Journal of Chromatography, 140 (1977) 71–76
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 10,161

SENSITIVE THIN-LAYER CHROMATOGRAPHIC METHOD FOR URINE SCREENING OF BARBITURATES*

J. A. VINSON**, J. E. HOOYMAN, H. KOHARCHECK and M. M. HOLMES

Research and Development Department, J. T. Baker Chemical Company, Phillipsburg, N.J. 08865 (U.S.A.)

(First received February 14th, 1977; revised manuscript received April 25th, 1977)

SUMMARY

A simple and sensitive thin-layer chromatographic (TLC) method is described for the detection of barbiturates and other sedatives at therapeutic levels in the urine. The method consists of an extraction followed by TLC on a single plate in a solvent system which separates the barbiturates from most of the other drugs. The upper portion of the plate is sprayed with a new visualization reagent, N,2,6-trichloro-*p*-benzoquinone imine, followed by heating. The barbiturates appear as blue spots. This procedure is capable of detecting barbiturates at levels of 0.1 mg/dl in urine.

INTRODUCTION

The development of poly-drug abuse in recent years has put an unusual burden on laboratories for the accurate identification of drugs in urine. Barbiturates remain one of the commonly abused drugs. The analysis of barbiturates requires procedures suitable for mass screening with rapidly available results. The method must be sensitive, give a minimum number of false positives and be low in cost.

Ultraviolet spectrophotometry (UV) at pH 10 and 13 is specific and useful as a screening tool for barbiturates and for quantitation[1] but is not sufficiently sensitive for detection of therapeutic levels. The UV method takes between 30 min and 1 h and is, therefore, not feasible for mass screening.

Colorimetric procedures can be performed in less than 10 min but are not very specific. The sensitivity of these methods is between 0.5 and 1 mg/dl[2,3] which is suitable for the detection of therapeutic levels of long-acting barbiturates in urine but is not sufficiently sensitive for the short-acting drugs. No information is possible on the identity of the barbiturate which is of importance in a hospital setting.

Gas chromatography (GC) is an extremely sensitive instrumental method for the analysis of drugs. This technique is not generally used for initial screening because

* Paper VI in the Series "Studies in Laboratory-Use Reagents." Presented at the Ninth International Congress on Clinical Chemistry, Toronto, Canada, July 1975.

** To whom correspondence should be addressed. Present address: Department of Chemistry, University of Scranton, Scranton, Pa. 18510, U.S.A.

of the expense and time involved. Rather, GC is utilized as a sensitive confirmatory tool, after preliminary identification by another technique, and for quantitation[4].

The newest development for the identification of barbiturates is immunoassay. This technique has recently been reviewed[5,6]. Critical comparisons were made of various immunoassays and thin-layer chromatography (TLC). Sensitivities of the Enzyme Multiplied Immunoassay Technique (EMIT) were 1 μg/ml in urine and for radioimmunoassay 0.5 μg/ml[7] which is acceptable. One problem with this method is the occurrence of false positives. EMIT gave 5% false positives for barbiturates as compared to TLC. Also, EMIT gave false negatives for glutethimide at therapeutic levels. Immunoassays are suitable for mass screening and they are used by the Air Force for this purpose. However, the cost per sample is moderate to high and thus may be prohibitive for commercial laboratories and hospitals. Due to non-specificity, results obtained by immunoassay should be confirmed by a non-immunological procedure.

TLC has been found to be the choice as the primary screening method for barbiturates[8]. It is a simple technique and considerably less expensive than immunoassay or GC[8]. One of the main drawbacks is the lack of sensitivity of the visualization reagent. Mercury-based sprays are the most commonly used visualizers for barbiturates. Single reagents such as mercuric sulfate or silver acetate produce white or gray spots which are difficult to see on a white TLC sheet. Spraying with mercuric salts followed by spraying with diphenylcarbazone produces pink or violet spots on a light background. This procedure gives better contrast with a sensitivity of 0.5 μg. However, at this low level the spots are fairly unstable and may disappear within minutes. In addition, mercury is expensive, presents a disposal problem, and is a health hazard due to its facility of entering the lungs in the aerosol form during spraying. The acidic nature of these visualization reagents also renders them hazardous and toxic. Another visualization method which is non-hazardous uses UV-quenching and allows barbiturates to be seen at microgram levels on TLC plates[9]. This method is not sensitive enough and not specific since many other drugs will produce spots due to UV-quenching.

We wish to report a new barbiturate visualization reagent, N,2,6-trichloro-*p*-benzoquinone imine (TCBI) for the detection of barbiturates in urine. TCBI has previously been used in combination with two other reagents as a three-spray system for the detection of barbiturates[10]. A preliminary report utilized a single spray containing TCBI[11] that gives blue colors with barbiturates and provides greater sensitivity than previously used visualization reagents.

MATERIALS AND METHODS

Human urines were obtained from the Center for Disease Control as unknowns in their proficiency testing program. Urines with known drugs and barbiturates were obtained from Warren Hospital, Phillipsburg, N.J., U.S.A. Blank urines containing no barbiturates were obtained from laboratory personnel.

Reagents

"Baker Analyzed" solvents and reagents were used from J. T. Baker (Phillipsburg, N.J., U.S.A.). Silica gel sheets (Bakerflex™ IB2), 200 μm, 20 × 20 cm (J. T. Baker) were used without activation. As screening solvent ethyl acetate–methanol–ammonia (100:18:1.5) was used.

The visualization reagent was prepared as follows. Dissolve 0.1 g of N,2,6-trichloro-*p*-benzoquinone imine in a mixture of 90 ml chloroform and 10 ml dimethyl sulfoxide. The dimethyl sulfoxide was previously saturated with sodium bicarbonate which was allowed to settle before decantation. This visualization reagent should be stored in a brown bottle in the refrigerator when not in use. The solution is stable for several months. Basic vapors such as ammonia and amines can darken the yellow colored solution which then should be discarded.

Procedures

Extraction. A single pH (9.5) liquid–liquid extraction procedure was used for the analysis[12]. Urine (10 ml) was taken through the extraction procedure and the evaporated extract reconstituted in 25 μl of methanol.

TLC method. Spot a half of the concentrated extract and a single barbiturate standard 1.5 cm from the bottom of the plate. Dry the spots at room temperature using a stream of air and develop 10 cm from the origin in an unsaturated tank ($8\frac{1}{2} \times 4 \times 9$ in.; Kontes, Vineland, N.J., U.S.A.) using the screening solvent. Dry the plate in a 110° oven for a few minutes to remove solvents and ammonia. Cover the plate below R_F 0.8 and spray the exposed portion of the plate with TCBI until just wet. Heat the plate in a 110° oven for a few minutes until the standard barbiturate is seen as a blue spot on a white background. If ammonia is not completely evaporated from the plate a light green background results which decreases sensitivity. A blue spot at, or slightly below, the R_F value of the standard indicates the presence of a barbiturate. A gray-green spot indicates glutethimide.

RESULTS AND DISCUSSION

The R_F values with the screening solvent for some common barbiturates and sedatives and approximate sensitivities with TCBI are shown in Table I. The barbiturates appear at R_F values above 0.90 and all give blue colors with TCBI except glutethimide which is gray-green. The sensitivity of the blue color is about 0.1 μg for all barbiturates except glutethimide. TCBI has greater sensitivity than the other barbiturate visualization agents. At extremely low levels approaching the detection limit the spots are best seen by looking from the rear of the plastic-backed TLC sheet with transmitted light. The blue spots are stable indefinitely although the background does change from white to a light tan color upon standing a few days in the laboratory.

Other sedatives including carbromal, mebutamate, ethchlorvynol and carisprodal are not visualized with TCBI. These drugs can be seen as white or gray spots above R_F 0.9 by dipping the plate, after spraying with TCBI, into a saturated aqueous solution of mercurous nitrate.

Forty-five drugs were tested for interferences in the method. Those drugs with R_F values greater than 0.8 in the screening solvent are listed in Table II. TCBI gives colors with a wide variety of drugs[11] but the blue color is indicative of barbiturates. Only one of the possible interferences, oxazepam, gives a blue color. This drug can be eliminated as a possibility by heating the developed plate for 5 min at 110°. Oxazepam, if present, turns a brown color, barbiturates remain blue. Individual barbiturates are best identified by using a GC method with the remaining portion of the extract.

Visualization of the other classes of drugs may be accomplished by spraying the

TABLE I

R_F VALUES AND SENSITIVITIES WITH TCBI FOR BARBITUTARES AND SEDATIVES

Color visualization sensitivity is the minimum amount necessary to see color. Spot visualisation sensitivity is the minimum amount needed in order to just discern a spot. The color of the spot cannot be determined at this lower level.

Drug	R_F	*Color*	*Sensitivity (µg)*	
			Color visualization	*Spot visualization*
Methyprylon	0.86	Blue	1.0	0.5
Pentobarbital	0.91	Blue	0.1	<0.05
Diphenylhydantoin	0.91	Blue	0.1	<0.05
Allobarbital	0.91	Blue	0.1	0.06
Phenobarbital	0.92	Blue	0.1	0.01
Barbital	0.92	Blue	0.1	0.04
Aprobarbital	0.92	Blue	0.1	<0.05
Hexital	0.93	Blue	0.1	0.05
Secobarbital	0.93	Blue	0.1	0.03
Butabarbital	0.93	Blue	0.1	0.01
Butalbital	0.93	Blue	0.1	0.01
Mephobarbital	0.94	Blue	0.1	<0.05
Methohexital	0.95	Blue	0.1	<0.05
Amobarbital	0.95	Blue	0.1	0.03
Glutethimide	0.98	Gray-green	0.5	0.2

lower portion of the plate, below R_F 0.8, with the usual reagents including iodoplatinate, sulfuric acid, ninhydrin and fluorescamine. In this way, narcotics, amphetamines and tranquilizers can be detected on the same plate as barbiturates. The R_F values of these other classes of drugs have been published previously[13].

Various extraction techniques were used to establish the applicability of the TLC method for urine analysis. These included a single pH (9.5) liquid–liquid extraction procedure[12], activated charcoal[14] and XAD-2 non-ionic resin[15]. All proved suitable with respect to naturally occurring interferences, *i.e.*, they have clean extracts in the barbiturate region of the plate.

A urine sample extract taken after ingestion of a therapeutic quantity of phenobarbital is shown in Fig. 1. In this case, only one-fifth of the concentrated extract from liquid–liquid extraction was used. The blue spots of the parent drug and metabolite are clearly visible on the TLC plate which was sprayed with TCBI over its entire area. Urine samples, after ingestion of other drugs including codeine, pentazocine, buta-

TABLE II

R_F VALUES OF POSSIBLE DRUG INTERFERENCES AND THEIR COLORS WITH TCBI

Drug	R_F	*Color with TCBI*
Dicyclomine	0.77	Green
Anileridine	0.79	Brown-green
Oxazepam	0.81	Blue
Ibogaine	0.90	Brown-green
Benzocaine	0.92	Orange-brown
Methapyrilene	0.93	Brown-green
Lidocaine	0.98	Green

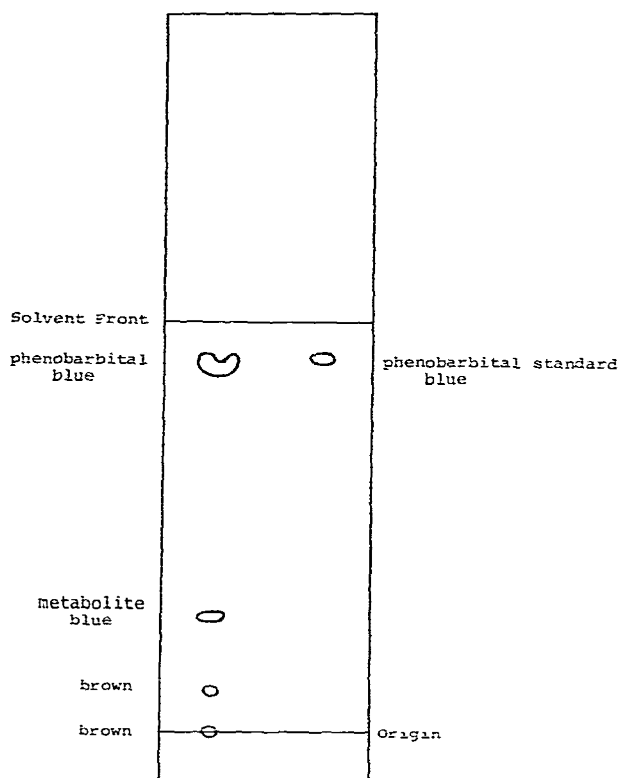

Fig. 1. TLC of urine sample extract taken 11 h after ingestion of 30 mg of phenobarbital.

zolidine, phenylbutazone and meperidine, gave no false positive results by this method. Ten proficiency urines which could contain barbiturates at levels of 1 μg/ml and above were tested using the liquid–liquid extraction and TLC. Five urines contained barbiturates and all were identified as positives by the method. No false posivites were found in the five urines containing no barbiturates but which were known to contain cocaine, amphetamines, methadone and morphine.

The detection of therapeutic levels of barbiturates by TLC is, of course, dependent on the extraction efficiency, fraction of the extract spotted, and the visualization reagent used. Great strides have been made in improving the extraction efficiency and cleanliness of the extract by the use of XAD-2 resin or charcoal. The practical limit of detection with XAD-2 and TLC in mass screening labs is about 2 μg/ml[16], although lower limits have been reported[17]. Thus, short and intermediate acting barbiturates may be difficult to detect in urine by previous methods as their levels in urine may be considerably less than 2 μg/ml.

Grove and Toseland[18] found 0.2 μg/ml of unchanged amobarbital, an intermediate acting drug, in urine by GC. The sample of urine was taken 3 days after ingestion of 200 mg of sodium amobarbital. Only GC and radioimmunoassay have been sensitive enough to detect this low level. As seen in Table III, with TCBI, very low levels of barbiturates can be detected in urine using a relatively inefficient liquid–liquid extraction technique and TCBI. Treatment of the plate with TCBI or mercurous

TABLE III

SENSITIVITY OF EXTRACTION AND TLC USING TCBI FOR THE DETECTION OF BARBITURATES IN URINE

Sensitivity was based on spiking 10 ml of urine with the drug and spotting one-half of the reconstituted extract; the concentration of drug was decreased until a dark spot was just visible on the TLC plate. The sensitivities of glutethimide and diphenylhydantoin were found by spraying with TCBI, heating the plate and dipping upper portion of the TLC sheet in a saturated mercurous nitrate solution. The resulting spots are gray.

Drug	*Urine concentration (mg/dl)*
Phenobarbital	0.1
Secobarbital	0.06
Glutethimide	0.2
Diphenylhydantoin	0.04

nitrate alone does not give as low a sensitivity for glutethimide and diphenylhydantoin as the combination of the two. Glutethimide and diphenylhydantoin are excreted in urine in only minute amounts[19] as the unchanged drug. Thus, the whole plate should be sprayed with TCBI and dipped in mercurous nitrate in order to see the polar metabolities which appear at R_F values lower than the unchanged drug.

In summary, the TCBI visualization reagent combined with any suitable extraction procedure has been found to be a simple, selective and extremely sensitive method for the screening of therapeutic levels of brabiturates in urine.

REFERENCES

1 P. Jatlow, *Amer. J. Clin. Pathol.*, 59 (1973) 167.
2 E. Z. Helman, *Clin. Chem.*, 16 (1970) 797.
3 F. Pehr, *Clin. Chem.*, 11 (1975) 1609.
4 M. Rubin, *Amer. J. Med. Technol.*, 39 (1973) 205.
5 J. H. Bidanset, *J. Chromatogr. Sci.*, 12 (1974) 293.
6 I. Sunshine, S. J. Mulé, M. Braude and R. F. Willette, *Immunoassays for Drugs Subject to Abuse*, CRC Press, Cleveland, Ohio, 1974.
7 S. J. Mulé, M. L. Bastos and D. Jukofsky, *Clin. Chem.*, 20 (1974) 243.
8 K. K. Kaistha, R. Tadrus and R. Janda, *J. Chromatogr.*, 107 (1975) 359.
9 M.-W. Hsuing, K. Itiaba and J. C. Crawhall, *Clin. Biochem.*, 7 (1974) 45.
10 J. R. Broich, D. B. Hoffman, S. Andryauskas, L. Galante and C. J. Umberger, *J. Chromatogr.*, 60 (1971) 95.
11 J. A. Vinson and J. E. Hooyman, *J. Chromatogr.*, 105 (1975) 415.
12 B. Davidow, N. L. Petri and B. Quame, *Amer. J. Clin. Pathol.*, 50 (1968) 714.
13 J. A. Vinson, J. E. Hooyman and C. E. Ward, *J. Forensic Sci.*, 20 (1975) 552.
14 J. M. Meola and M. Vanko, *Clin. Chem.*, 20 (1974) 184.
15 N. Weissman, M. L. Lowe, J. M. Beattie and J. A. Demetriou, *Clin. Chem.*, 17 (1971) 875.
16 B. Davidow, B. Quame, L. L. Abell and B. Lim, *Health Life Sci.*, 10 (1973) 329.
17 R. I. H. Wang and M. N. Mueller, *J. Pharm. Sci.*, 62 (1973) 2047.
18 J. Grove and P. A. Toseland, *Clin. Chim. Acta.*, 29 (1970) 253.
19 J. E. Wallace and S. L. Ladd, *Ind. Med.*, 39 (1970) 23.