives of phenobarbital and heptabarbital were synthesized in milligram amounts. Solutions in *n*-heptane were injected in the gas chromatograph fitted with a ^{63}Ni ECD. The minimum detectable amount was calculated as described previously[22].

Determination of phenobarbital in saliva

A 100-μl volume of saliva was mixed with 10 μl of 0.1 *M* trisodium phosphate in a test-tube (pH $\approx$ 9 in the solution). Then 0.5 ml of 0.1 *M* tetrabutylammonium hydrogen sulphate in phosphate buffer (pH 9) and 500 μl of 0.1 *M* PFB bromide in methylene chloride with heptabarbital (*ca.* 150 ng/ml) were added. The tube was shaken mechanically for 20 min. After the addition of 3.0 ml of *n*-heptane, the tube was shaken gently for a few seconds and centrifuged for 2 min at 2000 rpm.

A 1-μl volume of the organic phase was injected into the gas chromatograph then, 10 sec after the injection, valves 6 and 7 (Fig. 1) were closed for 20 sec.

RESULTS AND DISCUSSION

The venting system

The principle of the venting system is shown in Fig. 2. At the beginning of the chromatographic process, the system is run in the "open mode". When compounds of interest are eluted from the pre-column, valves 6 and 7 are closed and the system is run in the "closed mode" for a suitable period to allow the compounds to enter the analytical column. The valves are then re-opened in order to remove remaining sample components from the pre-column.

Tests with known samples of PFB derivatives of phenobarbital and the internal standard showed that the main part of the reagent and the solvent had passed the pre-column 10 sec after the injection and the PFB derivatives had passed the pre-column after a further 20 sec. Complete transfer of the PFB derivatives to the

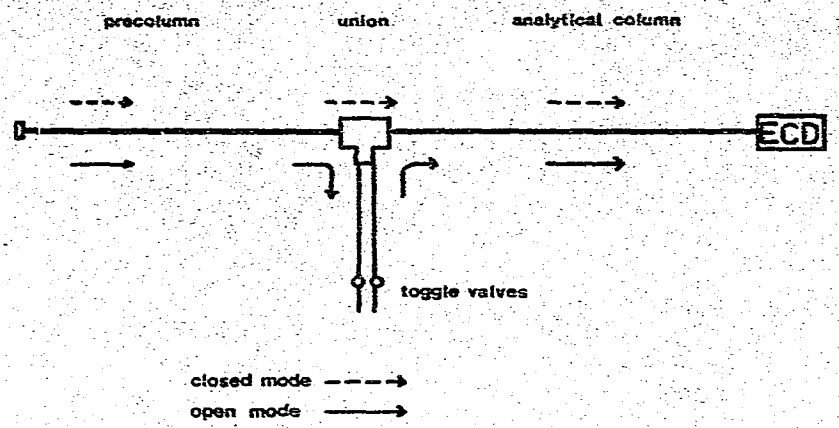

Fig. 2. Schematic diagram of flow in the GC venting system.

analytical column could obviously be obtained by running the system in the "closed mode" during the period 10–30 sec after the injection. This is the procedure recommended in the Experimental section.

The switching of the valves does not influence the separation efficiency of the system. Runs with *n*-heptane solutions of the PFB derivatives gave the same number of theoretical plates when the venting system was closed during the whole procedure and during the period 10–30 sec.

A typical chromatogram from a phenobarbital-containing saliva sample treated according to the technique described is given in Fig. 3a. A blank run is shown in Fig. 3b.

The importance of the venting system is demonstrated in Fig. 3c, which shows a chromatogram obtained with the same sample as in Fig. 3a when the chromatograph was run in the "closed mode" during the whole chromatographic process.

Tests showed that the venting system was capable of removing more than 10 times of the amounts of PFB bromide and methylene chloride that are present from the derivatization.

Extractive alkylation of phenobarbital

Extractive methylation of phenobarbital has previously been studied with carbon disulphide as organic phase, methyl iodide as alkylating reagent and different quaternary alkylammonium ions as extraction agents[3]; 0.01 *M* tetrahexylammonium ion and 0.03 *M* methyl iodide gave a quantitative reaction within 5 min.

The optimum extraction of phenobarbital as an ion pair with quaternary ammonium ions is obtained at pH 9–12, and an extraction constant of $10^{1.87}$ was found with tetrabutylammonium as counter ion and chloroform as organic phase[23].

In the present work, methylene chloride was preferred as the organic solvent. From the extraction data above, it could be estimated that 0.1 *M* tetrabutylammonium at pH 9 would give an extraction of at least 90% of phenobarbital as an ion pair[24].

The influence of the concentration of the alkylating reagent (PFB bromide) on the rate of PFB phenobarbital and PFB heptabarbital formation is demonstrated

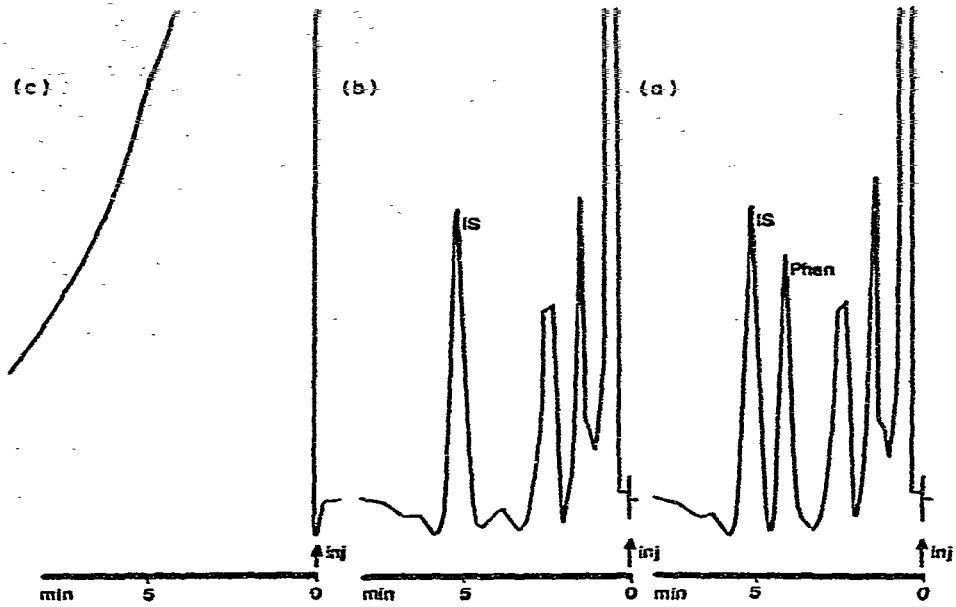

Fig. 3. Chromatograms from human saliva. (a) Sample spiked with 64 ng of phenobarbital (Phen) per 100 μl. (b) Blank chromatogram with internal standard (IS) heptabarbital. Injection of samples in "open mode". "Closed mode" for 20 sec, 10 sec after injection, then "open mode" again. (c) Same sample as in (a). "Closed mode" during the whole chromatographic process. Electrometer setting: $4 \cdot 10^{-10}$.

in Fig. 4. A quantitative yield was obtained with 0.1 *M* PFB bromide with a reaction time of 20 min or more.

Identity and properties of the derivatives

The derivatives were identified by MS. Walle[2] found that two PFB groups are coupled to the phenobarbital molecule by alkylation with PFB bromide. The MS studies confirmed that bis-PFB derivatives also were obtained by the extractive alkylation method used in this work.

The derivatives showed good chromatographic properties. No adsorption losses could be observed even when only a few picograms were injected, and peaks with good symmetry were obtained.

The minimum detectable amount was $5 \cdot 10^{-17}$ mole/sec for the PFB derivatives of both phenobarbital and heptabarbital. This corresponds to an injected amount of less than 1 pg, under the chromatographic conditions used, which is in good agreement with the results of Walle[2].

Determination of phenobarbital in saliva

Interest in the determination of drugs in saliva has increased recently, particularly as it is easy to collect a large number of samples from the same subject[25–28]. Heptabarbital was used as the internal standard as it reacts with PFB bromide at the same rate as phenobarbital (Fig. 4), and PFB heptabarbital has only a slightly longer retention time than PFB phenobarbital.

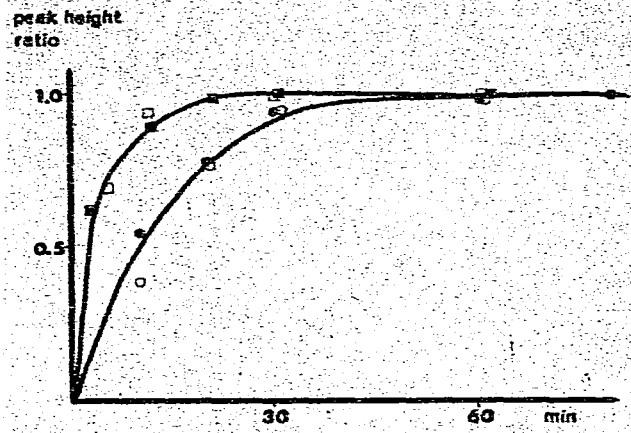

Fig. 4. Time course of pentafluorobenzylation of phenobarbital and heptabarbital. Procedure: see Experimental section. ●, 0.033 *M*; ■, 0.1 *M* PFB bromide (phenobarbital). ○, 0.033 *M*; □, 0.1 *M* PFB bromide (heptabarbital).

The standard graph was linear in the range 10–100 ng per 100 μl of saliva sample. Saliva samples and pure aqueous solutions gave identical standard graphs.

Quantitative determinations on saliva samples spiked with 60 ng per 100 μl were made with a standard deviation of 1.9% ($n = 10$). The recovery was 93%, determined by comparison with known amounts of pure PFB phenobarbital.

The time for a single analysis is about 45 min, and it is possible to perform more than 30 analyses per day.

ACKNOWLEDGEMENTS

Our thanks are due to Dr. Nils Kållberg for supplying heptabarbital and for valuable discussions on phenobarbital determinations. We also thank Dr. Thomas Walle for the preparation of crystalline bis-PFB phenobarbital.

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