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ANALYSIS OF TRACE AMOUNTS OF BARBITURATES IN SALIVA

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SUMMARY

A simple gas chromatographic method is presented for the determination of trace quantities (0.1–1.0 μg/ml) of amobarbital, pentobarbital and phenobarbital in saliva. The barbiturate is extracted with chloroform, alkylated with bis(2-chloroethyl) sulphate, and quantified using a non-polar (SE-30) column and electron-capture detection.

The procedure has been applied to the determination of the salivary half-lives of amobarbital and, for the first time, pentobarbital following administration of the drugs to human volunteers. The scale and sensitivity of detection are suitable for use in forensic or other medico-legal work.

INTRODUCTION

The alkylation of barbituric acids prior to gas chromatography (GC) leads to a significant improvement in their gas chromatographic properties. To date, detection has largely been confined to the flame ionization mode but more recent work[1–3] has described the alkylation of some barbituric acids with pentafluorobenzyl bromide prior to electron-capture detection.

Although a highly sensitive response was reported for the pure pentafluorobenzyl derivatives of several barbituric acids[1], the method described for the pentafluorobenzylation of phenobarbital extracted from saliva[2] has the disadvantage of requiring a pre-column venting system for the removal of excess reagent which would otherwise produce a large detector response and make quantification of the phenobarbital an impossibility. It appears, also, that the method would not be suitable for the determination of barbiturates with retention times smaller than that of phenobarbital, due to the presence of two large unidentified peaks seen in the chromatogram of the extracted saliva. Such a limitation would, of course, exclude many barbiturates of interest. In a later report[3] describing the pentafluorobenzylation of pentobarbital isolated from serum, excess reagent did not appear to be a problem and there was no

evidence of interfering peaks. In this case, however, prolonged heating of the reaction mixture (4 h) was necessary, in addition to time consuming extraction, washing and concentration procedures.

In this paper, we describe the determination of the barbiturates amobarbital, pentobarbital and phenobarbital in human saliva, after alkylation of the isolated acid with bis(2-chloroethyl) [BCE] sulphate, and electron-capture detection. From previous work[4–6] the easy formation of the BCE derivatives, as well as the structure and relative electron-capture response of the BCE derivatives of barbiturates and some anticonvulsant compounds, has been demonstrated. Furthermore, these pure derivatives have been used to validate the efficient recovery of the barbiturates from saliva.

In an extension of the work, the analytical method has been applied to a pharmacokinetic study of pentobarbital and amobarbital where, after administration of these barbiturates to volunteers, salivary barbiturate concentrations were monitored. From plots of concentration *versus* time, estimates of the *in vivo* biological half-lives of these drugs were obtained.

EXPERIMENTAL

Apparatus

A Hewlett-Packard Model 5750 gas chromatograph, with a 2 mCi ^{63}Ni electron-capture detector operated at 240°, was used. The chromatographic column consisted of a borosilicate glass coil (0.56 m × 6.4 mm O.D.) packed with 3% (w/w) SE-30 on Chromosorb 750 (100–120 mesh) and resilanized with hexamethyldisilazane prior to use. A mixture of argon–methane (95:5) was maintained at a flow-rate of 100–104 ml/min. A pulsed voltage was applied to the detector (amplitude 30 V, period 50 μsec, width 0.75 μsec).

Reaction mixtures were heated in small, graduated Pyrex tubes (5 ml, length 99 mm) seated in an aluminium heating-block (150 × 52 × 36 mm) whose temperature was carefully controlled by means of a continuously variable regulator. A shallow recess (depth 21 mm) was used for refluxing purposes and a deeper recess (81 mm) for the evaporation of solvents and triethylamine.

Reagents and chemicals

The barbituric acids (May & Baker, Dagenham, Great Britain) were recrystallized from benzene and their purity confirmed by microanalysis. All solvents were of analytical reagent quality and re-distilled from an all-glass system. Stock solutions of barbituric acids and derivatives were made in ethyl acetate or ethanol. Traces of acid found in the reagent BCE sulphate (Eastman-Kodak, Rochester, N.Y., U.S.A.) were removed by washing with water and an ether solution of the reagent was dried with anhydrous sodium sulphate, prior to use. Triethylamine (TEA) was also re-distilled (b.p. 89°). Graduated glass syringes (100 μl, SGE) were employed for delivering reagents in quantities less than 0.1 ml.

Conversion efficiency

Following the procedure given below, percentage conversions for the derivatization reaction were obtained by comparing peak height ratios for the conversion product with standard solutions of the pure derivative, chromatographed immediately

before and after the product. BCE cyclobarbital was used as internal standard for the quantification of amobarbital and pentobarbital. For work with phenobarbital, the internal standard was BCE amobarbital. In both cases, working concentrations of the internal standard ranged from 0.4 to 5.0 μg/ml.

Extraction procedure and recovery efficiency

Fresh, mixed and unstimulated saliva was centrifuged for 2 min to remove debris. Supernatant fluid (4.0 ml) was removed and to it was added 100 μl of the standard barbituric acid solution (4–40 μg/ml in ethanol). After mixing well, the solution was acidified (1 *M* HCl, 0.1 ml) and extracted with chloroform (3 × 6 ml) by shaking gently in a glass-stoppered centrifuge tube (capacity 30 ml). Emulsification was usually overcome by centrifuging and any protein precipitate, which occasionally extended into the organic layer, was removed by centrifuging the tube in the inverted position. The organic layer was removed by passing a Pasteur pipette down through the aqueous phase and protein precipitate, into the chloroform layer.

The combined phases were evaporated to dryness (rotary vacuum evaporator at 40°) and the residue quantitatively transferred to a 5-ml reaction tube with small amounts of ethyl acetate. The contents of the tube were evaporated to near dryness, as before, and the residual solution (*ca.* 100 μl) refluxed at 97° for 5 min with TEA (200 μl) and BCE sulphate (50 μl, 1.3 *M* in ether). The bulk of the excess TEA and ethyl acetate was removed by evaporation (10 min) on the heating-block and any last traces, especially of TEA, were removed by evaporation under reduced pressure (40°, 4 min).

Ethyl acetate (2.0 ml) was now added to the residue, preparatory to the removal of quaternary ammonium salts by washing with water (≈ 1.5 ml). The organic layer was then recovered, the aqueous layer washed again with ethyl acetate (1.5 ml) and the combined ethyl acetate extracts evaporated to dryness under reduced pressure in a round-bottom flask (capacity ≈ 20 ml) at 40°. Finally, ethyl acetate (1.0 ml) was added to the residue, followed by internal standard (1.0 ml), then the flask stoppered and the contents mixed well prior to chromatographic examination.

Solutions of standards were prepared by subjecting 100 μl of ethyl acetate to the same chloroethylation procedure but, in place of the ethyl acetate, 1.0 ml of a standard solution containing an equivalent amount of the appropriate BCE barbiturate derivative was added before the internal standard.

Estimation of salivary half-life for barbiturates

Pentobarbital and amobarbital were administered orally to two healthy adult males and unstimulated, mixed saliva (5 ml) was collected at specified time intervals, not less than 3 h after a meal. The samples were then frozen (at −15°) until analyzed as described above. From plots of concentration *versus* time, the salivary half-life was obtained from the slope of the terminal, linear phase of the elimination curve after calculating the regression line by the method of least squares. To allow for non-linear elimination of drug from the body, drug concentrations measured within 5 h of ingestion were not included in the calculations.

Three trials were conducted with pentobarbital, used as the sodium salt (Petab, Knoll Labs., Arncliffe, Australia; 100 mg tablet) and was repeated by one subject after an interval of 6 weeks. With amobarbital, a single tablet of the free acid (Amytal, Eli Lilly, West Ride, Australia; 120 mg) was ingested by each subject.

RESULTS AND DISCUSSION

The chloroethylation of amobarbital, pentobarbital and phenobarbital proceeds readily under the conditions described and is suited to adaptation for the derivatization of microgram quantities of these compounds. There is little interference from excess reagents or other compounds and the procedure is relatively quick with reproducible and nearly quantitative conversions for the three barbiturates. No variation in the yield of BCE amobarbital was observed when the reaction mixture was refluxed for longer periods (5–35 min), or when the volume of the amine was varied from 200 to 300 μl.

Although widely used as a mildly basic catalyst, potassium carbonate has been implicated[1] with the hydrolysis of barbituric acids. Certainly, its use in the chloroethylation of barbiturates[4–6] resulted in very poor yields and may have been due to hydrolytic reactions. TEA was a suitable alternative. However, removal of all traces of TEA and its salts was necessary to prevent excessive tailing of the solvent peak during GC .This was satisfactorily accomplished by evaporation under reduced pressure followed by aqueous washes of the residue.

In order to observe any contribution to measured peak heights by excess reagents or reaction by-products, standards were prepared by subjecting a "blank" solution to the same chloroethylation procedure as used for the sample but the appropriate BCE barbiturate derivative was added to it just before GC. Replicate studies of the efficiency of the derivatization, gave yields of 96.0% for 1.0–2.0 μg amobarbital (sample, $n = 6$; standard deviation, S.D. $= \pm 1.6\%$), 88.0% for 0.50–1.0 μg pentobarbital ($n = 6$; S.D. $= \pm 2.6\%$) and 86.0% for 1.0–2.0 μg phenobarbital ($n = 6$; S.D. $= \pm 3.0\%$).

Because pK_a values of the barbituric acids fall in the range 7.4–8.3, their extraction from biological fluids is influenced by the pH of the aqueous system and the polarity of the extracting solvent. The barbiturates chosen for this study, are of low lipophilicity, and mildly polar solvents such as ether and chloroform have been used for their extraction after acidification of the sample. Furthermore, it has been shown that in blood, the hydroxylated metabolites of the barbiturates are present only as a very small fraction[7] of the total intake. The presence of these metabolites in saliva has not yet been documented, but since the transfer of most drugs from plasma to saliva appears to be a passive process[8,9], it is feasible that the transfer would be paralleled by the metabolites. However, studies of the 3-hydroxy metabolites of amobarbital[10,11] and pentobarbital[12,13], and the *p*-hydroxy metabolite of phenobarbital[14,15], have indicated that poor solubility and a low partition coefficient prevent their recovery from blood or aqueous solutions when chloroform is the extracting solvent. For this reason, it was the solvent of choice for the extraction of unchanged barbiturate from saliva. In fact, no evidence of hydroxy-barbiturates (whose retention times can approximate that of the internal standard) was observed.

Table I presents the results for the recovery of barbituric acids added to saliva, using the experimental procedure based on chloroethylation.

Turning to the pharmacokinetic study, the saliva concentrations of both barbiturates, measured during the first 5 h after ingestion, show the familiar rapid rise during the absorption phase, as well as the steady decline during the elimination phase[16]. Of greater interest, and also shown in Fig. 1, the terminal elimination phase,

TABLE I

RECOVERY OF BARBITURATES ADDED TO SALIVA

Barbiturate	*Quantity added (μg)*	*Number of replicates*	*Mean recovery (± S.D.)*
Amobarbital	0.40–4.0	13	96.2 ± 4.0
Pentobarbital	0.50–4.0	9	86.8 ± 3.3
Phenobarbital	0.50–1.0	6	86.1 ± 1.8

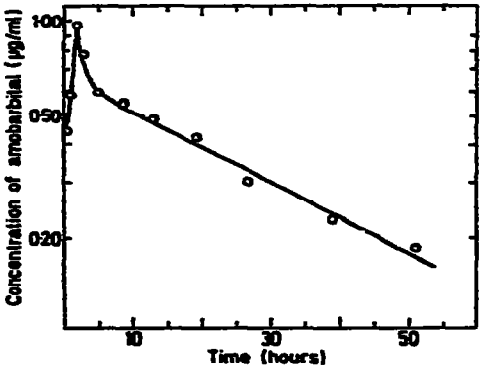

Fig. 1. A typical elimination curve for amobarbital obtained from saliva analyses.

when drug concentrations fall in a linear fashion as a result of metabolism and excretion, was closely observed in all trials conducted. Typical chromatograms obtained during the course of this work are presented in Fig. 2.

Salivary concentrations of amobarbital in both subjects are comparable to those quoted by Inaba and Kalow[17]. However, for both volunteers, the half-life of amobarbital was greater than those measured previously[17] for which average values were given as 20.0 ± 1.7 h.

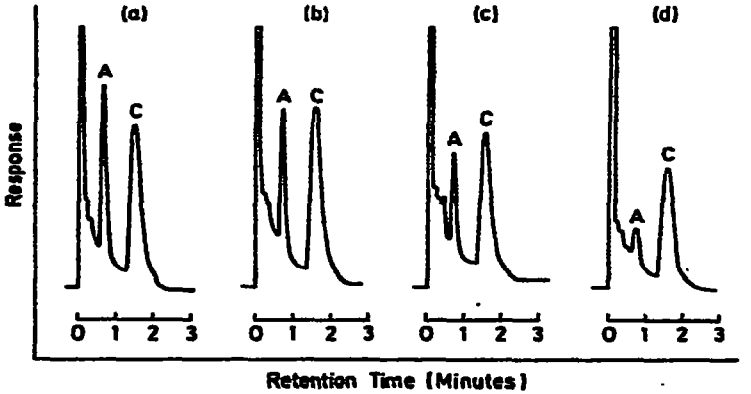

Fig. 2. Chromatograms of chloroethylated saliva extracts following ingestion of amobarbital. Concentrations (μg/ml saliva) and time intervals represented are (a) 0.77 at 3 h, (b) 0.48 at 13 h, (c) 0.30 at 27 h, and (d) 0.18 at 51 h. A = BCE amobarbital; C = BCE cyclobarbital. Concentration of internal standard, 2 ng per injection.

Table II presents data obtained during the pharmacokinetic study, and shows that for both subjects, the half-life of amobarbital is greater than for pentobarbital. The difference may be related to the lipid solubility of the drug[18] and the form in which it was administered[19] (that is, as the sodium salt or free acid). Correlation coefficients for the regression analysis of data for both drugs, were between 0.990 and 0.997 indicating a close fit of experimental observations with the calculated regression line and establishing the value of the analytical method.

TABLE II

PHARMACOKINETIC DATA FOR PENTOBARBITAL AND AMOBARBITAL

x = Time (h) and y = log of concentration (μg/ml) in saliva; r is the correlation coefficient.

Barbiturate	*Subject*	*Equation of regression line*	*Half-life (h)*	*r*
Pentobarbital	A (trial I)	$y = -0.158 - 0.0163x$	18.5	0.990
Pentobarbital	A (trial II)	$y = -0.221 - 0.0158x$	19.1	0.992
Pentobarbital	B	$y = -0.198 - 0.0173x$	17.4	0.993
Amobarbital	A	$y = -0.174 - 0.0268x$	25.9	0.992
Amobarbital	B	$y = -0.182 - 0.0135x$	22.3	0.997

Finally, although this study has been restricted to the barbiturates, the advantages of chloroethylation also apply to other acidic drugs[4–6]. When combined with the additional practical advantages of working with saliva, we believe this analytical procedure may be valuable in areas of forensic chemistry and related medico-legal work.

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