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AN IMPROVED METHOD FOR RAPID, LARGE-SCALE THIN-LAYER CHROMATOGRAPHIC URINE SCREENING FOR DRUGS OF ABUSE

J. R. BROICH, D. B. HOFFMAN, S. ANDRYAUSKAS, L. GALANTE AND C. J. UMBERGER
Department of Toxicology, Office of the Chief Medical Examiner New York, N.Y. 10016 (U.S.A.)

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SUMMARY

This report describes a rapid, reliable, thin-layer chromatographic screening method, suitable for large scale operations, for the detection of drug abuse. By utilizing microdot sample application on 100 mμ thin-layer silica gel plates, sample size has been diminished with an increase in sensitivity. Drugs are detected using a separate series of chromophoric spray reagents for the acid and basic drugs, including a new reagent N,2,6-trichlorobenzoquinoneimine for the visualization of cyclic imide type compounds (barbiturates). Confirmation of drug identification to eliminate false positive results, is achieved, using the technique of microcrystallography.

INTRODUCTION

The increased awareness in both the public and private sectors of the social and economic consequences of drug abuse has necessitated the development of a rapid, reliable method for the testing of large numbers of urines for the possible presence of drugs of abuse. Such a method should involve both sensitivity in the microgram range and minimizing the possibility of false positive drug identification. This latter factor assumes increased importance as urine screening becomes a routine aspect of standard medical examinations. Based on an observation by STEVENSON[1] that appreciable quantities of barbiturates may be extracted into organic solvents from aqueous buffer solutions of pH > 9, DAVIDOW *et al.*[2] reported a method for urine screening involving the simultaneous extraction of acid, neutral and basic drugs in a single solvent system. These authors noted, however, that pH-conditions for the extraction of barbiturates and many basic drugs were not optimal and thus for example, secobarbital was barely detectable.

The present paper describes a rapid, efficient dual extraction system which avoids the above mentioned problem as well as some of the potential complications in drug identification arising from the presence of different classes of drugs in the same extract. In addition, a new, highly sensitive procedure involving the N,2,6-trichlorobenzoquinoneimine as a chromophoric spray reagent for the detection of submicrogram quantities of barbiturates is described.

MATERIALS

All chemicals and solvents employed are of reagent grade quality and are used without further purification.

The spray reagents may be conveniently prepared as follows: (A) Ninhydrin: 0.1% in methanol. (B) Iodoplatinate reagent: Mix 1 ml of 5% platinic chloride solution (prepared in concentrated hydrochloric acid) with 9 ml of aqueous 10% potassium iodide solution, and dilute to 50 ml with distilled water. (C) Dragendorff reagent: Add a solution containing 1.3 g bismuth subnitrate in 60 ml water and 15 ml glacial acetic acid to 12 g of potassium iodide in 30 ml water, then dilute with 100 ml water and 25 ml glacial acetic acid. (D) Methanol sodium bicarbonate: Suspend 5 g sodium bicarbonate in 100 ml methanol. Agitate before use. (E) N,2,6-trichlorobenzoquinoneimine reagent: 0.1% in chloroform. This solution is stable when kept refrigerated. (F) Mercurous nitrate solution, 1%: Dissolve 1 g of solid mercurous nitrate in concentrated nitric acid and dilute to 100 ml with distilled water. (G) Vanillin solution: Dissolve 1 g vanillin to 100 ml methanol and add 2 ml concentrated sulfuric acid. (H) Furfural solution: Dilute approximately 0.5 ml furfural to 50 ml with methanol and add 2 ml concentrated hydrochloric acid.

The following apparatus were used: Eberback Shaker; Eastman Chromatogram Sheet 6060—silica gel with fluorescent indicator; Linderstrom-Lang Levy micropipettes, 5 λ capacity; "Mineralite" long-wave length UV lamp; "Mineralite" short-wave length UV lamp; test tubes, Kimax 16 × 125 mm; beakers, 10 ml; Whatman filter paper, No. 1, 7 cm.

METHODS

Extraction and preparation of sample

Two 2 ml aliquots of each urine sample are placed in separate 16 × 125 mm test tubes. In this manner the extractions of the acid-neutral and basic fractions are performed on separate portions of each sample. To each tube in first set (A) obtained as described above is added one drop of concentrated sulfuric acid and 5 ml of chloroform. To each tube in the second row (B) are added in succession two drops of concentrated ammonium hydroxide solution, sufficient solid sodium bicarbonate to attain a just saturated solution of pH 8,5–9.0 and 5 ml of a chloroform–isopropanol solution (4:1). The tubes, held in racks, are then thoroughly agitated for 5 min in an Eberback Shaker. After complete separation of the two layers, the upper aqueous phase is removed by aspiration. To each of the A beakers is now added 1 ml of an aqueous saturated solution of sodium bicarbonate. After briefly reagitating the tubes, the separated, upper aqueous phase is aspirated off*. The remaining organic layers in both the A and B tubes are filtered into 10 ml beakers. The filter papers are rinsed with small quantities of the appropriate solvent. The beakers from tubes A contain the acid/neutral (A/N) drugs, and those from B contain the basic (B) drugs. The

* If detection of the possible presence of salicylates is desired, the aqueous layer should be carefully removed using a syringe or medicine dropper, acidified with hydrochloric acid, and extracted with ether or chloroform. The filtered, evaporated residue from the organic phase can then be tested chromatographically or chemically (1% aqueous ferric chloride solution) for the presence of salicylates.

solvent is carefully removed by evaporation on a steam bath or hot plate with the following special provision for the B beakers. After approximately half of the solvent has been evaporated (residual ammonia having also been boiled off) one drop of concentrated hydrochloric acid is added. Care should be taken to remove all the beakers as soon as all solvent has been evaporated off.

Chromatography

The residue in each beaker is carefully dissolved in a small but sufficient volume of the appropriate solvent, *i.e.* chloroform for the A beakers and methanol for the B fraction. The beakers are then tilted at a 45° angle to allow evaporation of the solvent to about 5 μl. If necessary, this amount of solvent may be added to redissolve the concentrated residue. Each entire extract is then spotted using five λ micropipettes on an Eastman Chromatogram Sheet 6060 (silica gel with fluorescent indicator). To facilitate application of the extract, the thin-layer chromatographic (TLC) plate may be placed on a hot plate whose surface temperature is regulated at 35°–40°, or alternately, a stream of hot air may be used for the same purpose. The diameter of a single spot should not exceed 1 mm. In this manner it is possible to conveniently place upwards thirty five spots on one side of a plate. Both sides of a single plate may be used to chromatograph samples of a given type fraction, but we have also found that where smaller numbers of samples are involved the A/N and B fractions may be spotted on opposite sides of a single plate. Each fraction may be developed independently of the other, and it is only necessary to cover one half of the plate when visualizing the other half with spray reagents. The developing systems are: A/N plate—hexane–ethanol (93:7); B plate—ethyl acetate–methanol–conc. ammonium hydroxide (85:10:1) (modified Davidow). Each plate is developed to a distance of 5 cm from the origin, removed from the tank and thoroughly dried by means of a stream of hot air.

Visualization of the TLC plates

Acid/neutral. The developed, dried plate is first examined under short-wave (254 mμ) UV light, and the areas of those drugs which appear dark due to absorbtion of light are marked (Table I). To enhance this effect, the plate may be briefly exposed to ammonia fumes before examination under UV light. Salicylic acid and salicylamide both yeild a sharp blue fluorescence. To facilitate subsequent application of the color reagents, the area under that of the barbiturate standard with the lowest R_F (phenobarbital when present) is marked off by means of a line drawn across the plate. The area below the indicated line is now covered while the plate is developed with the N,2,6-trichlorobenzoquinoneimine reagent as follows:

Reagent D is applied and after drying the plate with a stream of air, dimethylsulfoxide is sprayed until the surface appears just wet. Reagent E is applied and the plate headed with a stream of hot air for approximately one minute to allow full color development. Barbiturates and the structurally related compounds glutethimide and diphenylhydantoin give purple or violet colors on an almost colorless background. The entire plate is now sprayed with the mercurous nitrate solution (F) resulting in intensification and/or darkening of the aforementioned colors as well as the appearance of color for other compounds (methyprylon, ethinamate, carbromal) not reactive with N,2,6-trichlorobenzoquinoneimine (Table I). Meprobamate may now be visualiz-

TABLE I

Compound	R_F	*UV (254 mμ)*	*TCBI*	$HgNO_3$
Amobarbital	0.47	Abs[a]	P[a]	dk BV[a]
Aprobarbital	0.42	Abs	P	dk BV
Barbital	0.39	wk, Abs	lt B	lt B
Bromural	0.41	Abs	n	dk BV
Butabarbital	0.41	Abs	P	dk BV
Butabital (Sandoptal)	0.43	Abs	P	PB
Carbromal	0.51	Abs	n	GyBl
Carisoprodol	0.36	none	w	W
Cyclobarbital	0.41	Abs	lt BP	BP
Diphenylhydantoin (Dilantin)	0.34	Abs	P	BP
Ethinamate	0.41	none	n	BrBl
Glutethimide (Doriden)	0.53	Abs	P	V
Heptabarbital	0.59	Abs	V	V
Hexabarbital	0.55	Abs	V	V
Mephobarbital	0.55	Abs	V	V
Meprobamate	0.13	none	n	
Methabarbital	0.60	Abs	V	V
Methocarbamol	0.10	Abs	n	n
Methyprylone (Noludar)	0.53	Abs	n	GyBl
Pentobarbital	0.43	Abs	BP	PB
Phenobarbital	0.32	Abs	BP	PB
Primidone	—	none	n	n
Salicylic acid	—	fl	n	
Salicylamide	0.30	fl	GrB	GrB
Secobarbital	0.48	Abs	P	PB
Thiopental	0.53	Abs	Y	Y

[a] Abs = absorbance; fl = fluorescence; n = no reaction; lt = light; dk = dark; wk = weak; B = blue; Br = brown; Bl = black; Gr = green; Gy = grey; P = purple; V = violet; W = white; Y = yellow.

ed by applying the vanillin solution (G) and gently heating until appearance of a characteristic clear yellow color. Additional evidence for the presence of this drug may be obtained by applying, with subsequent heating, the furfural solution (H) which transform the yellow color to black.

Basic. The dried plate is first sprayed with the ninhydrin reagent (A) and gently heated with a stream of hot air, amphetamine appearing as yellow spot. Upon exposure of the plate to long wave (360 mμ) UV light for 10 min amphetamine and methamphetamine now appear as red violet and light violet spots, respectively. The plate is now sprayed with iodoplatinate reagent (B), air dried, and then sprayed with Dragendorff reagent (C). After 10 min the colors obtained are recorded and the R_F values calculated (Table II).

Confirmation for drug identification

Whenever possible it is of course highly desirable that the presence of illicit drugs of abuse as indicated by TLC be confirmed by additional independent methods. It should be emphasized that conclusions as to the definitive presence of one or more specific drugs in any given sample are not to be inferred solely from chromatographic data. In this regard, the presence of chromatographically non-specific spots reflects

TABLE II

Compound	R_F	*Ninhydrin*	*UV (360 mμ)*	*Iodoplatinate*	*Dragendorff*
Codeine	0.52	—	—	V[a]	RO[a]
Dilaudid	0.24	—	—	V	RO
Methadone	0.94	—	—	V	O
Meperidine (Demerol)	0.84	—	—	V	RO
Morphine	0.32	—	—	B	RO
Nalorphine (Nalline)	0.60	—	—	V	O
Amitriptyline (Elavil)	0.86	—	—	V	O
Amphetamine	0.64	wk Y[a]	RV[a]	RV	O
Imipramine (Tofranil)	0.82	—	—	V	O
Methamphetamine	0.58	—	wk V	VW	RO
Chlordiazepoxide (Librium)	0.72	—	—	VO	O
Chlorpromazine (Thorazine)	0.82	—	—	V	BrO
Diazepam (Valium)	0.92	—	—	V	O
Prochlorperazine (Compazine)	0.64	—	—	V	BrO
Promazine (Spasine)	0.70	—	—	V	BrO
Thioridazine (Mellaril)	0.84	—	—	VY	O
Trifluoperazine (Stelazine)	0.70	—	—	V	BrO
Cocaine	0.96	—	—	V	O
Lidocaine	0.92	—	—	BV	BrO
Pentocaine	0.84	—	—	V	O
Procaine	0.84	—	—	V	O
Propoxyphene (Darvon)	0.96	—	—	V	O
Methaqualone (Quaalude)	0.92	—	—	V	O
Cyclazocine	0.82	—	—	V	O
Methapyrilene	0.86	—	—	BV	RO
Pentazocine	0.88	—	—	V	O
Phenazocine	0.92	—	—	V	O
Chloroquine	0.46	—	—	V	O
Nicotine	0.78	—	—	BG	RO
Quinine	0.52	—	—	V	RO
Quinidine	0.58	—	—	V	RO

[a] wk = weak; Br = brown; O = orange; B = blue; Gr = green; R = red; V = violet; W = white; Y = yellow.

another instance of the necessity for further analysis involving microcrystallography, spectrophotometry, etc. on material eluted from the TLC plate or obtained from extraction of aliquots of the remaining sample. The microcrystal identification of drugs provides a convenient method for confirmatory analysis. The procedures followed are essentially those reported by FULTON *et al.*[3,4] using a polarizing microscope. Of the various basic drugs tested, it was found that submicrogram quantities of morphine, codeine, methadone, amphetamine, methamphetamine, propoxyphene (Darvon) and quinidine could be confirmed after methanolic elution from TLC plates previously treated with the ninhydrin, iodoplatinate and Dragendorff spray reagents as described in this paper. On the other hand, dilaudid, meperidine (Demerol) and quinine could not be identified. It should be pointed out that quinidine could not be identified using the standard procedure in the reference cited but could be confirmed because of the formation of circular needle-like clusters found in the presence of a gold bromide/phosphoric acid reagent. It should be pointed out that elimination of the Dragendorff reagent will enable almost all the basic drugs listed in Table II to be readily identified by the aforementioned microcrystal techniques. Contrary to the claims present in the literature, our laboratories have so far been unsuccessful in

using Reinecke salt to identify dilaudid from TLC plates. It may be noted that although both amphetamine and methamphetamine are easily identified and distinguished using gold bromide/phosphoric acid reagent, periods ranging from 1–24 h are required for crystal formation.

The presence of morphine and quinine may also be confirmed using urine samples of 0.1 ml or less by the fluorometric procedure of BROICH[5].

RESULTS AND DISCUSSION

The data presented in Tables I and II illustrate the applicability of the procedures described in the paper for the detection in urine specimens of a wide variety of acid, neutral and basic compounds. The significant advantage of this technique as compared to that involving a one-step simultaneous extraction of acid and basic drugs as described by DAVIDOW *et al.*[2] is the attainment of a greater degree of sensitivity in the limits of drug detection. In general this method will routinely permit the chromophoric detection of drugs present in urine in an approximate concentration of 1 µg/ml and many drugs can be readily detected at even lower levels. Glutethimide, however, is not readily visualized if present in concentrations less than 5 µg/ml. Because of its intense fluorescence quinine may be detected under UV light at a concentration of 0.01 µg/ml. DOLE *et al.*[6] have reported the use of ion-exchange paper for the separation of drugs from urine and more recently, MULÉ[7] has described and compared the identification of such drugs of abuse using both the direct extraction and ion-exchange paper techniques. Using radioactive assays the latter researcher reported that whereas morphine, pentobarbital and *d*-amphetamine were obtained in recoveries of 21.7%, 2.4% and 2.1%, respectively, using the ion-exchange paper method, the direct extraction method of these same drugs from undiluted urine afforded recoveries of 61%, 86% and 61%. Furthermore, the ion-exchange method as described require the use of 50 ml of urine as opposed to the 2 ml volumes that are employed in the much more rapid direct extraction procedures given in the present paper.

The simple but expedient procedure of using separate two ml aliquots of each urine sample for the detection of the acidic/neutral and basic fractions permits optimal condition for the extraction of both types of drugs as well as the use of independent systems for the chromatographic development and visualization of the two fractions. This factor is of particular importance in the case of the A/N fraction since it permits application of the highly sensitive N,2,6-trichlorobenzoquinoneimine reagent, the use of which would preclude subsequent application of the basic spray reagents if the DAVIDOW method were followed. Furthermore, the use of Eastman Chromatogram Sheet 6060 which possesses an adsorbant layer of only 100 mµ thickness enables the entire extract from an initial volume of only 2 ml urine to be conveniently spotted and chromatographed using the microdot technique.

We have found that accurate and reproducible results can be obtained without prior heat activation of these plates. Slight daily variations in the reported R_F value may occur with changes in the ambient humidity and hence, care should be taken to store all unused TLC sheets in a dry atmosphere. It is of critical importance that the level of developing solvent in the chromatographic tank be kept well below the level of the spotted microdots. Care should also be taken not to flood the chromatogram

when applying the spray reagents but rather to apply a light stream of fluid until the colors of the standards appear or, for those cases where heating is required for color development, until the surface of the plate is just covered.

Inclusion of the step involving a bicarbonate wash of the chloroform solution containing the A/N drugs eliminates much interfering material that would otherwise obscure the visualization of the barbiturate and neutral drugs. N,2,6-trichlorobenzoquinoneimine (TCBI) as a spray reagent for the detection of barbiturates has been found to be more satisfactory than mercury solutions alone or in combination with diphenyl carbazone for the visualization of chromatographed barbiturates present in microgram or submicrogram quantities. Upon subsequent application of the mercurous nitrate reagent, the existing chromophorus become more intense thus adding to the sensitivity threshold, and certain drugs, *i.e.* bromural, carbromal, ethinamate and methyprylon (Noludar) which are not visualized with the TCBI or mercury reagents alone, now appear as discrete spots. Consideration of the structure activity relationships of the compounds listed in Table I reveals that in general a positive reaction with the TCBI reagent itself requires the cyclicimide ring structure possessed by the barbiturates and certain related compounds, *e.g.*, glutethimide (Doriden) and diphenylhydantoin (Dilantin). Amides (methyprylone, primidone); Carbamates (meprobamate, carisoprodol, ethinamate, methacarbamol) and ureides (bromural, carbromal) are non-reactive. Salicylamide reacts under the test condition described but the color developed is both distinctly different and less stable than that obtained with the barbiturates. The reactions of quinone haloimides with phenols under basic conditions to form deeply colored, highly conjugated salts is well known. It is quite possible that the interaction of the TCBI with barbiturates occurs via a similar type reaction involving the imide ion.

Many basic fractions have been found to contain ninhydrin positive materials of varying R_F values which, however, do not give any further color development with the iodoplatinate or Dragendorff reagents. The presence of such compounds does not interfere with the determination of any of the drugs listed in Table II. To the authors' knowledge the procedures given in this paper are the first which permit chromatographic distinction between dilaudid and morphine, and quinine and quinidine.

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