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ISOLATION AND IDENTIFICATION OF MORPHINE FROM *POSTMORTEUM* TISSUES

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SUMMARY

A procedure for the determination of morphine in forensic material has been investigated with the result that a rather rapid and reliable method is available now. Specific recommendations are made using a certain filter paper in order to reduce the loss of the alkaloid. It was further established that a concentration of greater than 5% of hydrochloric acid resulted in an appreciable loss of morphine when used to cleave the glucuronide. Modifications in the solvents used for the thin-layer chromatographic separations facilitated the isolation of morphine. The free morphine was determined by ultra violet spectroscopy and gas–liquid chromatography. It was further established that urine and bile are the specimens of choice, followed by liver and kidney. The concentrate of the alkaloid in blood and spleen is relatively low and samples of spinal and ocular fluid were found to be free of morphine in most cases tested.

INTRODUCTION

Deaths due to morphine intoxication seem to be on the increase. This fact is brought to light by the rather large number of cases encountered in some Coroner's laboratories. In Cook County, Ill., there were about 150 such cases in 1969 and 220 in 1970. It became necessary to have available for routine analysis a method of isolation and detection of morphine in various body fluids and tissues. Furthermore, information concerning the relative amount of the opiate in different body tissues would be of value in deciding which are the most generally useful tissues for routine analysis. This communication describes the determination of morphine in forensic material where (a) certain steps in the generally recommended extraction[1] are eliminated, (b) solvents used in the thin-layer chromatographic (TLC) steps are modified and (c) gas–liquid chromatography (GLC) is used as an additional means of identification.

EXPERIMENTAL

Materials and methods

Solvents. The following solvents are all Analytical Reagent Grade unless otherwise specified: Chloroform, isoamyl alcohol or isobutyl alcohol, isopropyl alcohol, methyl alcohol (spectral A.R.), petroleum ether (b.p. 30–60°), acetonitrile.

Reagents. Phosphoric acid, concentrated 85%, sodium hydroxide solution 30% w/w, *M*/15 dibasic sodium phosphate (9.40 g/l) pH 8.7, ammoniacal silver nitrate reagent (concentrated ammonium hydroxide is added dropwise to 10% w/v silver nitrate solution until a precipitate is formed and the addition is then continued until the precipitate just dissolves), sodium carbonate (A.C.S. certified) anhydrous, chromatogram sheets (silica gel E.K. Co.) without fluorescent indicator, iodine crystals U.S.P., morphine alkaloid U.S.P., filter paper 2v (Whatman No. 12), morphine standard stock solution (containing 100 mg of morphine per 100 ml of solution in 0.5 *N* hydrochloric acid which is diluted to the proper concentrations for the determination of standard reference curve), stock solution in methanol: 100 mg of morphine alkaloid are dissolved in 100 ml of methanol.

Equipment

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

A Barber Colman gas–liquid chromatograph (GLC) series 5000 with dual hydrogen flame ionization detector was used. The columns were of U-shaped borosilicate glass, 4 mm I.D., 6 ft. long and packed with 100/200 mesh Delta scientific ABS (silanized, acid and base-washed) Chromosorb W, coated with a liquid phase 3% OV-1 or 3% QF-1.

The detector temperature was 280°, the injection temperature 260°, and column temperature 230°. Nitrogen was used as a carrier gas with a flow rate of 35 ml/min with an inlet pressure of 40 lbs. The ratio of hydrogen to air was 5:2 and the attenuation used was 512.

Extraction of samples

All the samples used in this study were submitted by the pathologists of the Cook County Coroner's Laboratories.

Isolation of morphine from urine. A 25-ml sample of urine contained in a 250-ml erlenmeyer flask was treated with 2.5 ml of concentrated hydrochloric acid by autoclaving under 15-lbs. pressure for 45 min. After cooling, the mixture was filtered through Whatman No. 12 filter paper. The filtrate was then transferred to a separatory funnel and extracted with 50 ml of chloroform to remove the interfering chromogens and other possible interfering "Acidic Medicinals" such as aspirin or phenacetin. After shaking the mixture vigorously for 3 min, the organic phase was filtered through Whatman No. 12 filter paper and the resulting filtrate was evaporated on a steam bath. (The residue consists primarily of acid drugs and is free of morphine[2,3].) The aqueous layer was then adjusted to pH 11.5 with *ca.* 5 ml of 30% sodium hydroxide. 100 ml of the chloroform–isobutanol (4:1) mixture was added and the mixture was shaken again for 3 min. After separation of the two phases the organic layer was filtered through a Whatman No. 12 filter paper into an evaporating dish and

evaporated on the steam bath. (It should be noted here that the residue does not contain morphine.) The aqueous layer was then acidified with concentrated phosphoric acid and slowly brought to pH 8.7 by adding solid sodium carbonate, and then transferred to a separatory funnel where 200 ml of the chloroform–isobutanol (4:1) mixture was added. This was followed by the addition of 2.0 ml of ammoniacal silver nitrate to remove the purines and other substances which still may be present. The mixture was shaken manually for 3–5 min. After separation of the two phases the organic layer containing relatively pure morphine was filtered through Whatman No. 12 filter paper into a 500-ml separatory funnel. In order to remove water soluble bases completely the organic phase was washed with three 50-ml portions of phosphate buffer pH 8.7 and again filtered through a Whatman No. 12 filter paper. The filtrate was evaporated on a steam bath. This residue contains morphine in a relatively pure state. Morphine thus isolated was dissolved in hot methanol (60°) and 3 ml of the solution was placed in a 1-cm quartz cell and the UV spectrum was obtained using 3 ml of hot methanol (60°) in the reference cell (340–210 nm). If morphine is present, a broad peak is noticed at 285 nm and on addition of alkali the peak is shifted to 295 nm. Such a shift is very characteristic of sodium phenoxides. A second maximum peak at 250 nm was observed when the methanolic solution was made alkaline. The absorbance can be quantitated against a standard morphine solution (Fig. 1).

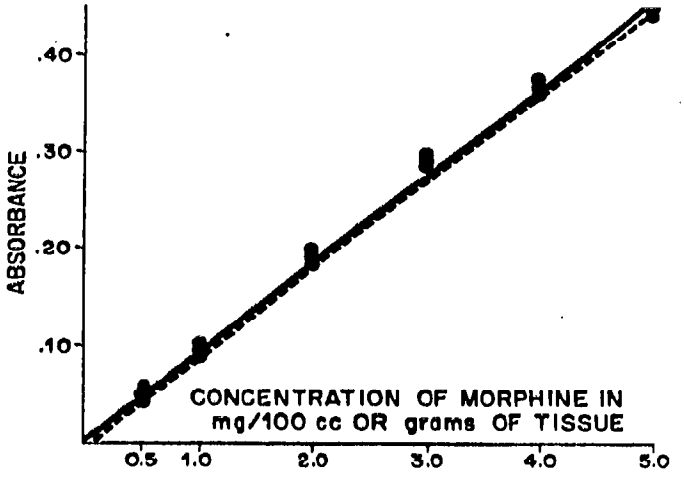

Fig. 1. Plot of absorbance *versus* concentration. ●—●, Standard curve; ●---●, Curve obtained when stock solution was treated as recommended in extraction procedure.

Isolation of morphine from bile. The procedure used for urine can be applied here unless a smaller quantity than 25.0 ml of bile is available in which case enough water is added to adjust the volume to 25 ml prior to the autoclaving.

Isolation of morphine from liver, kidney, brain, and spleen. To 25 grams of minced liver in a 400-ml beaker, 10 ml of distilled water plus 2.5 ml of concentrated hydrochloric acid was added. The mixture was then autoclaved under 15-lbs. pressure for 45 min. After cooling the slurry was transferred to a separatory funnel and washed twice with 100-ml portions of petroleum ether (b.p. 30–60°) to remove any lipid materials. The petroleum ether was discarded and from here on the procedure of extracting morphine is the same as that used for urine.

Isolation of morphine from blood. To 250 ml of blood was added *ca.* 20 g of sodium bicarbonate and 8 drops of concentrated ammonium hydroxide; the sample was then homogenized for about 10 min. It was then transferred to a 2-l glass bottle and 1500 ml of an ether–acetonitrile (2:1) mixture was added. The bottle was then shaken in an automatic shaker for 1 h. The mixture was allowed to separate and the organic solvent filtered into a 2-l beaker. (By then the blood is usually in a "dry", granulated state, and the filtering offers no problems.) Concentrated hydrochloric acid (15 drops) was added to the filtrate in the beaker, and the latter was placed on a steam bath and the organic solvent allowed to evaporate in a stream of air or nitrogen.

When evaporation was completed the contents of the beaker were transferred to a 250-ml separatory funnel using ether and 0.2 *N* hydrochloric acid successively. Several rinsings of the beaker were necessary but the total volume of ether used should be *ca.* 200 ml and the total volume of 0.2 *N* hydrochloric acid about 50 ml.

The separatory funnel was shaken for 1 min. After standing, the lower aqueous acidic layer was separated and then centrifuged and the final clear aqueous solution transferred to another 250-ml separatory funnel. The ether was discarded. The aqueous solution after adjusting to pH 8.7 (using 30% sodium hydroxide and sodium bicarbonate and phosphoric acid if necessary) was extracted with 200 ml of a chloroform–isobutanol mixture (4:1).

The solvent, after separation, was filtered and evaporated to dryness. The residue consists of morphine, which was dissolved in methanol and the resulting solution was scanned in the UV.

Isolation of morphine from spinal, ocular and amniotic fluids. Spinal fluid, ocular fluid, and amniotic fluid are relatively free from chromogens and there is no need for the purification steps.

After hydrolysis the pH is adjusted to 8.7 and extraction is effected with chloroform–isobutanol (4:1). The organic layer is filtered and evaporated on the steam bath. The quantitation is then done the same way as that of urine. Unfortunately, very little morphine is found in the above mentioned fluids.

When it is necessary to further identify and purify the sample of morphine obtained from any of the above tissues or fluids, the following procedure is recommended. The methanol solution of the morphine extracted from various tissues is spotted on a silica gel plate and chromatographed using ethyl acetate–methanol–ammonium hydroxide (85:10:5) as the developing solvent. An authentic sample of morphine is also spotted on the same plate for the purpose of reference. The plate is dried under the hood for 10 min and the spots are developed in iodine vapors.

In cases (very rare) where additional evidence may be necessary the following procedure is recommended. The TLC separation is repeated and only the reference sample of morphine is exposed to iodine vapors. By comparison, spots are located on the plate without spraying with iodine and the silica gel with the morphine is scraped off into a 30-ml beaker and a few drops of phosphate buffer (pH 8.7–9.0) were added. 10 ml of the solvent chloroform–isobutanol (4:1) are added and warmed on the steam bath to assure complete elution. This is repeated twice. The eluate is filtered and then evaporated on the steam bath. The residue is dissolved in hot methanol and scanned at the UV again for purposes of qualitative detection.

Additional qualitative evidence can be obtained by use of GLC separation

where, in most instances, after quantitation by the UV method and after TLC isolation, enough residue is left to demonstrate the presence of morphine by the following method:

The residue is dissolved in 1 ml of hot methanol and transferred to a 3-ml

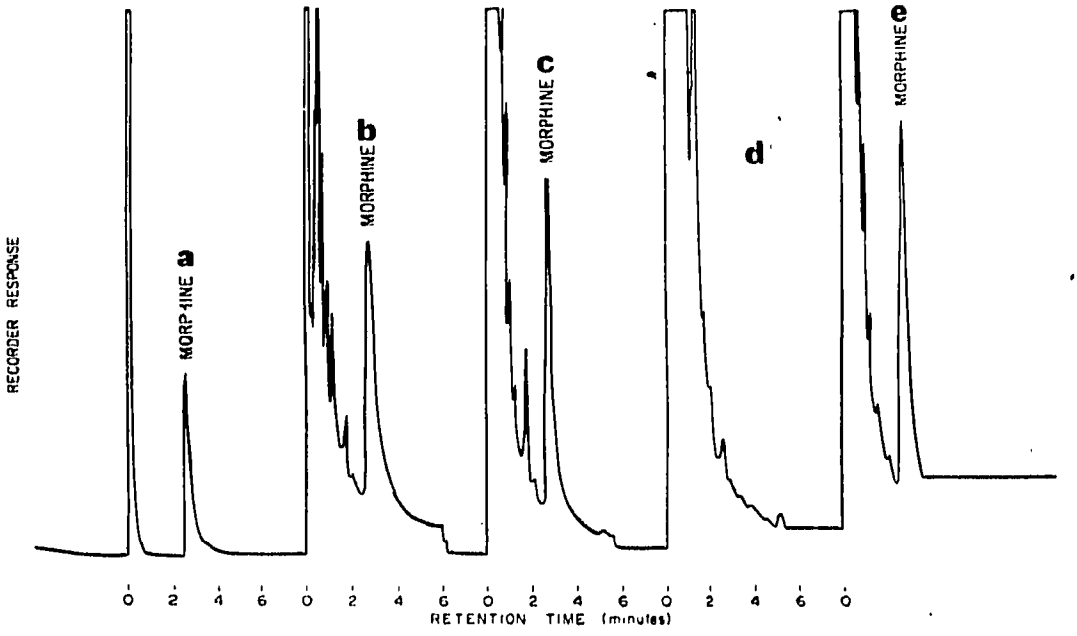

Fig. 2. GLC of a positive case: (a) morphine stand; (b) bile extract; (c) urine extract; (d) negative case; (e) liver extract. (Before purification.)

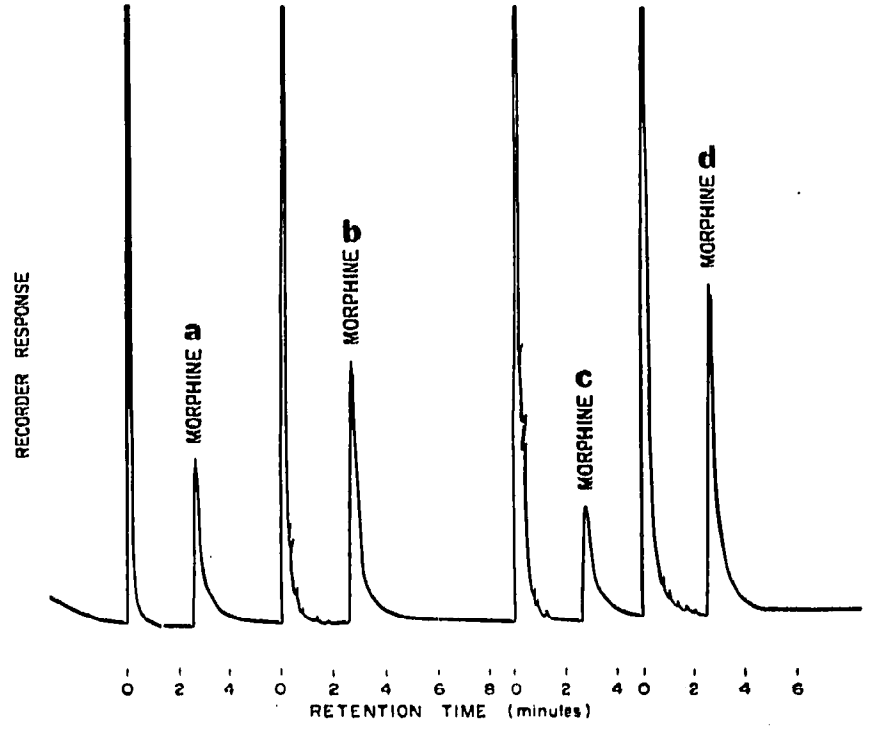

Fig. 3. GLC of (a) morphine stand; (b) bile extract; (c) urine extract; (d) liver extract. (After purification.)

conical vial. After evaporating the methanol on a steam bath two drops of isopropanol are added to dissolve the residue. 5 μl of this solution are then injected into the gas chromatograph. The results obtained by the use of either one or both of the columns recommended (OV-1, QF-1) may serve as final evidence for the presence or absence of morphine (Figs. 2, 3). Using isopropanol in place of methanol will prevent bleeding of the columns.

Separation of medicinals found in some tissue samples containing morphine

Occasionally one finds in addition to morphine, medicinals such as codeine, pentazocine, apomorphine, and quinine. These are the most frequently encountered

TABLE I

THIN-LAYER CHROMATOGRAPHY DATA

S_1 = Ethyl acetate–methanol–ammonia (85:10:5); S_2 = ethanol–benzene–dioxane–ammonia (5:50:40:5); S_3 = ethanol–pyridine–dioxane–water (50:20:25:5); S_4 = chloroform–diethylamine (9:1); S_5 = methanol–butanol–benzene–water (60:15:10:15); S_6 = ethanol–acetic acid–water (60:30:10).

Compound	*R_F values*[a]					
	S_1	S_2	S_3	S_4	S_5	S_6
Morphine	0.59	0.36	0.50	0.48	0.29	0.50
Codeine	0.78	0.72	0.65	0.59	0.35	0.58
Pentazocine	0.93	0.89	0.85	0.65	0.50	0.78
Quinine	0.87	0.78	0.75	0.90	0.65	0.63
Heroin	0.65	0.95	0.90	0.89	0.75	0.70
Apomorphine	0.98	0.45	0.95	0.90	0.80	0.67

[a] Mean values after six determinations.

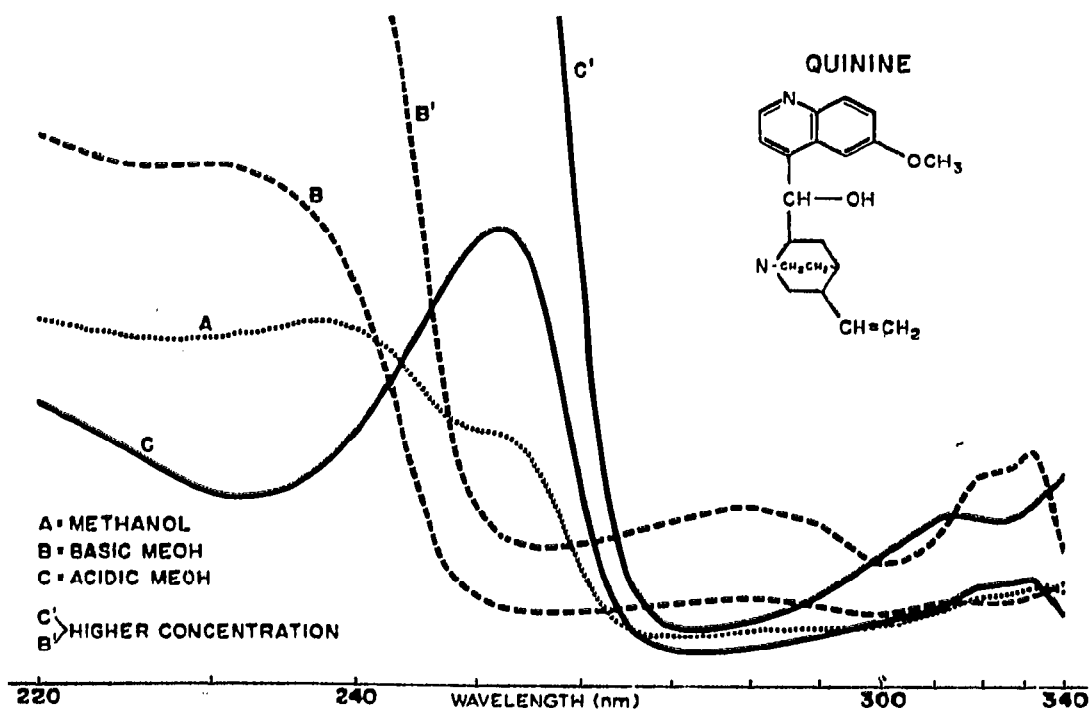

Fig. 4. A typical UV curve of quinine.

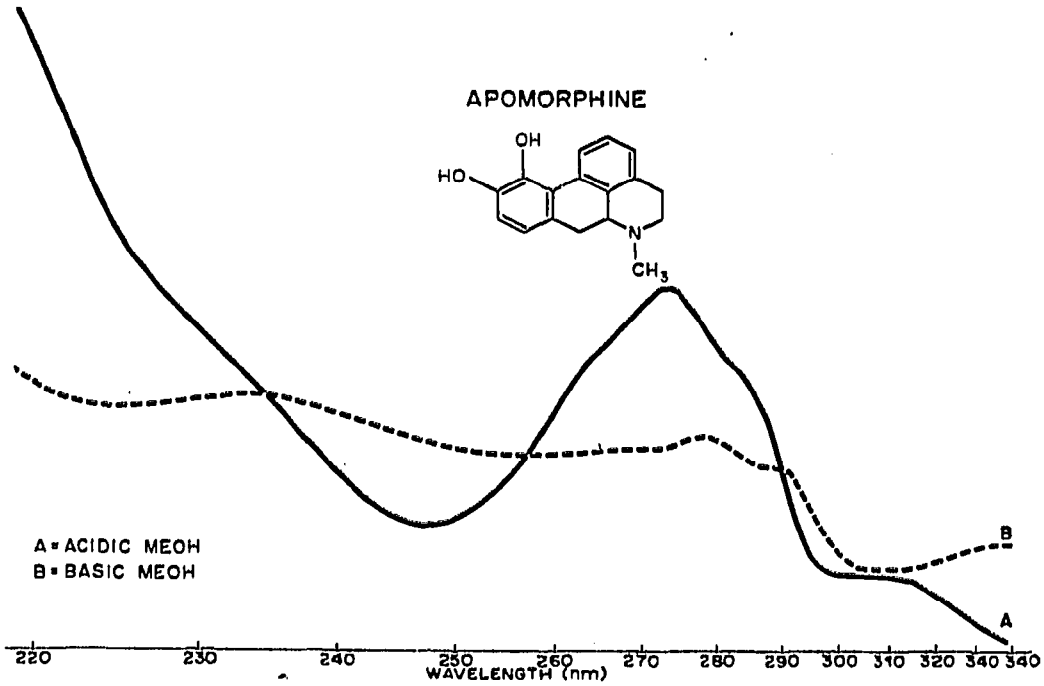

Fig. 5. A typical UV curve of apomorphine.

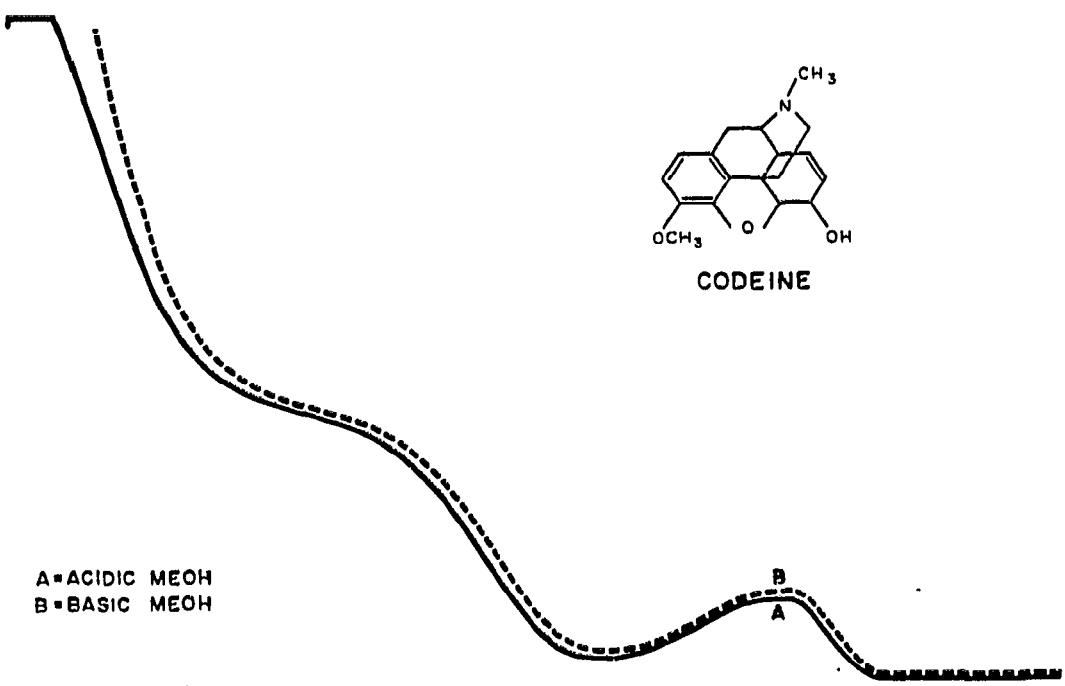

Fig. 6. A typical UV curve of codeine.

substances in samples containing morphine. Any one of these medicinals can be separated from morphine by use of TLC using several solvent systems. Results are shown in Table I.

Untreated spots of a concurrently run sample can be eluted with chloroform–isobutanol (4:1) as the eluate. After evaporation of the solvent the residue was dissolved in hot methanol and the UV curves are obtained as shown in Figs. 4–7.

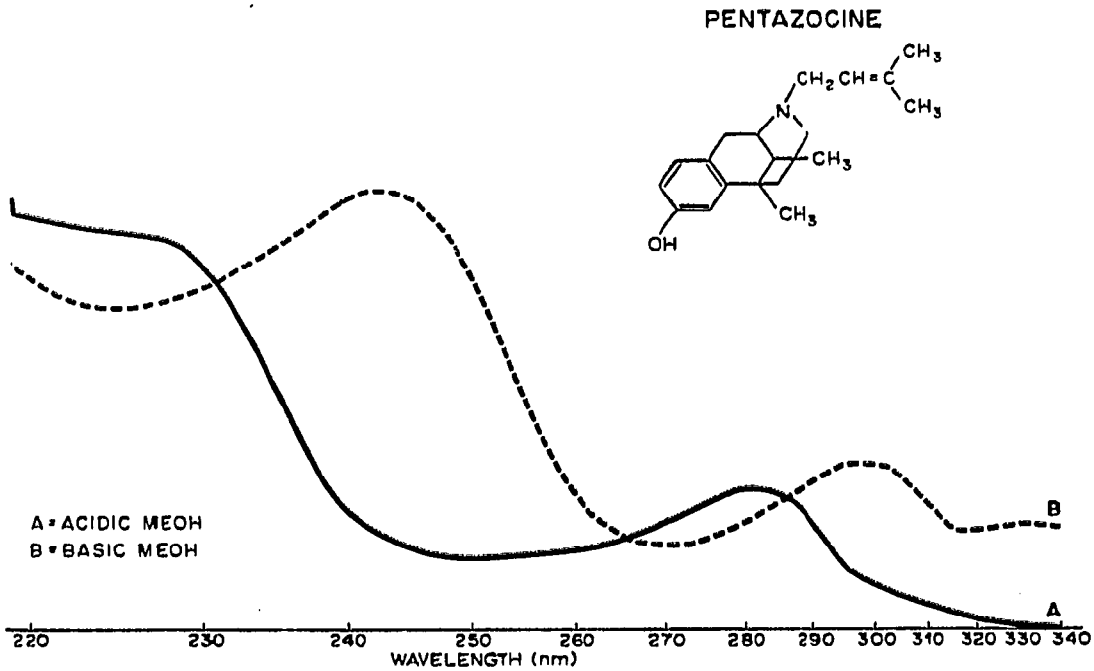

Fig. 7. A typical UV curve of pentazocine.

Quantitation of morphine

For the determination of absorbances certain aliquots of methanolic solution are diluted with methanol so that the dilution may vary from 5 to 50 μg per ml of solution. The absorbance is measured at 295 nm. A typical reference curve is shown in Fig. 1, where a straight line is obtained when absorbance *vs.* concentration is plotted, indicating the Beer-Lambert law is obeyed. In order to check any possible loss in

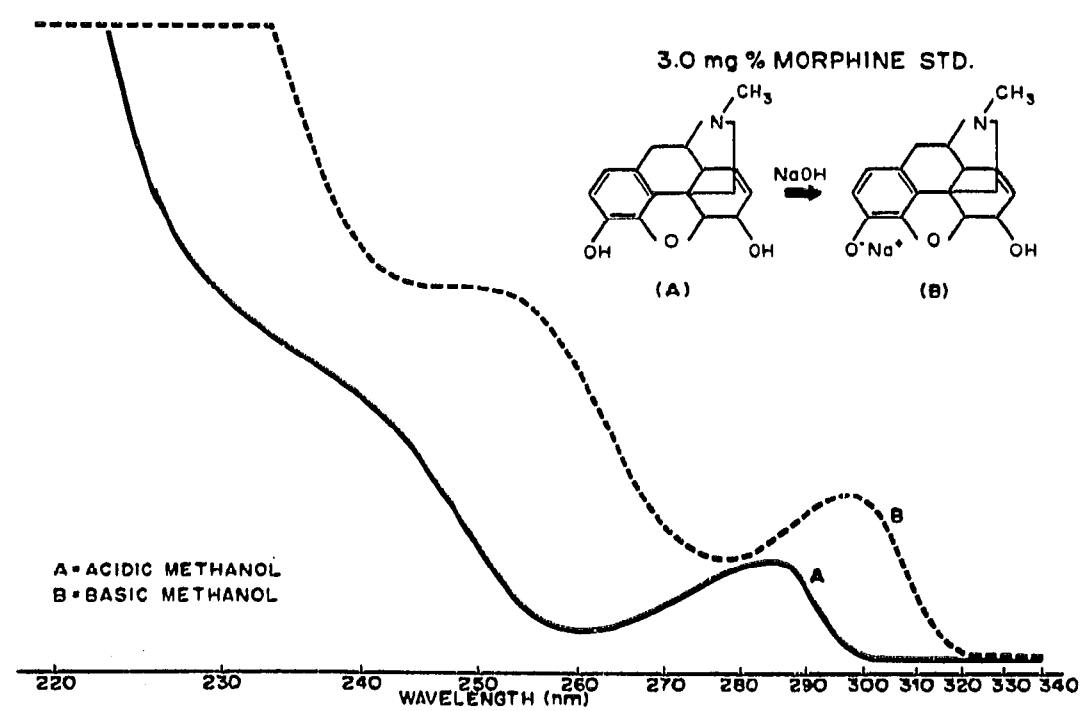

Fig. 8. A typical UV curve of morphine.

the recovery of the alkaloid during the procedure of isolation of the morphine from body fluids one of the following methods are recommended.

0.5, 1.0, 2.0, 3.0 and 5.0 ml of acid stock solution are diluted to 100 ml with water. 25 ml of each of the dilutions were used as a blank in all determinations. A typical reference curve obtained by this procedure is shown in Fig. 1 indicating that there may be a loss of 3 to 5% morphine during the extraction procedure. This curve should be used with all unknowns. A typical UV curve of 30 μg per ml standard morphine is shown in Fig. 8.

A second method that can be used is the standard addition method which could be employed if enough sample is available to run at least three determinations. One aliquot is used to run the determination in the usual manner and two more aliquots to which different but known amounts of morphine have been added (see Table II).

A typical reference curve applying the above methods is shown in Fig. 9.

TABLE II

THE STANDARD ADDITION METHOD

Case No.	*Sample*	*Absorbance at 295 nm*[a]	*mg% morphine added*[a]
70-2157	Urine	0.04	0.0
70-2157	Bile	0.06	0.0
70-2045	Urine	0.10	0.5
70-2045	Bile	0.13	0.5
70-2038	Urine	0.16	1.0
70-2038	Bile	0.19	1.0
70-1970	Urine	0.26	2.0
70-1970	Bile	0.27	2.0
70-2028	Urine	0.36	3.0
70-2028	Bile	0.38	3.0
70-2095	Urine	0.29	0.0
70-2985	Bile	0.36	0.0
70-2103	Urine	0.41	1.0
70-2103	Bile	0.48	1.0
70-2216	Urine	0.53	2.0
70-2216	Bile	0.59	2.0

[a] Mean values after three determinations.

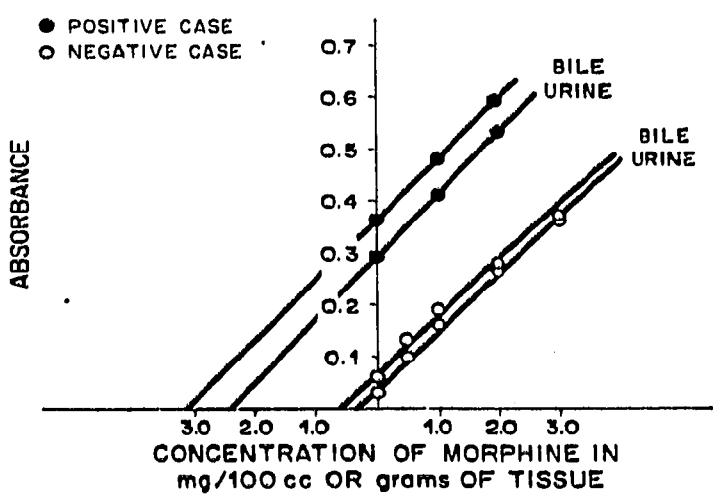

Fig. 9. Measurement of morphine concentrations in bile and urine by standard addition method.

DISCUSSION

Methods generally used for the identification as well as determination of morphine in biological materials make use of TLC, GLC, and spectrophotometry.

In general, the morphine is excreted in urine mainly as the glucuronide. For purpose of isolation of the free alkaloid the urine is heated with hydrochloric acid and heated under 15-lbs. pressure for about 30–45 min (Table III). A number of the methods advise the use of 15% hydrochloric acid without any consideration as to what may happen to morphine under these conditions[4,5].

TABLE III

TYPICAL RESULTS OF TWELVE SAMPLES OF URINE SELECTED AT RANDOM

Case No.	*mg% morphine[a] before hydrolysis*	*mg% morphine[a] after hydrolysis*
70-2211	1.6	6.8
70-2216	0.8	4.9
70-2251	1.9	5.9
70-2268	3.0	14.5
70-2305	1.2	5.0
70-2306	0.5	3.6
70-2329	9.9	29.9
70-2334	0.0	1.8
70-2335	2.9	19.5
70-2337	1.0	5.6
70-2351	0.0	0.0
70-2360	0.0	0.0

[a] Mean values for three determinations.

TABLE IV

EFFECTS OF HYDROCHLORIC ACID CONCENTRATION AND THE TIME OF HEATING ON HYDROLYSIS OF THE CONJUGATES IN URINE

% Hydrochloric acid	*Autoclaving time (min)*	*3 mg% morphine[a] standard*	*mg% morphine per 100 mg urine (positive urine)*	*3 mg% morphine[a] added (positive urine)*
0	0	3.00	2.10	4.90
2	15	2.95	4.30	6.90
2	30	2.96	6.80	9.80
2	45	2.98	6.90	9.90
3	15	3.00	6.00	9.10
3	30	3.10	6.90	9.90
3	45	3.10	6.80	10.12
5	15	3.10	6.60	9.40
5	30	2.85	6.50	9.20
5	45	2.85	6.40	9.25
10	15	2.55	5.80	8.40
10	30	2.40	5.44	7.84
10	45	2.40	5.44	7.83
15	15	1.75	3.90	5.70
15	30	1.60	3.74	5.34
15	45	1.65	3.74	5.40

[a] Mean value after nine determinations.

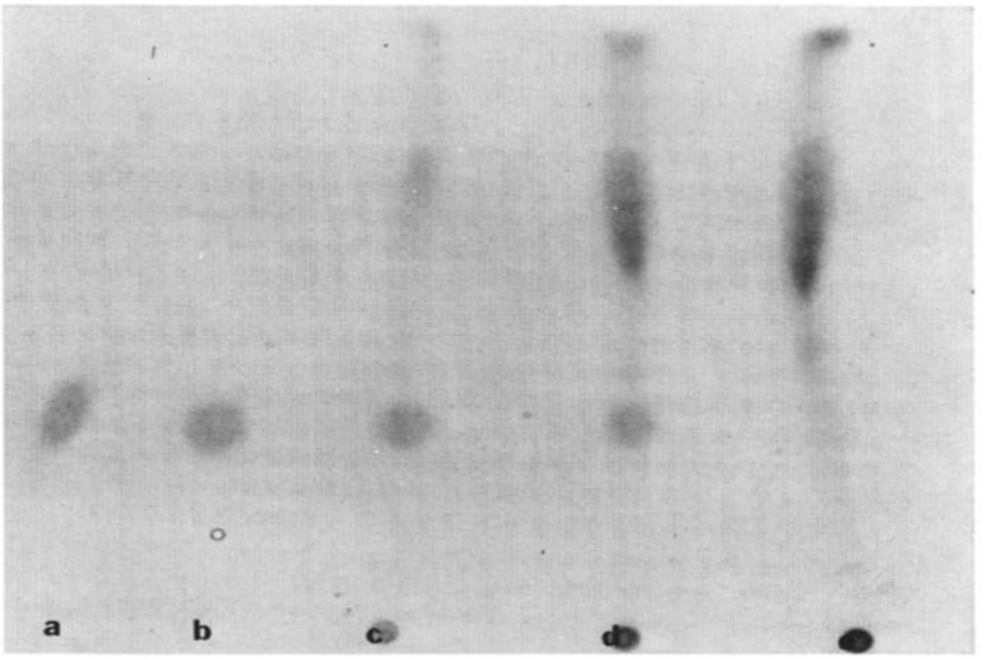

Fig. 10. TLC of (a) morphine stand.; (b) 5% HCl; (c) 10% HCl; (d) 15% HCl after hydrolysis; (e) apomorphine. Solvent: ethyl acetate–ammonia (9:1).

It is not too surprising to find a loss of morphine using this concentration of the acid (Table IV). We found that any concentration greater than 5% would destroy morphine with the resulting formation of apomorphine (Table IV, Fig. 10).

The filter paper used or recommended in certain published procedures[1,6] was Whatman No. 32 and No. 42. Both of these permit rather rapid filtration but unfortunately a certain amount of morphine was retained on the paper. This could and did result in a number of "negative samples" (Table V). We found that this loss could be eliminated using Whatman's paper No. 12, although the filtration was not as rapid.

It should be noted that once the alkaloid is extracted by the organic solvent at pH 8.7 this solvent is filtered and evaporated and the resulting residue dissolved in methanol at 60°. This is at variance with the recommended procedures by several

TABLE V

EFFECT OF FILTER PAPER USED FOR MORPHINE IDENTIFICATION

Case No.	*Sample*	*Filter paper used*	*Results mg% morphine*[a]
70–2334	Bile	Whatman No. 3	—
70–2334	Bile	Whatman No. 4	—
70–2334	Bile	Whatman No. 12	0.50
70–1434	Urine	Whatman No. 3	0.90
70–1434	Urine	Whatman No. 4	0.40
70–1434	Urine	Whatman No. 12	1.70
70–1434	Urine	Whatman No. 32	1.40
70–1434	Urine	Whatman No. 41	1.10
70–1434	Urine	Whatman No. 50	1.30

[a] Mean values for six analyses.

authors[1,4,5,7] who advise the extraction of the morphine from the organic solvent by use of 1 *N* or 2 *N* sulfuric acid. It was found that there is a rather appreciable loss of morphine in this step as observed by the fact that the organic solvent still contains some of the alkaloid (Table VI). In the recommended procedure washing the organic solvent with phosphate buffer (pH 8.7), followed by filtration and evaporation as mentioned above, results in no loss of morphine.

TABLE VI

EFFECT OF SOLVENT EVAPORATION *vs.* EXTRACTION WITH 1 *N* SULFURIC ACID OF MORPHINE ON THE FINAL STEP OF QUANTITATION

Case No.	*Sample*	*Residue after evaporation mg% morphine*[a]	*1 N H_2SO_4 extract mg% morphine*[a]
70–2211	Urine	6.8	5.95
70–2216	Urine	4.9	4.3
70–2251	Urine	5.9	5.0
70–2268	Urine	14.5	12.8
70–2305	Urine	5.0	4.3
70–2306	Urine	3.6	3.1
70–2329	Urine	30.0	25.5
70–2335	Urine	19.5	17.2
70–2337	Urine	5.6	4.7

[a] Mean values for three determinations.

In forensic work the number of body tissues and fluids are, or may be, limited at times. For the detection of morphine, either urine or bile are the tissues and fluids of choice. Both of these have been shown to have the highest concentration of the alkaloid (Table VII).

TABLE VII

DISTRIBUTION OF MORPHINE IN FATALITIES

Sample	*Amount of tissue (mg/100 grams)*				
	Case No.				
	70–1914	*70–1928*	*70–908*	*70–1004*	*70–1255*
Bile	7.6	9.0	3.50	14.5	7.0
Urine	4.2	5.4	7.60	9.0	4.5
Liver	1.1	1.2	0.50	2.5	0.5
Kidney	0.8	1.0	.05	1.9	0.7
Spleen	Trace	Trace	—	—	—
Blood	Trace	Trace	—	0.4	—
Spinal fluid	Trace	Trace	0.30	0.5	1.0
Amniotic fluid	—	—	—	0.7	—
Ocular fluid	—	—	None	None	None

Comparing the concentration of morphine in urine with that in bile, one may establish the cause of death in regard to morphine poisoning. It is assumed that if the concentration of the opiate is higher in urine, death has occurred due to chronic

poisoning or a relatively higher concentration in the bile would indicate acute poisoning.

However, should there be no specimen of urine or bile available for analysis, it is recommended that liver be used. In the absence of liver, samples of kidney could be used as a last resort.

Extremely large quantities of blood, spleen, and brain are required to demonstrate the presence of morphine in these tissues. Spinal fluid and ocular fluid were also investigated because these fluids are clear and free of the many chromogens that the other tissues have. It proved to be very disappointing that in ocular fluid no morphine was found in known morphine fatalities, where in spinal fluid only a small amount of morphine was found.

TABLE VIII

GAS–LIQUID CHROMATOGRAPHY DATA

Compound	*Retention time (min)*			
Temperature (°C)	*225°*	*250°*	*225°*	*225°*
Inlet pressure (ml/min)	*40*	*70*	*36*	*36*
Column	*1% SE-30*	*5 % SE-30*	*3 % QF-1*	*3 % OV-1*
Morphine	2.8	4.0	2.5	2.9
Codeine	2.0	2.9	2.0	2.3
Pentazocine	1.5	2.0	1.8	2.0
Quinine	10.8	13.0	6.8	5.0
Heroin	5.0	9.0	3.8	3.3
Apomorphine	8.0	11.0	5.6	4.9

The free and purified morphine is determined by several instrumental procedures. One of these is the determination of the UV spectrum which is sufficient in most cases. Should additional purification be necessary, TLC separation could be employed. This serves not only as further purification but also as a method of identification. If forensic evidence is needed, the thin-layer plate could be retained as evidence for legal purposes. A third method used for the detection and estimation of morphine, is a GLC procedure using 3% QF-1 and 3% OV-1 columns (Table VIII).

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