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# A SENSITIVE THIN-LAYER CHROMATOGRAPHIC TECHNIQUE FOR DETERMINING MORPHINE IN URINE

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## SUMMARY

A screening procedure is described for determining morphine and other narcotics in acid-hydrolyzed urine. A simple pH adjustment and extraction are followed by washing the extracting solvent with a phosphate buffer which effectively removes hydrolytic products that normally produce inferior chromatograms. Interfering substances are discussed, with special reference to methadone, nicotine, caffeine, and phenothiazine compounds.

## INTRODUCTION

The analytical toxicologist must detect both toxic and subtherapeutic levels of psychotropic agents in biologic fluids and tissues. No single instrumental technique is suitable for the analysis of all drugs; therefore, effective screening tests that identify the presence of groups of drugs are desirable. Thin-layer chromatography (TLC) has proven to be a rapid method that provides high sensitivity and exhibits separation characteristics equivalent to other chromatographic systems. Specificity can be enhanced by appropriate control of three conditions: (a) the extraction procedure, (b) the solvent system for developing the chromatogram, and (c) the chromatographic spray reagent. We have found that TLC techniques described in the literature lack validity in the analysis of acid-hydrolyzed specimens.

This paper describes a unique extraction procedure for analyzing opiates and other alkaline drugs of abuse in extracts from acid-hydrolyzed urine. An earlier report[1] not only substantiated the need for hydrolysis in determining total morphine in urine but distinctly demonstrated that exclusion of that step in the analytical

EXPERIMENTAL

*Materials*

*Extraction solvent.* 10 ml of *tert.*-butanol (Mallinckrodt Chemical Works No. 2998, or equiv.) were mixed with 90 ml of chloroform (Mallinckrodt No. 440G, or equiv.).

*Phosphate buffer.* 35 g of dibasic potassium phosphate (J. T. Baker Chemical Co. No. 3252 or equiv.) were dissolved in 100 ml of water. The pH was adjusted to 10.1 ± 0.1 by the further addition of dibasic anhydrous sodium phosphate (Mallinckrodt No. 7917G, or equiv.).

*Borate buffer.* A saturated solution of sodium borate (Borax®) with a pH of 9.5 was prepared by placing 6 g of sodium borate decahydrate in 100 ml of water.

*Chromatographic solvents.* Solvent A consisted of 1-butanol (Fisher Scientific Co. No. A-3999, or equiv.), water, and glacial acetic acid (J. T. Baker Co. No. 9507, or equiv.) in a ratio of 4:2:1. Solvent B contained 1-butanol in concentrated hydrochloric acid (Fisher Scientific Co. No. A-144, or equiv.) in a ratio of 9:1. The mixture was saturated with water and the aqueous phase discarded. Solvent C consisted of methanol (Mallinckrodt No. 3016G, or equiv.), water, glacial acetic acid, and benzene (Merck No. 7216, or equiv.) in a ratio of 80:15:2:5.

*Thin-layer chromatographic apparatus.* Chromatographic plates (Uniplate®) Silica Gel G, 250 $\mu$, 20 cm × 20 cm (Analtech, Inc., Newark, Del.).

*Chromatographic tanks.* Glass tanks 3.0 × 10.5 cm, 9.0 cm deep with glass plate lid sealed with vacuum grease.

*Potassium carbonate.* Anhydrous, analytical reagent (Mallinckrodt No. 6814, or equiv.).

*Iodoplatinate reagent.* 1 g of chloroplatinic acid (platinic chloride) was dissolved in 25 ml of water. 10 g of potassium iodide were added to 300 ml of water. The two solutions were combined and diluted to 400 ml with water. This reagent was stored under refrigeration. For the working spray the stock solution was diluted 1:2 with water. The working spray is stable for two to three days if stored in a brown glass bottle.

*Procedure*

10 ml of urine (or 10 g of homogenized tissue) and 1 ml of 6 *N* hydrochloric acid solution were pipetted into a 125-ml erlenmeyer flask. The flask and contents were autoclaved for 15 min at 120° and allowed to cool. The hydrolyzed urine was adjusted to pH 8.5–9.0 by the addition of anhydrous potassium carbonate and (or) saturated borate buffer and filtered through Whatman No. 1 or equivalent, filter paper into a glass-stoppered 50-ml graduated cylinder. This filtration step of the aqueous sample removes approximately 20% of the opiate and may be omitted. 30 ml of solvent — 10% *tert.*-butanol in chloroform — were added, and the flask was shaken for approximately 3 min. After phase separation, which occasionally required centrifugation if the above filtration step was omitted, the aqueous layer was discarded. The solvent was washed twice with 10 ml of 35 % $K_2HPO_4$, the phosphate wash was discarded, and the solvent was filtered through a double layer of filter paper into a 125 ml (# 24/40) flask. The flask was attached to a rotary vacuum

ness, four to six drops of isopropanol were added, and the flask rotated to allow solution of the residue. For the analysis of a large number of specimens the chloroform–alcohol solvent can be evaporated from several test tubes simultaneously by the application of heat and by passing a stream of air into each tube via a suitable manifold. With micropipets (50–100 $\mu$l), one or more standards (1 mg/ml solution) were applied to the TLC plates. The use of a heat source expedited the spotting. With biologic samples as much extract as possible was spotted without the addition of more isopropanol to the flask. Care was taken to keep all spots equidistant from the bottom edge of the chromatographic plate. An appropriate amount of developing solvent was placed in a chromatography jar and the lid positioned to allow equilibration of solvent vapors within the tank. Several plates were placed in the tank in a way to prevent contact over the area in which separation and migration was desired. When the solvent was ascended sufficiently (preferably at least 15 cm), the plate was removed and dried. The plate was then sprayed with iodoplatinate reagent, and the positions and colors of the developed spots noted. Any acid or solvent remaining on the plate made the iodoplatinate reagent less specific because the deep blue color associated with morphine became darker and indistinguishable from other basic drugs. Maximum development of color required 3 to 4 h.

## RESULTS

The loss of the opiate in the various steps of the extraction procedure was determined by use of [$^{14}$C]morphine (Table I). Maximum recovery into the chloroform was achieved provided the acidified urine was not filtered. As expected, double extraction of the urine provides a significant increase in morphine recovery. $R_F$ values from two chromatographic solvent systems are presented for the major excretion product of twenty pharmacologic compounds in Table II. Urine from human subjects, who for various reasons had taken the pharmacologic agents, served as a source of biologic specimens. The urine was analyzed by the methods described in this report. Each $R_F$ value represents the average of at least five determinations. To compensate

TABLE I

MORPHINE DISTRIBUTION IN EXTRACTION PROCEDURE

| | *No initial filtration* | | *Initial filtration* | | *Double extraction of aqueous phase*[c,d] |
|---|---|---|---|---|---|
| | *Washed flask*[a] | *Unwashed flask* | *Dry filter* | *Wet filter*[b] | |
| Amount on filter paper (No. 1) | — | — | 22.3 | 15.3 | — |
| Amount remaining in initial flask | 0.2 | 6.0 | 3.6 | 3.2 | 0.2 |
| Amount remaining in aqueous phase | 10.0 | 13.0 | 7.2 | 10.5 | 1.1 |
| Amount in buffer wash (twice) | 3.0 | 2.4 | 1.9 | 3.2 | 2.6 |
| Amount on filter paper (No. 2) | 4.0 | 4.0 | 3.7 | 3.2 | 3.7 |
| Amount in chloroform | 82.8 | 74.6 | 61.3 | 64.6 | 92.4 |

[a] Flask washed twice with 1 ml of water.
[b] Filter paper moistened with water prior to filtration.
[c] No initial filtration; washed flask.

TABLE II

$R_F$ VALUES[a] OF DRUGS IN CHROMATOGRAPHIC SOLVENT SYSTEMS A AND B

| *Drug* | *Solvent system A* | *Solvent system B* |
|---|---|---|
| Antazoline | 0.68 | 0.64 |
| Chlorpromazine[b] | 0.66 | 0.65 |
| Propoxyphene | 0.64 | 0.64 |
| Methadone[b] | 0.64 | 0.57 |
| Amitriptyline | 0.64 | 0.60 |
| Mephentermine | 0.63 | 0.66 |
| Dextromethorphan | 0.62 | 0.61 |
| Hydroxyzine | 0.60 | 0.48 |
| Quinine | 0.58 | 0.40 |
| Meperidine | 0.56 | 0.52 |
| Prochlorperazine[b] | 0.54 | 0.55 |
| Chlorpheniramine | 0.50 | 0.17 |
| Atropine | 0.50 | 0.44 |
| Cocaine | 0.48 | 0.34 |
| Morphine | 0.46 | 0.42 |
| Codeine | 0.46 | 0.37 |
| Chloroquine | 0.38 | 0.25 |
| Nicotine | 0.26 | 0.04 |
| Caffeine (metabolite) | 0.06 | 0.04 (0.32) |

[a] $R_F$ values adjusted for morphine standard developed on the same plate.
[b] Major excretion product of drug.

TABLE III

COLOR EXHIBITED BY IODOPLATINATE POSITIVE COMPOUNDS

The descriptions are based on solvents A and C. Differences noted when developed in solvent B are: (a) Hydroxyzine, homogeneous blue; (b) Nicotine, no trail; (c) Caffeine, two spots — upper one large pale blue, bottom one blue-gray.
The colors are only approximations; for example, amitriptyline, methadone, and propoxyphene are all listed as "purple" but when viewed together are distinguishable.

| | Morphine blue (deep) | | |
|---|---|---|---|
| Amitriptyline | purple | Compazine | blue-purple |
| Antazoline | blue-purple | Dextromethorphan | blue-purple |
| Atropine | blue-purple | Hydroxyzine | purple center, blue fringe |
| Caffeine | blue (light) | Meperidine | purple |
| Chloroquine | purple (trails) | Mephentermine | blue-gray |
| Chlorpheniramine | blue-purple | Methadone | purple |
| Chlorpromazine | blue-purple | Nicotine | gray-black (trails) |
| Cocaine | purple | Propoxyphene | purple |
| Codeine | blue-purple | Quinine | blue-purple |

for differences between individual plates, morphine standards were spotted on each plate and the individual $R_F$/morphine $R_F$ ratios were adjusted to a composite mean morphine $R_F$ value upon which all $R_F$ data were based. Colors for the various compounds developed by the iodoplatinate spray reagent are listed in Table III.

## DISCUSSION

The proposed procedure is capable of analyzing a majority of the alkaline-

rehabilitation programs. Some heat-labile or volatile alkaline drugs, such as the amphetamines, will not withstand the acid hydrolysis and are lost or destroyed prior to the solvent extraction. Nevertheless, hydrolysis greatly enhances the sensitivity for detecting morphine and codeine[1-3]. Drugs that withstand the hydrolysis often do not develop color upon treatment with iodoplatinate. Most of these compounds develop strong brown spots when sprayed with iodine solution[4].

An interesting observation is that only small amounts of morphine are lost by washing the extracting solvent with the phosphate buffer. The wash provides the primary mechanism for obtaining excellent chromatograms of extracts from acid-hydrolyzed urine. It is obvious that better recovery could be obtained by increasing the volume of extraction solvent, but a considerable increase in evaporating time would ensue. The extracting technique as described is appropriate for simultaneous screening of a large number of specimens.

The relation between the observed color and the time of the iodoplatinate spraying can be an important consideration. Morphine intensifies in color for several hours following exposure to the iodoplatinate spray. Immediately after spraying, total morphine in urine is not detected below 0.5 μg/ml, but levels of 0.15 μg/ml (10-ml specimen) or less can routinely be detected if the plates are observed after a period of 3 to 4 h. Conversely, some drug metabolites exhibit morphine-like colors initially, but fade. Many compounds on the plate change color with time. Methadone and propoxyphene initially exhibit identical shades of violet-purple, but within minutes propoxyphene develops a rust-color hue.

The effects of metabolism should be kept in mind when interpreting TLC plates. Methadone standards are revealed as single spots, but with solvent system A *in vivo* specimens will exhibit two spots of similar color and intensity corresponding to the unchanged drug and the principal metabolite[5,6]. Caffeine does not react with iodoplatinate, but the principal caffeine metabolites do. Since approximately 90 % of ingested caffeine is excreted as metabolites, the familiar "caffeine" spots appear on plates only after extraction of urine specimens. Nicotine, a common interfering substance with most TLC systems, does not introduce any problem with the technique proposed in this report. Phenothiazine compounds and their metabolites generally appear as a series of four to five components and can be quickly characterized.

The interpretation of the TLC $R_F$ value is a comparative art rather than an absolute one[7]. Consequently evaluations of an unknown are best made by comparison with standards developed on the same plate rather than by reference to tables of $R_F$ values; however, the latter may be of definite value in eliminating drugs of widely differing migration rates. The evaluation of an unknown by TLC should be based on development in at least two or three different solvent systems. For morphine and codeine the solvent systems utilized in this study have proven most effective in analyzing acid-hydrolyzed specimens. All previously published TLC methods do not effectively separate the opiates from the high concentration of hydrolytic by-products obtained when urine is acid-hydrolyzed.

The proposed TLC method was observed to be approximately ten times more sensitive than a modified KUPFERBERG technique[8,9] based on spectrofluorometry. Comparative studies with the automated turret spectrofluorometric method of

two techniques appear comparable. The XAD-2 resin column method described in a recent report by MULÉ *et al.*[11] does not recover morphine glucuronide from urine; consequently, the sensitivity of that TLC procedure for determining total morphine in urine should be considerably less than the method of this report[1].

In this laboratory, a TLC procedure for rapidly determining morphine in urine has been utilized as a versatile, economic and relatively rapid tool for analyzing large numbers of specimens, with confirmatory analysis being made primarily by gas chromatography[3]. The technique has proven to be most effective as a screening mechanism for opiates in the United States Air Force world-wide drug abuse screening program in which more than 1000 specimens per day are analyzed. Simultaneous development on two chromatographic systems (Table II) provides an extremely low false positive rate as determined by several confirmatory methods.

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