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ROUTINE IDENTIFICATION OF DRUGS OF ABUSE IN HUMAN URINE

III. DIFFERENTIAL ELUTION OF THE XAD-2 RESIN

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SUMMARY

The differential elution of the XAD-2 resin consisted of a sequential solvent extraction of drugs from the resin as follows: after passing the urine over the resin, the columns were transferred to centrifuge tubes containing 0.1 *N* hydrochloric acid and eluted with 10 ml of isopropyl ether. The tubes were thoroughly shaken and the phases were separated by freezing the aqueous layer and pouring off the isopropyl ether. This phase was evaporated to dryness and the residues containing primarily the acidic and neutral drugs were applied to Polygram silica gel sheets and developed in chloroform–methanol–ammonia (90:10:1). The XAD-2 resin columns saved for the second elution were placed on the original centrifuge tubes containing the frozen acidic aqueous phase. The resins were eluted with two 10-ml portions of chloroform–isopropanol (3:1). One millilitre of 0.125 *M* borax solution was added to each tube (pH 8–9.5), it was shaken and the phases were separated. The organic phases were evaporated to dryness and the residues containing primarily the basic and some neutral drugs were applied to silica gel thin-layer plates and developed in ethyl acetate–methanol–water–ammonia (85:10:3:1). The acidic, basic, and neutral drugs were detected by sequential chromogenic spraying of the chromatographic sheets and plates.

Total recovery of the barbiturates was about 81% and for the basic drugs, it ranged from about 80 to 100%. The percentage increase in drugs detected with the differential elution technique as compared to the single elution method ranged from 2.4 to 100%. A decrease was observed only with D-propoxyphene (50%) and phenylpropanolamine (6.2%).

INTRODUCTION

The XAD-2 resin column method[1–5] for extracting drugs subject to abuse

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from urine usually involves a single organic solvent extract that contains acidic, neutral and basic drugs. Thin-layer chromatographic (TLC) analysis of this total extract often presents difficulties in detection and identification of the various drugs. To overcome these problems a sequential spray sequence was applied to each TLC plate[6] in order to achieve greater specificity, or the extract was divided into equal portions and applied on two different TLC plates with sequential spraying emphasizing either the acidic or basic drugs[2,7] on each plate. Although the latter approach provided reasonable TLC data it was not fully satisfactory in terms of sensitivity. To obtain maximal sensitivity as well as the capability of detecting a variety of drugs subject to abuse, a differential elution of the XAD-2 resin was developed. With this technique, acidic, neutral, and basic drugs may be differentially eluted from the same resin using the solvents isopropyl ether and chloroform–isopropanol (3:1) in sequence. This communication describes the results of these experiments.

MATERIALS AND METHODS

All chemicals were of reagent grade. The XAD-2 resin, columns, hydraulic flow control apparatus (HFCA) as well as the spray reagents and drug standards were as described previously[2,7] except where noted.

Procedure

The columns containing the XAD-2 resin were transferred to the HFCA and partially submerged in water while 25 ml of urine was poured into each of the columns[2]. The urine flow-rate was controlled through the HFCA to provide an extraction time of 20–25 min, and air pressure was applied to remove residual urine from the columns. All the columns were transferred to 50-ml centrifuge tubes containing 1 ml of 0.1 *N* HCl and eluted with 10 ml of isopropyl ether. Air-pressure if required was used to start the flow of the solvent through the columns and discontinued once elution started. The columns were saved for a subsequent solvent elution. The centrifuge tubes containing the isopropyl ether and aqueous phases were thoroughly shaken using a Genie Vortex mixer. The two phases separated on standing and the centrifuge tubes were partially dipped into a freezing mixture (dry ice–acetone) so that the lower aqueous phases were completely submersed and the upper layers remained above the level of the freezing mixture. In about 60 sec the liquid content of the test tubes was poured into wide-mouth 50-ml centrifuge tubes and the frozen aqueous layers remained in the original centrifuge tube. The centrifuge tubes containing the isopropyl ether extract were transferred to a water-bath at 80° and the solvent was evaporated under a stream of air. The remaining residues contained primarily the acidic and some neutral drugs.

The XAD-2 resin columns saved for the second elution were placed on the original centrifuge tubes containing the frozen acidic aqueous layers. The resins were then eluted with 20 ml of chloroform–isopropanol (3:1) in two equal 10-ml portions. Following elution the columns were discarded and 1 ml of 0.125 *M* borax solution was added to each centrifuge tube to insure a final pH in the range 8–9.5. The organic and aqueous phases in the centrifuge tubes were well shaken using a Genie Vortex mixer. The phases separated on standing and the upper aqueous phases were removed by aspiration. About 200 μl of 6 *N* HCl in methanol were added to each tube and the

organic solvent was evaporated to dryness in a water-bath set at 80° under a stream of air. These residues contained primarily the basic drugs and some neutral drugs.

Thin-layer chromatography

Acidic drugs. The extracts obtained following evaporation of isopropyl ether were dissolved in about 50 μl of methanol and applied to 0.15-mm Silica Gel F_{254}Q5WF thin-layer plates (Quantum Industries, Fairfield, N.J.) or 0.25-mm Polygram silica gel sheets made by Macherey, Nagel & Co., Düren, G.F.R. (Brinkmann Instruments, Westbury, N.Y.), and the plates were developed in chloroform–methanol–ammonia (90:10:1) without saturating the chamber. The plates were air-dried and subjected to the following spray reagent sequence: mercuric sulfate reagent; 0.1% diphenylcarbazone in chloroform (DPC) followed by air drying of the chromatogram; iodine acid solution*; and finally furfural alcoholic solution** and HCl. In many instances the TLC plates were simply sprayed with 0.01% DPC, followed by mercuric sulfate and resprayed with 0.1% DPC. All spray reagents not noted were prepared as described previously[2].

Basic drugs. The extracts obtained following evaporation of the chloroform–isopropanol mixture were dissolved in about 50 μl of methanol and applied to 0.25-mm silica gel thin-layer plates made by E. Merck AG, Darmstadt, G.F.R. (Brinkmann Instruments, Westbury, N.Y.), and the plates were developed in ethyl acetate–methanol–water–ammonia (85:10:3:1) solvent system. The plates following development and drying were subjected to the following sequence of spray reagents: 0.3% ninhydrin acid followed by heating the plate in an oven at 100° for 5 min; 5% sulfuric acid and heating with hot air for 5 min; iodoplatinate reagent and after 15 min spraying with *p*-nitroaniline (PNA) reagent followed by a heavy spray with 25% alcoholic NaOH solution. All spray reagents were prepared as described previously[2].

RESULTS

The recovery of various drugs from human urine after differential elution of the XAD-2 column with isopropyl ether followed by chloroform–isopropanol (3:1) in comparison to the single elution technique with chloroform–isopropanol (3:1)[2,7] is given in Table I. The isopropyl ether eluent contains, after washing with HCl, about 80% of barbiturates, 44% of meprobamate, and small amounts of weakly basic drugs like caffeine, benzodiazepam derivatives and a chlorpromazine metabolite. The recovery of barbiturates with 10 ml of the first elution solvent (isopropyl ether) is about 15% less than observed with a single solvent elution of two 10-ml fractions of chloroform–isopropanol (3:1). However, since the total extracted quantity of drug is applied to a single thin-layer plate, instead of splitting the extract[2,7] and applying it to two different thin-layer plates, the sensitivity is greatly increased. Actually the isopropyl ether and the residual water that mixes with the eluate contained 78.1% of methadone, 73.6% of meprobamate, 21% of cocaine, 19.8% of nicotine, 13.4%

* Iodine acid solution was prepared by mixing 2 g of iodine with 50 ml of 95% ethanol. 2 g of KI is dissolved in 16.2 ml of water and both solutions are mixed until clear. Finally, add 33.8 ml of conc. HCl with mixing. Store at room temperature.

** 10% furfural solution was prepared by diluting 10 ml of furfural to 100 ml with ethanol. Store in refrigerator.

TABLE I

RECOVERY OF DRUGS FROM HUMAN URINE (pH 6.5) FOLLOWING ELUTION OF THE XAD-2 COLUMNS

Elution solvents used: (A) isopropyl ether; (B) chloroform–isopropanol (3:1).

Compound	*Amount in urine (μg/ml)*	*Recovery* after single elution with eluent B, 2 × 10 ml (% ± S.D.)*	*Total recovery after differential elution***		
			Eluent A followed by Eluent B		
			1 × 10 ml (% ± S.D.)	*1 × 10 ml (% ± S.D.*	*2 × 10 ml (% ± S.D.)*
[^{14}C] Amphetamine	2.0	84.0 ± 4.7	0.4	82.5 ± 1.7	93.1 ± 0.7
[^{14}C] Caffeine	11.0		2.4 ± 0.2	82.4 ± 0.8	86.9 ± 1.2
[^{14}C] Cocaine	1.0		0.8	89.0 ± 2.0	92.1 ± 1.7
[^{14}C] Meperidine	1.0		0.3	96.2 ± 4.9	102.6 ± 1.2
[^{14}C] Mescaline	1.0		0.3	80.0 ± 1.5	90.9 ± 0.4
[^{3}H] Methadone	2.0	86.3 ± 4.2	0.3	77.9 ± 7.5	85.1 ± 3.5
[^{14}C] Morphine	2.2	84.0 ± 5.6	0.0	79.5 ± 2.1	86.2 ± 1.9
[^{14}C] Meprobamate	1.0		44.0 ± 0.3	41.4 ± 0.7	42.0 ± 0.5
[^{14}C] Nicotine	11.0		0.1	97.1 ± 1.8	98.9 ± 1.3
Quinine***	10.0		0.0	84.5 ± 4.7	96.2 ± 1.0
[^{14}C] Pentobarbital	2.0		80.6 ± 0.9	2.5 ± 0.1	2.5 ± 0.1
[^{14}C] Phenobarbital	2.0	95.7 ± 2.8	80.3 ± 3.2	4.1 ± 1.2	4.3 ± 1.2
D-Propoxyphene§	10.0		0.0	74.9 ± 4.8	79.1 ± 4.9

* Method as described previously by Mulé *et al.*[2] and Bastos *et al.*[7].

** Urine samples analyzed as described under Materials and methods, and previously[2] for the radioactive techniques.

*** Quinine analyzed using the Farrand Mark I spectrophotofluorometer; E_x, 350 nm; E_m, 450 nm.

§ D-Propoxyphene (Darvon) analyzed by gas chromatography as described previously by Mulé[8].

of meperidine, and 8.0% of D-propoxyphene. The basic drugs, however, remain in the aqueous acidic phase and the ether extract remains substantially free of the basic drugs. No loss of drug was observed by using the acetone–dry ice freezing method for separating the aqueous and isopropyl ether phases.

The final chloroform–isopropanol (3:1) extract, containing the drugs eluted from column and recovered from the alkaline aqueous washing solution, contains almost all the basic drugs except for 42% of meprobamate and minor amounts of barbiturates (2.5 and 4.3%). The data in Table I also show quite clearly an increase in the recovery of basic drugs by eluting the columns twice with 10 ml of chloroform–isopropanol (3:1) as compared to once with the same volume of eluent. The recovery of methadone and morphine (85.1 and 86.2%, respectively) were similar to those observed with the single elution technique (Table I) using 2 × 10-ml fractions of chloroform–isopropanol (3:1) as described previously[7]. However, the recovery of amphetamine (93.1%) was much better than obtained previously (84%). Other highly basic compounds such as nicotine and mescaline, were better extracted at higher pH with the double elution technique as compared to the previous methods[2,7].

Table II shows the results obtained after analyzing 1258 urine samples with the XAD-2 column extraction technique using both the single elution method previously described[2,7] and the new double elution technique described under Materials and methods. An increase in the number of positive samples was detected

TABLE II

COMPARISON OF RESULTS OBTAINED IN THE ROUTINE ANALYSIS OF 1258 URINE SAMPLES USING THE SINGLE* AND DIFFERENTIAL ELUTION TECHNIQUE**

Drug	*No. of samples*		*Percentage increase or decrease in No. of drug samples detected with the differential elution technique*	*Statistical significance*
	Single elution technique	*Differential elution technique*		
Quinine	75	106	41.3	$Z = 2.39$; $P < 0.05$
Morphine	22	23	4.5	
D-Methorphan	11	13	18.2	
Methadone	579	593	2.4	
D-Propoxyphene	10	5	−50.0	
Chlorpromazine	12	13	8.3	
Amitriptyline	0	4	undetermined	$Z = 2.00$; $P < 0.05$
Amphetamine	0	7	undetermined	$Z = 2.65$; $P < 0.01$
Phenylpropanolamine	16	15	−6.2	
Phenobarbital	29	58	100.0	$Z = 3.16$; $P < 0.01$
Secobarbital and pentobarbital	30	32	6.6	
Diphenylhydantoin	5	10	100.0	

* Mulé *et al.*[2]; Bastos *et al.*[7].

** Urine samples analyzed as described under Materials and methods.

by the new method primarily because both acidic and basic drugs were extracted and chromatographed separately. Significant increase in drugs detected was observed with amitriptyline ($P < 0.05$), phenobarbital (100%, $P < 0.01$), diphenylhydantoin (100%), amphetamine ($P < 0.01$), and quinine (41.3%, $P < 0.05$). The detection of other drugs was improved by 2–18%. A reduction in drugs detected was only observed with D-propoxyphene and phenylpropanolamine.

DISCUSSION

The results obtained with the double elution technique indicated better recoveries for most drugs as well as a more logical approach to extraction and subsequent TLC.

Isopropyl ether as a solvent provided pure extracts, was less dangerous than ethyl ether, and easily volatilized. Washing the ether extract with aqueous HCl was indispensable. Had this step been omitted, the isopropyl ether solvent having extracted some basic drugs would have defeated the purpose of the differential elution technique as well as cause a significant reduction in recovery of the organic bases in the chloroform–isopropanol (3:1) extract.

The separation of the acid aqueous and isopropyl ether phases can be done mechanically with a Pasteur pipette or separatory funnel but for the analysis of large numbers of samples, the separation was greatly facilitated by freezing the aqueous layer and pouring off the ether phase. The isopropyl ether extract was evaporated without washing the extract with sodium bicarbonate to eliminate strongly acidic

drugs such as salicylic acid that are often extracted from urine at low pH. However, this washing step may be instituted if considered necessary.

The chloroform–isopropanol (3:1) extract contained most of the basic drugs and was enriched with the basic compounds trapped in acid aqueous solution used for washing the isopropyl ether fraction. This extract appeared to be purer than the extract obtained with the single elution technique[2,7].

A comparison of both techniques indicated that improving sensitivity for the detection of drugs of abuse in urine by concentrating the basic or acidic drugs in appropriate solvents and subsequent application of the extract to individual chromatograms will not always result in an increase in the number of samples detected as positive. However, in all but two cases (phenylpropanolamine and D-propoxyphene) there was an increase in the number of positive samples. In all probability greater statistical significance would have been observed with the differential elution technique had the analysis been performed on a larger number of samples.

Cross-over information obtained with a double chromatogram system using a split extract does in some cases help to overcome the decreased sensitivity inherent in splitting the extract between two chromatograms. This fact was observed on several occasions with drugs present in large amounts on a single thin-layer chromatogram that did not develop properly (streaking). Thus, it is necessary to complement this method as well as all TLC methods with a routine screening assay. In fact, it is suggested that all urines be screened first by another analytical technique (*i.e.* immunoassay, fluorometry) and then subjected to confirmation by the differential elution technique as described in this report.

The significant improvement in detecting acidic drugs, amphetamines, and the advantages of separating acidic from basic drugs are important enough to recommend this new method of using the XAD-2 resin to other branches of analytical toxicology, whose complexity often demands a classic separation of drugs.

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