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Note

Separation of barbiturates and morphine analysis from putrefied *post mortem* tissue

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Very often the forensic toxicologist is confronted with analytical problems when he receives *post mortem* tissues that have a variable degree of putrefaction ranging from an early to an advanced stage. The causes of tissue putrefaction involve changes resulting from autolysis, enzymolysis, and bacterial alterations[1,2].

In this study methods are described for the purification and extraction of body fluids and tissues. The barbiturates and morphine present in the extraction products are separated and identified by ultraviolet (UV) absorbance followed by gas–liquid chromatography (GLC) and thin-layer chromatography (TLC).

EXPERIMENTAL

Instruments and conditions

A Beckman DK-2A ratio recording spectrophotometer was used for all UV determinations.

GLC was carried out using a Barber Colman Series 5000 gas–liquid chromatograph with a dual hydrogen flame ionization detector. The columns were of U-shaped borosilicate glass, 4 ft. × 4 mm I.D., and packed with 100–120 mesh Delta Scientific ABS (silanized and acid- and base-washed) Chromosorb W, coated with 3% OV-1. The detector temperature was 280°, the injection temperature 260°, and the column temperatures for barbiturates and morphine were 190° and 220°, respectively. Nitrogen was used as the carrier gas with a flow-rate of 35 ml/min and an inlet pressure of 40 lbs./sq.in. The hydrogen to air ratio was 5:3. The attenuation used was 4.0.

For TLC 20 × 20 cm Eastman Chromagram silica gel sheets (No. 6061) were used. The solvent tank was saturated for at least 1 h prior to development. The plate was developed at room temperature for about 18 cm. The solvent systems used for the separation of barbiturates and morphine were chloroform–acetone (88:12) and ethanol–acetic acid–water (60:30:10), respectively.

Separation of barbiturates

Purification and extraction. Ten milliliters of body fluid or 10 g of putrefied tissue are transferred to a steam distilling tube. The contents are acidified with 0.5 ml concentrated hydrochloric acid and the mixture is steam distilled. Between 150 to 200 ml of the distillate are collected. The distillate is discarded and the residue is

extracted according to the technique recommended by Broughton[3] for fresh body fluids. Fig. 1 shows typical absorbance curves for non-putrefied tissue and Fig. 2 shows curves for "positive" putrefied blood samples before (a and b) and after purification (a′ and b′). From the UV absorbance curves a and b of Fig. 2 it cannot be ascertained whether the samples contain barbiturates. Curves a′ and b′, however, show the specific UV absorbance of barbiturates.

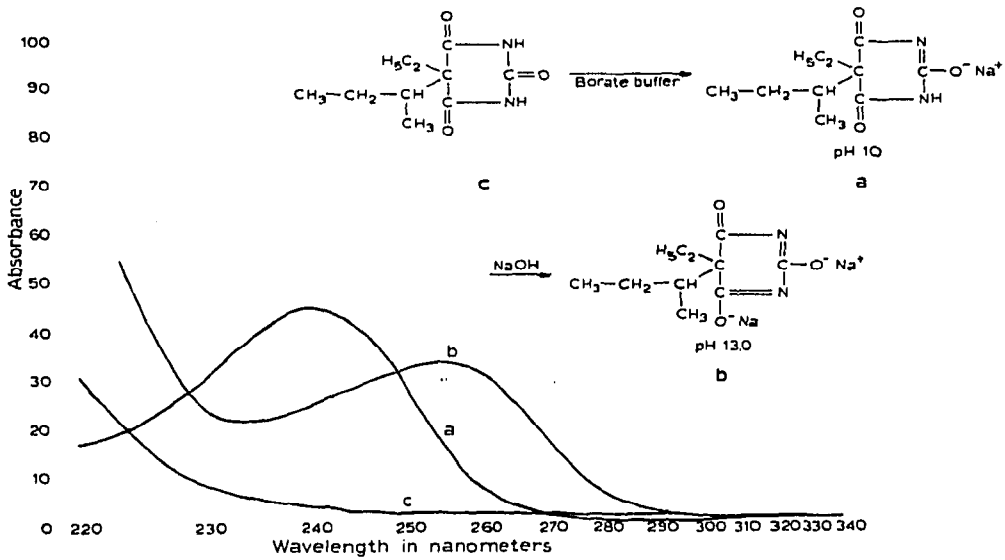

Fig. 1. Typical UV curve showing the separation of butabarbital from non-putrefied blood. a = Borate + sample; b = Sodium hydroxide + sample; c = After acidification.

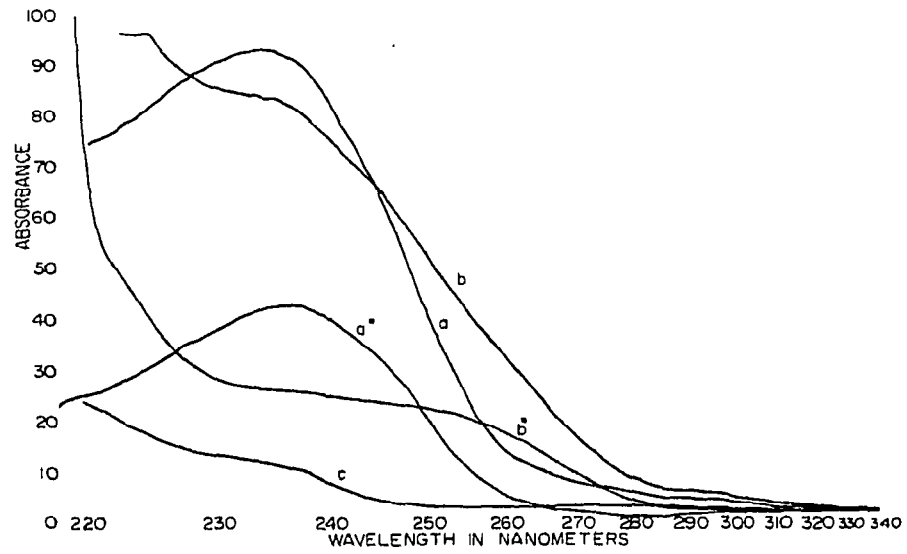

Fig. 2. Typical UV curve of putrefied blood suspected of barbiturate poison. a = Borate + sample before purification; b = sodium hydroxide + sample before purification; a′ = borate + sample after purification; b′ = sodium hydroxide + sample after purification; c = after acidification.

Gas–liquid chromatography. For the purpose of the qualitative and quantitative determination of the individual barbiturates, the following procedure is recommended: The alkaline extract used for the quantitation by UV is acidified and extracted with chloroform. The chloroform is evaporated to dryness on a steam-bath. The residue is dissolved in a minimum amount of isopropanol (about 1 ml). The isopropanol solution is transferred to a calibrated vial and concentrated on a sand-bath to 50 μl. Fifty microliters of the internal standard (glutethimide) in isopropanol (known concentration) are added to a calibrated vial and mixed. Five microliters of the mixture are injected into the gas–liquid chromatograph for the qualitative and quantitative determination of barbiturates. Results are shown in Fig. 3.

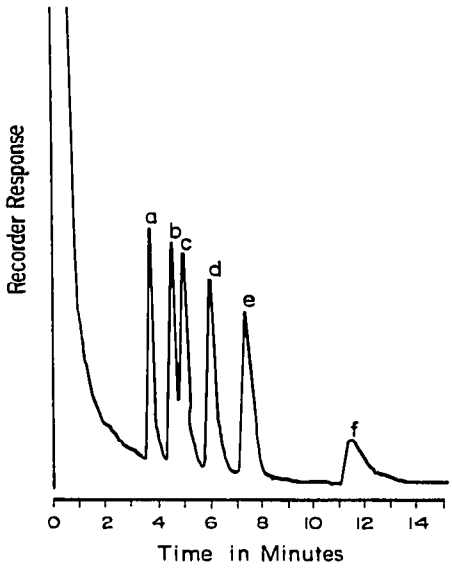

Fig. 3. Separation of five barbiturates with glutethimide as internal standard. Column, 3% OV-1; temperature, 190°, flow-rate, 35 ml/min. a = butabarbital; b = amobarbital; c = pentobarbital; d = secobarbital; e = glutethimide; f = phenobarbital.

Thin-layer chromatography. For the purpose of additional identification, the following procedure is recommended. Aliquots of the isopropanol solution used for GLC are spotted on a silica gel plate. The plate is developed at room temperature in a saturated tank for about 18 cm using chloroform–acetone (88:12) as the solvent system. An authentic sample of the common barbiturates is spotted on the same plate for the purpose of reference. The plate is dried under a hood for 10 min and the spots are identified as follows: The dry plate is exposed to chlorine in a TLC tank for about 2 min. (Chlorine is generated by adding concentrated hydrochloric acid to potassium permanganate.) The chlorine is removed from the plate by leaving it under the hood for 5 min. The plate is subjected to UV light (short wavelength) for 3 min. Then it is sprayed lightly with a saturated solution of benzidine dihydrochloride. The barbiturates present show as blue spots on a brown background[4] (Fig. 4).

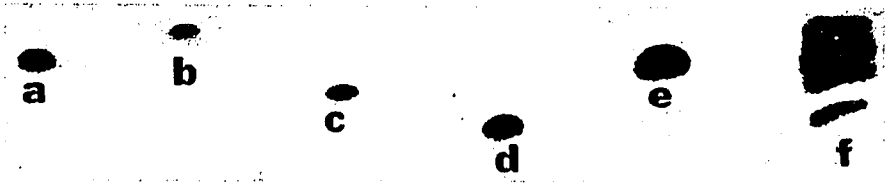

Fig. 4. TLC separation of five barbiturates. a = Pentobarbital; b = secobarbital; c = phenobarbital; d = butabarbital; e = amobarbital; f = mixture of a, b, c, and e. Solvent, chloroform–acetone (88:12).

Separation of morphine

The method of quantitation of morphine from fresh tissues and fluids is that of Christopoulos and Kirch[5]. For putrefied tissues the same method of extracting and purifying is used followed by additional TLC to eliminate false positive morphine. For this TLC procedure the solvent system ethanol–acetic acid–water (60:30:10) is used.

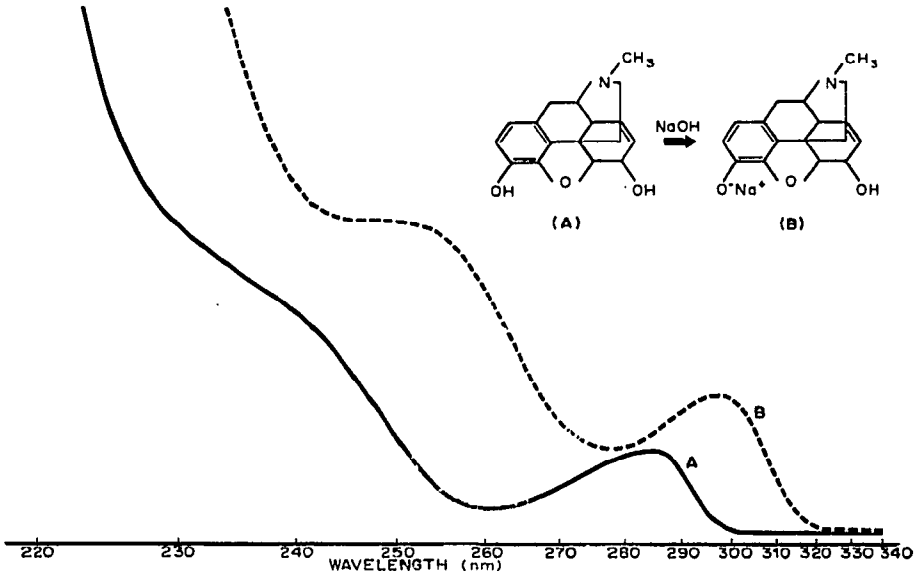

Fig. 5. Typical UV curve of 3.0 mg% standard morphine. A = Sample in methanol + 0.1 *N* hydrochloric acid; B = sample in methanol + 0.45 *N* sodium hydroxide solution.

In Fig. 5 a typical UV curve of 3.0% of standard morphine is shown. Fig. 6 shows the UV spectrum of putrefied tissue, Fig. 7 shows the thin-layer chromatogram (using Davidow's[6] system), and Fig. 8 shows the gas–liquid chromatogram. The false positive morphine shown by Figs. 6–8 is eliminated by the additional TLC procedure (Fig. 9).

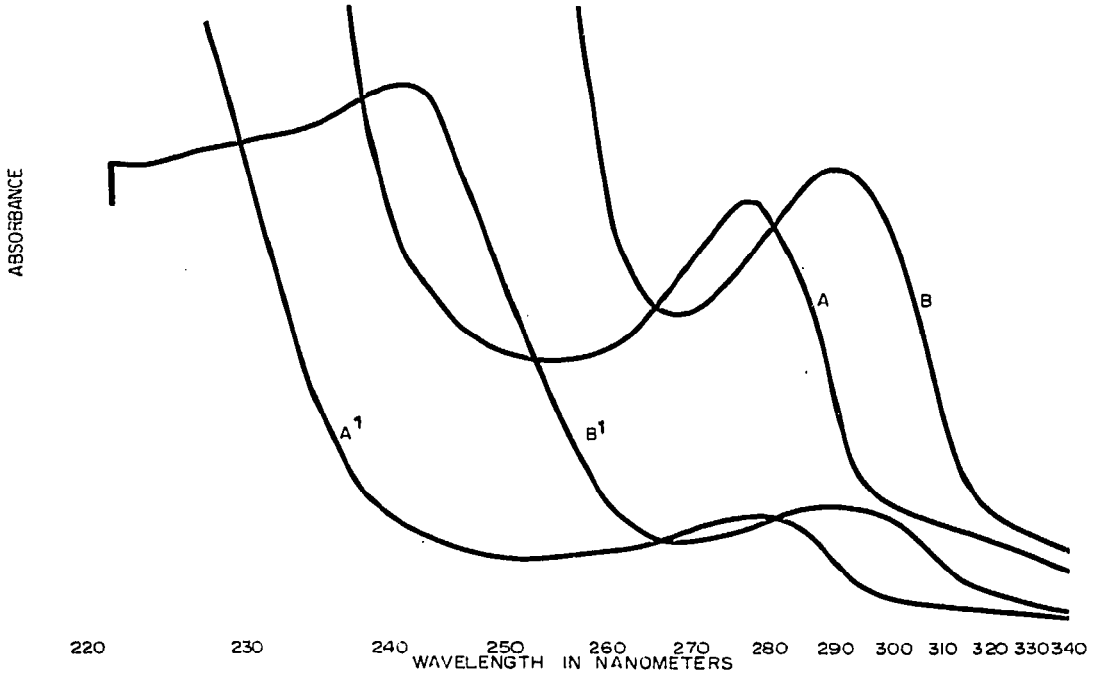

Fig. 6. Typical UV curve of putrefied liver. A = Sample in methanol + 0.1 *N* hydrochloric acid; B = sample in methanol + 0.45 *N* sodium hydroxide solution; A′ = diluted sample in methanol + 0.1 *N* hydrochloric acid; B′ = diluted sample in methanol + 0.45 *N* sodium hydroxide solution.

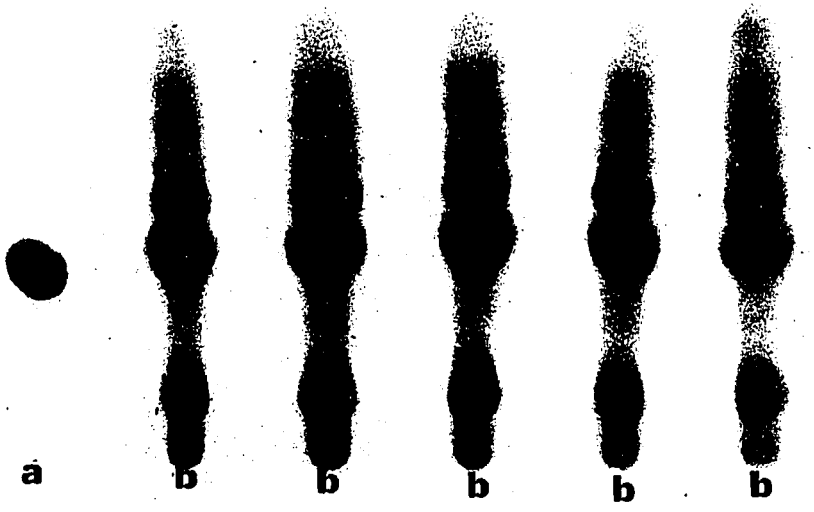

Fig. 7. Typical thin-layer chromatogram of putrefied liver. a = Morphine; b = liver extract. Solvent, ethyl acetate–methanol–ammonium hydroxide (85:10:5).

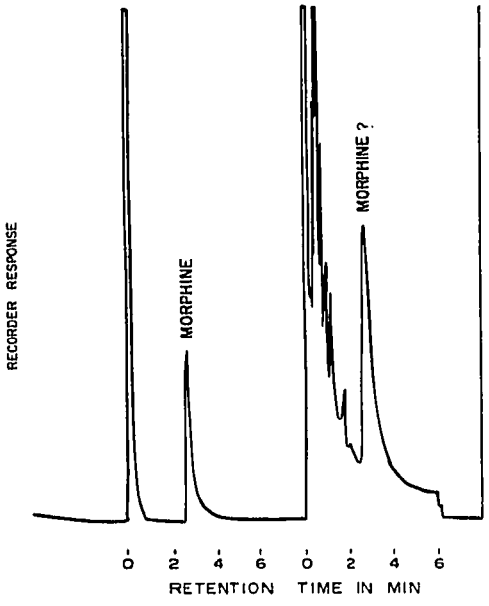

Fig. 8. Typical gas–liquid chromatogram of putrefied liver. Column, 3% OV-1; temperature, 220°; flow-rate, 35 ml/min.

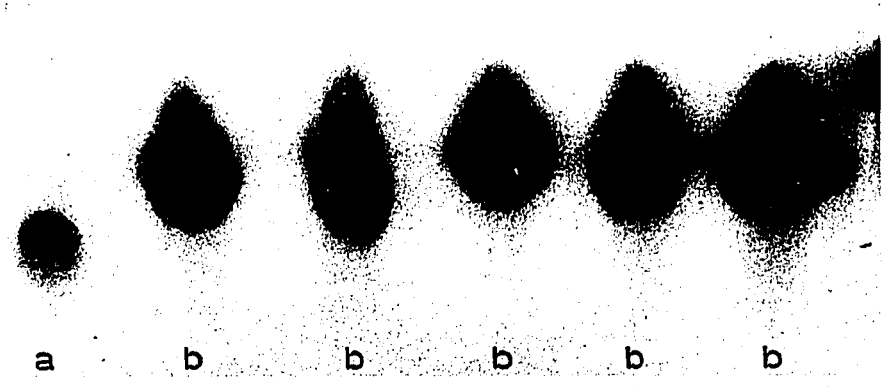

Fig. 9. Typical thin-layer chromatogram of putrefied liver. a = Morphine, b = liver extract. Solvent, ethanol–acetic acid–water (60:30:10).

RESULTS AND DISCUSSION

In Cook County Coroner's Laboratories, as a routine procedure for the identification and estimation of barbiturates in *post mortem* tissue, UV spectroscopy is used for quantitation and occasionally TLC is applied as an additional tool in such identification. However, GLC can be used for quantitation, as has been described above. In a non-purified putrefied specimen the UV spectrum is not conclusive, as shown by curves a and b in Fig. 2 and the gas–liquid chromatogram also shows too many peaks where the retention time for the interference peaks could be misinter-

preted (Fig. 10). However, when the purification and extraction procedure described above is applied to putrefied specimens, the UV spectrum is very conclusive (curves a′ and b′ of Fig. 2) and the gas–liquid chromatogram clearly shows only two peaks (Fig. 11).

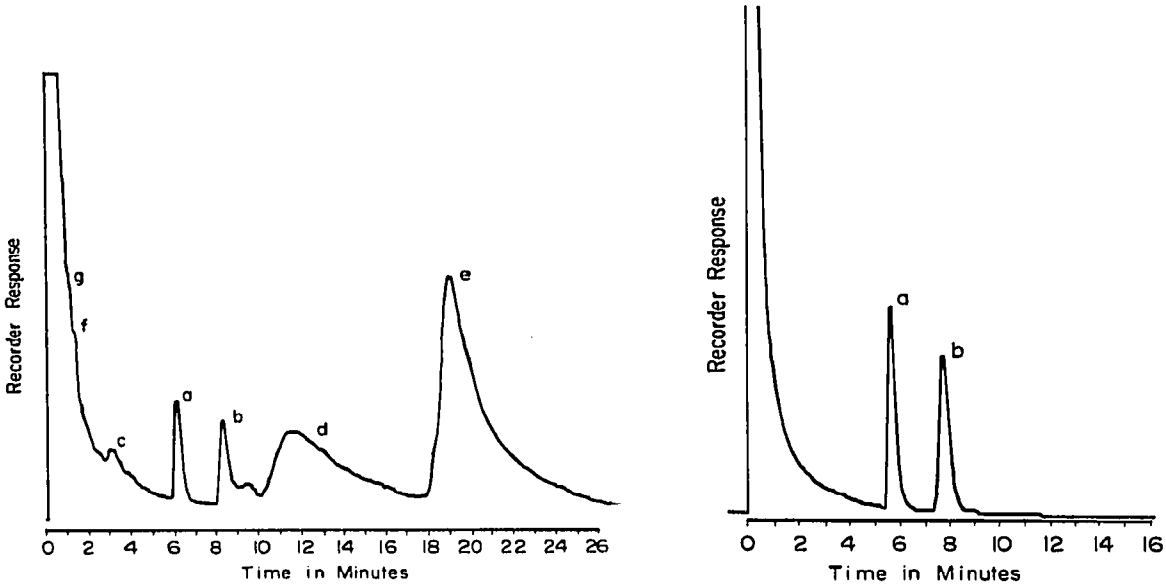

Fig. 10. GLC of non-purified putrefied material in a case of tuinal overdose. a = Amobarbital; b = secobarbital; a, b = tuinal; c, d, e, f, g are interferences (unidentified). Column, 3% OV-1; temperature, 200°; flow-rate, 30 ml/min.

Fig. 11. Separation of tuinal from a putrefied case of blood after purification. a = Amobarbital; b = secobarbital. Column, 3% OV-1; temperature, 180°; flow-rate, 55 ml/min.

Table I shows the per cent recoveries of five common barbiturates applying the procedure described above for putrefied tissues and fluids. The recoveries range from 88 to 96%. As can be seen, the barbiturates are stable to the steam distillation.

Table II shows the effects of putrefaction on barbiturate concentration. The

TABLE I

RECOVERY OF FIVE COMMON BARBITURATES

Barbiturate standard	*Before steaming*	*After steaming*	
	Concentration (mg%)*	*Concentration* (mg%)*	*Recovery (%)*
Butabarbital	1.86	1.78	96
Amobarbital	1.72	1.62	94
Pentobarbital	1.48	1.40	95
Secobarbital	1.37	1.21	88
Phenobarbital	1.92	1.74	91

* Mean value of three determinations. Quantitation by UV.

TABLE II

EFFECT OF PUTREFACTION ON BARBITURATE CONCENTRATION DETERMINATION

Barbiturate	*Specimen*	*Initial concentration (mg%)*	*Concentration after*			
			3 days	*6 days*	*18 days*	*36 days*
Secobarbital	Blood	2.85*	2.99	3.28	3.79	3.80
	Liver	6.74	7.10	8.30	9.10	9.40
	Brain	3.01	3.10	3.30	3.30	3.40
Phenobarbital	Blood	7.5	7.9	8.50	9.30	9.50
	Liver	15.9	16.3	17.9	18.5	21.5
	Brain	9.3	9.8	11.3	10.5	10.9
Pentobarbital	Blood	3.95	4.20	4.70	5.0	5.65
	Liver	6.50	7.20	7.70	9.90	9.62
	Brain	4.20	4.70	4.90	5.10	5.08

* Mean value of three determinations. Quantitation by UV and GLC.

specimens selected were overdose cases of secobarbital, phenobarbital and pentobarbital. The concentrations were determined as received in blood, liver and brain. Twelve equal portions of blood, liver and brain were weighed out and allowed to putrefy at room temperature. Triplicate analyses were performed on these samples after 3, 6, 18, and 36 days of decomposition. From Table II, one can conclude that putrefication increases the concentration of barbiturates. The increases range from 17% in brain tissue to 48% in liver tissue. From these findings one can assume that the protein-bound barbiturates are released.

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