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THE DETERMINATION OF PENTOBARBITAL AND OTHER BARBITURATES IN BLOOD PLASMA BY GAS—LIQUID CHROMATOGRAPHY WITH ON-COLUMN AND PRE-COLUMN BUTYLATION

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SUMMARY

Two g.l.c. methods for the determination of pentobarbital and other barbiturates are reported. In the first method the plasma samples are extracted with toluene; the toluene layer is back-extracted with a small volume of a tetrabutylammonium hydroxide solution of which an aliquot is injected into the gas chromatograph. This method is simple and rapid, and sensitive enough for monitoring pentobarbital plasma concentrations in the therapeutic range. A number of barbiturates are not well extracted from toluene by tetrabutylammonium hydroxide because of ion-pair partitioning. The extraction efficiency of barbiturates from toluene is much improved by back-extraction of the toluene layer with a tetramethylammonium hydroxide solution; clean chromatograms are obtained from blank plasma when the toluene layer is back-extracted, instead of evaporated, prior to derivative formation. This modification is introduced in the second method; an aliquot of the tetramethylammonium hydroxide layer is subjected to a pre-column butylation procedure which converts the barbiturates completely without any decomposition in the injection port; the sensitivity is at least as good as that of the on-column technique.

Barbiturates are often converted to derivatives before passage through a gas chromatographic column. In a much-used on-column flash methylating method [1—3], the drug is extracted from the plasma with toluene and back-extracted with a small volume of a methanolic solution of trimethylphenylammonium hydroxide (TMPAH) or tetramethylammonium hydroxide (TMAH), of which an aliquot is injected into the gas chromatograph. The compound is methylated in the injection port at high temperatures. The simplicity of the method is attractive. Its main disadvantages are: the formation of decomposition products at the high temperatures in strongly alkaline medium during the methylation process; the re-methylation of drugs that are biologically de-methylated, resulting in the same end-product for the parent compound and the metabolite; and the possibility of a reduction in column life caused by multiple injections of strongly alkaline solutions.

Butylation of the compounds eliminates the problem of the formation of

the same end-product from different compounds. However, on-column flash butylation [4, 5] with tetrabutylammonium hydroxide (TBAH) also gives rise to the formation of more than one product during conversion. This can be overcome by the alkylation of the compounds before injection into the gas chromatograph, as described by Greeley [6] and by IJdenberg [7]. In this paper, determinations of pentobarbital in plasma by an on-column flash butylation technique and by a pre-column butylation technique based on Greeley's method [6] are described. The extraction of other barbiturates in both procedures is also investigated.

EXPERIMENTAL

Apparatus

A Varian-Aerograph Model 2700 gas chromatograph equipped with flame ionization detectors was used for the on-column butylation experiments. The glass column (180 cm × 6 mm o.d.) was packed with 3% OV 17 on 100—120-mesh Gas-Chrom Q (Applied Science Labs., State College, Pa.), conditioned overnight at 250°C (40 ml min^{-1} nitrogen flow), and silylated at 250°C by injecting five 10-μl portions of Silyl 8 (Pierce, Rockford, Ill., U.S.A.). The operating conditions were: injection port temperature, 325°C; column temperature, 215°C; detector temperature, 260°C; carrier gas (nitrogen) flow-rate, 40 ml min^{-1}; hydrogen flow-rate, 40 ml min^{-1}; air flow-rate, 350 ml min^{-1}.

A Packard-Becker Model 419 gas chromatograph equipped with flame ionization detectors was used for the pre-column butylation experiments. The glass column (150 cm × 2 mm i.d.) was packed with 3% OV 17 on 100—120-mesh Chromosorb WHP (Chrompack, Middelbury, The Netherlands), and conditioned overnight at 250°C (20 ml min^{-1} nitrogen flow). The operating conditions were: injection port temperature, 230°C; column temperature, 195°C; detector temperature, 230°C; carrier gas (nitrogen) flow-rate, 20 ml min^{-1}; hydrogen flow-rate, 25 ml min^{-1}; air flow-rate, 250 ml min^{-1}. Peak areas were obtained with the chromatographic Data Analyser System IV B (Spectra Physics, Santa-Clara, CA). Ultraviolet absorption of barbiturate solutions was measured with a Unicam SP 500 spectrophotometer with matched 1-cm cells.

Reagents and materials

Commercial pentobarbital, secobarbital, heptobarbital, phenobarbital, mephobarbital and hexobarbital were used without purification. TBAH (1 M in methanol) was obtained from Eastman-Kodak, TMAH (20% in methanol) and 1-iodobutane from Aldrich Chemicals, and dioctyl sodium sulfosuccinate (DOSS) from Merck. Other solvents and reagents were of analytical grade.

Procedure A. Determination of pentobarbital in plasma with on-column butylation

Prepare an internal standard solution (8 μg ml^{-1}) of mephobarbital in

toluene, containing 1% (v/v) of methanol. Prepare the TBAH solution by mixing equal volumes of the methanolic 1 M solution and water. Add 1 ml of 1 M hydrochloric acid and 5.00 ml of the internal standard solution to 1.00 ml of plasma in a centrifuge tube and shake for 15 s with a vortex-type mixer. Centrifuge for 3 min at 2500 G, transfer the toluene layer to a centrifuge tube, and shake for 1 min with 50 μl of TBAH solution. After centrifugation for 1 min at 2500 G, inject 2.5 μl of the TBAH layer slowly (5 s) into the gas chromatograph.

Procedure B. Determination of pentobarbital and mephobarbital in plasma with pre-column butylation

Prepare an internal standard solution (10 μg ml^{-1}) of heptobarbital in toluene containing 1% (v/v) of methanol. Add 0.1 ml of 4 M hydrochloric acid and 3.00 ml of the internal standard solution to 1.00 ml of plasma in a centrifuge tube. Shake for 30 s (vortex-type mixer), centrifuge for 3 min at 2500 G, transfer the toluene layer to a centrifuge tube, and shake for 1 min with 25 μl of 20% TMAH in methanol. Centrifuge for 2 min at 2500 G, and transfer 10 μl of the TMAH layer to a glass capillary tube (5 cm × 3 mm i.d.); add 35 μl of N,N-dimethylacetamide (DMA) and 10 μl of 1-iodobutane. Mix the contents of the tube; after 5 min, centrifuge for 1 min at 2500 G. Inject 2—4 μl of the clear supernatant solution into the gas chromatograph.

Procedure C. Extraction of barbiturates from water with toluene

Prepare separate solutions (40 μg ml^{-1}) of pentobarbital, secobarbital, heptobarbital, phenobarbital, mephobarbital and hexobarbital in water. Add 3.00 ml of 1 M hydrochloric acid to 10.00 ml of the barbiturate solution and shake for 1 min with 30.0 ml of toluene containing 1% (v/v) of methanol. Centrifuge for 5 min at 2500 G, and mix 5.00 ml of the clear aqueous phase with 1.15 ml of 1 M sodium hydroxide and 3.85 ml of 0.05 M borax solution (pH 10). Measure the absorbance of the resulting solution at 240 nm against a blank solution prepared by treating 10 ml of water in the same manner. Prepare standard solutions by adding, to 5.00 ml of a mixture of 3.00 ml of 1 M hydrochloric acid and 10.00 ml of barbiturate solution, 1.15 ml of 1 M sodium hydroxide and 3.85 ml of 0.05 M borax solution (pH 10). Measure the absorbance at 240 nm.

Procedure D. Extraction of barbiturates from toluene with TMAH

Prepare barbiturate standard I by dissolving pentobarbital, hexobarbital and phenobarbital in DMA (3 mg ml^{-1} for each compound). Prepare barbiturate standard II by dissolving secobarbital, mephobarbital and heptobarbital in DMA (3 mg ml^{-1} for each compound).

For barbiturate solution I, dissolve 30 mg of each of pentobarbital, hexobarbital and phenobarbital in 250 ml of toluene. For barbiturate solution II, dissolve 30 mg of each of secobarbital, mephobarbital and heptobarbital in 250 ml of toluene.

Internal standard solution I contains 1 mg ml^{-1} of heptobarbital in toluene; internal standard solution II contains 1 mg ml^{-1} of phenobarbital in toluene.

Shake 5.00 ml of barbiturate solution I (vortex-type mixer) for 1 min in a centrifuge tube with 100 μl of 20% TMAH in methanol. Centrifuge for 3 min at 2500 G, and transfer 5.0 μl of the TMAH layer to a glass capillary tube (5 cm × 3 mm i.d.) Add 30 μl of internal standard solution I and 10 μl of DMA. Mix, add 10 μl of 1-iodobutane, mix, and after 5 min centrifuge for 1 min at 2500 G. Inject 1—2 μl of the clear supernatant solution into the gas chromatograph.

Mix 10.0 μl of barbiturate standard I in a glass capillary tube with 30 μl of internal standard solution I and 5 μl of 20% TMAH in methanol. Add 10 μl of 1-iodobutane, mix, and after 5 min centrifuge for 1 min at 2500 G. Inject 1—2 μl of the clear supernatant solution into the gas chromatograph. Follow the same procedure with barbiturate solution II, internal standard solution II, and barbiturate standard II.

Procedure E. Extraction of barbiturates from toluene by TBAH

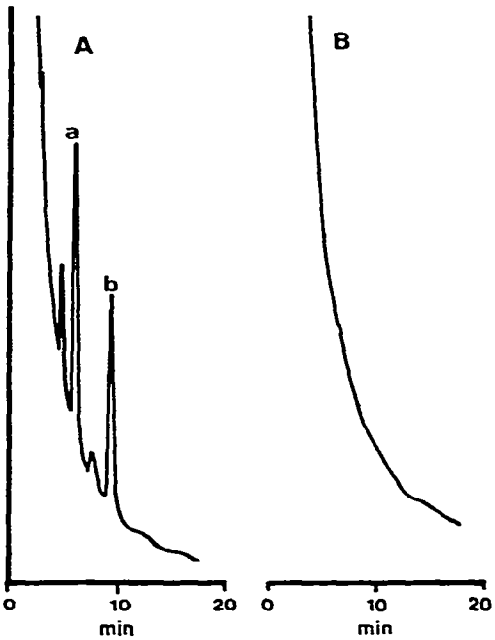
Method E1. Follow procedure D, but use a mixture of equal volumes of 1 M TBAH in methanol and water instead of the TMAH solution (centrifugation after the addition of 1-iodobutane is not necessary).

Method E2. For each of the six barbiturates investigated, prepare a solution by dissolving 30 mg of the compound in 250 ml of toluene. Shake 50.0 ml of barbiturate solution with 1.00 ml of the TBAH solution in methanol/water described above for 1 min. Filter the toluene layer through phase-separating paper (Whatman 1PS). Evaporate 40.0 ml of the clear toluene solution under reduced pressure. Dissolve the residue in 5 ml of a 3 M sodium acetate—acetic acid buffer solution (pH 2.8) and add 10 ml of water, 45 ml of chloroform, and 5 drops of a solution of methyl yellow in ethanol (0.05%). Shake well, and titrate with 0.005 M DOSS solution, shaking the mixture after each addition, until the yellow colour of the chloroform layer changes to orange. Prepare and titrate blank solutions by treating 50-ml portions of toluene in the same manner.

RESULTS AND DISCUSSION

The determination of pentobarbital in plasma with on-column flash butylation described (Procedure A), is analogous to MacGee's method [1] with TMAH replaced by TBAH. The TBAH solution in methanol had to be diluted with water to reduce its miscibility with toluene. Slow injection of the TBAH layer produced higher peaks, in agreement with MacGee's findings [8]. Typical chromatograms obtained with blank plasma and with plasma to which pentobarbital and mephobarbital were added, are shown in Fig. 1. Both barbiturates give rise to a major peak and at least one minor peak, as reported previously[5].

A calibration plot was constructed by applying the proposed procedure to



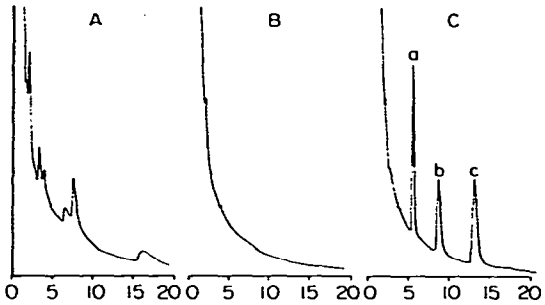


Fig. 1. On-column flash butylation. Chromatogram B was obtained from an extract of blank plasma, and chromatogram A from an extract of blank plasma spiked with pentobarbital (a) and mephobarbital (b). For chromatographic conditions and extraction procedure, see Procedure (A).

Fig. 2. Pre-column butylation. Chromatogram A was obtained by injection of the butylated residue of the toluene extract of blank plasma; chromatogram B from blank plasma; chromatogram C from blank plasma spiked with pentobarbital (a), mephobarbital (b), and heptobarbital (c). For chromatographic conditions and extraction procedure, see Procedure B.

plasma samples (1.0 ml) spiked with 0.5—15 μg of pentobarbital. Following chromatography, the heights of the peaks corresponding to the butylated products of pentobarbital and mephobarbital (Fig. 1, peak a and peak b, respectively) were measured and the peak-height ratios were plotted against the concentration of pentobarbital in the sample. The results are summarized in Table 1. The calibration plot was a straight line through the origin. The sensitivity of the method is sufficient for monitoring pentobarbital plasma concentrations in the therapeutic range. The absolute recoveries of pentobarbital and mephobarbital were determined by comparing the peak areas following this procedure with the peak areas obtained by injection of an aliquot of a solution of pentobarbital and mephobarbital in the TBAH solution. No correction was made for the volume change of the TBAH layer after extraction. The (apparent) absolute recoveries were 51% for pentobarbital (n = 10, c.v. = 8%) and 20% for mephobarbital (n = 10, c.v. = 11%). In order to determine the cause of these rather poor recoveries, the efficiency of the

TABLE 1

On-column flash butylation. Peak-height ratios of pentobarbital to mephobarbital (internal standard), and coefficients of variation (c.v.), obtained with plasma samples (1 ml) containing 0.5—15 $\mu g\ ml^{-1}$ of pentobarbital

Concentration ($\mu g\ ml^{-1}$)	p.h.r.[a]	p.h.r./concentration	c.v. (%)
0.5	0.065	0.130	16
2.5	0.338	0.135	5.5
5	0.649	0.130	3.1
7.5	1.007	0.134	3.6
10	1.358	0.136	2.7
15	2.051	0.137	3.8

[a]p.h.r. = Peak-height ratio. Each ratio is the mean value of 6 determinations.

extraction procedure was investigated for pentobarbital, mephobarbital and four other barbiturates. The percentage of barbiturate extracted from acidified water with toluene, containing 1% of methanol, was calculated from the u.v. absorbances of barbiturate solutions before and after extraction (Procedure C). Of the six barbiturates, only heptobarbital (23%) is poorly extracted: phenobarbital was extracted to the extent of 60% and the others at >85%. The extraction of heptobarbital was much improved with diethyl ether—toluene mixtures (results not shown).

The extraction of barbiturates from toluene with a small volume of TBAH solution was investigated by a gas chromatographic technique (Procedure E, method 1). Following chromatography, the percentage of the extracted drug was calculated from the resulting peak-area ratios (barbiturate/internal standard). A correction was made for the 10% reduction in volume of the TBAH layer with extraction. The results (Table 2) indicate that the rather poor recoveries from plasma of pentobarbital and mephobarbital, obtained with the extraction procedure preliminary to the on-column flash butylation,

TABLE 2

Extraction of barbiturates from toluene with solutions of TBAH and of TMAH

Compound	Extraction with TBAH (%)		Extraction with TMAH (%)
	Determined by titration[a]	Determined by g.l.c.[a]	
Pentobarbital	59	52	94
Secobarbital	43	38	96
Heptobarbital	97	85	93
Phenobarbital	98	93	93
Mephobarbital	24	19	87
Hexobarbital	28	25	77

[a]Each result is the mean of 4 pairs of determinations.

are caused by the back-extraction step of the compounds from toluene with TBAH. Borg and Schill [9] found that barbiturates can be extracted into chloroform, in their monovalent anionic form, as ion-pairs, from aqueous alkaline solutions. The influence of ion-pair partitioning on the extraction efficiency of the barbiturates in the toluene—TBAH system was investigated (Procedure E, method 2) by titration of the TBA^+ ions in the toluene layer after extraction by a modification of an earlier procedure [10, 11]. The results correspond well with those of the gas chromatographic technique (Table 2), indicating that, after extraction, the barbiturates remain in the toluene mainly as ion-pairs with TBA^+ as the counter-ion. Borg and Schill [9] concluded that ion-pairs were not formed with the divalent anions of the barbiturates. In strongly alkaline solutions, the second dissociation equilibrium of the non-*N*-methylated barbiturates therefore influences the extraction of the monovalent barbiturate anion with TBA^+, resulting in an optimum pH for the extraction of the barbiturate from alkaline solutions. An optimum pH for the extraction (as ion-pairs) of *N*-methylated barbiturates (e.g. hexobarbital and mephobarbital) does not exist; ion pairs of these compounds are equally well extracted from strongly alkaline solutions, and this may explain the poor extraction of these compounds from toluene with the TBAH solution.

The extraction of the barbiturates from toluene can be much improved by the use of a methanolic TMAH solution instead of TBAH, as shown by the results in Table 2 (Procedure D; a correction was made for the 25% reduction in volume of the TMAH layer with extraction.)

With Greeley's pre-column butylation method [6] the barbiturates are almost completely converted to one product. The excess of TMAH in the reaction mixture is removed by the addition of an excess of 1-iodobutane, yielding 1-butanol and TMA-iodide (precipitate). The resulting solution is therefore practically neutral. An obvious procedure for the determination of barbiturates in plasma with the use of pre-column butylation involves extraction of the plasma with toluene, evaporation of the organic solvent instead of back-extraction, and butylation of the residue. A typical chromatogram of blank plasma, treated in this manner, is shown in Fig. 2A. A number of potentially interfering peaks in the chromatogram can be eliminated by extraction of the compounds from the toluene layer with TBAH solution (Fig. 1) or with TMAH solution (Fig. 2). A procedure for the determination of pentobarbital and mephobarbital in plasma was based on Greeley's pre-column butylation method [6] following extraction of the compounds and internal standard from acidified plasma with toluene, and back-extraction of the toluene layer with methanolic TMAH solution (Procedure B).

A typical chromatogram is shown in Fig. 2C. A calibration plot was constructed from plasma samples spiked with pentobarbital and mephobarbital (0.5—10 $\mu g\ ml^{-1}$) by plotting the peak-height ratios for pentobarbital/internal standard and mephobarbital/internal standard against the pentobarbital and mephobarbital concentrations, respectively. The results are summarized in Table 3. For both compounds the calibration was a straight line through the

TABLE 3

Pre-column butylation. Peak-height ratios of pentobarbital to heptobarbital (internal standard), and of mephobarbital to heptobarbital, and coefficients of variation (c.v.), obtained with plasma samples (1 ml) containing 0.5—10 $\mu g\ ml^{-1}$ of pentobarbital and mephobarbital

Concentration ($\mu g\ ml^{-1}$)	Pentobarbital			Mephobarbital		
	p.h.r.[a]	p.h.r./concentration	c.v. (%)	p.h.r.[a]	p.h.r./concentration	c.v. (%)
0.5	0.186	0.372	7.4	0.084	0.168	7.8
2.5	0.973	0.389	5.2	0.415	0.166	3.1
5	1.908	0.382	4.2	0.828	0.166	3.5
7.5	2.872	0.383	3.6	1.256	0.167	3.6
10	3.677	0.377	4.6	1.677	0.168	4.1

[a]p.h.r. = Peak-height ratio. Each ratio is the mean value of 6 determinations.

origin. The precision at a concentration of 0.5 $\mu g\ ml^{-1}$, which is about the lowest measurable pentobarbital plasma level with the on-column flash butylation technique, is still fairly good because of the better extraction efficiency and the much smaller solvent peak.

The determination of barbiturates in plasma by the pre-column butylation procedure, compared with the on-column technique, involves more manipulations and the time needed for the analysis of 10 samples is about 20 min longer. However, decomposition of the products during the butylation procedure does not occur; the lower injection port temperature, and the injection of solutions from which the excess of quaternary ammonium base has been removed, may prolong the column life.

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