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Extraction of Drugs from Biofluids and Tissues with XAD-2 Resin

Good toxicological procedure necessitates the analysis of biological fluids and tissue for a wide range of drugs. The forensic toxicologist must also consider the analytical problems associated with the isolation and quantitation of drugs present in low concentrations. The usual approach is to use a solvent extraction procedure or one of its numerous modifications.

The recent introduction of some types of resins capable of adsorbing drugs and other substances from aqueous solution, particularly urine [*1-4*], has turned our interest to their usefulness in the extraction of various types of drugs from other biological materials. The XAD-2 resin adsorption method presented in this paper utilizes a reasonably small sample of biological material, such as blood, urine, bile, gastric contents, brain, liver, kidney, and other tissues, and results in drug recoveries which, in general, are sufficiently pure for identification and quantitation.

Materials and Methods

Column Preparations

A portion of the dry XAD-2[3] (16-50 mesh) resin was suspended in distilled water for at least 24 h prior to use. Glass columns 30 cm high and either 1.5 cm in diameter for liquids or 2 cm in diameter for tissue analysis were used. A plug of glass wool was placed in the bottom of the column and the resin slurry (about 12 g for liquids and 20 g for tissues) was then poured into the column and allowed to settle to a height of 10 cm. This procedure produced a self-packing column. A flow rate of less than 20 ml/min was used in these studies. This was accomplished by varying the size of the glass wool plug or constricting the end of the column or both.

Prior to using the column for analysis, the resin bed was washed with 50 to 100 ml of distilled water followed by 50 to 100 ml of methanol, and again with 50 to 100 ml of water, depending on the size of the column. Stoppering the column filled with water

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after washing the resin or between analyses maintains the resin in a hydrated state, which is necessary for efficient drug extraction.

Sample Preparations

Urine—Five to 20-ml samples of urine were used for screening purposes. The pH of the urine was adjusted to approximately 7.5 to 8.0 with 10 percent NaOH.

Blood—Ten millilitres or more of blood were utilized for screening purposes. The blood was mixed with five volumes of water and this mixture was agitated for one minute to lyse the red cells. This was filtered through Whatman No. 40 filter paper with a Buchner funnel.

Serum—Serum requires no special preparation prior to analysis. After measuring the sample available, the pH was adjusted to 7.5 to 8.0 if necessary.

Bile—A 10-ml sample was mixed with one tenth its volume of concentrated HCl and then autoclaved at 15 psi (103 kPa) for 30 min. After cooling, the mixture was filtered through Whatman No. 40 filter paper and the pH adjusted to approximately 7.5 to 8.0.

Gastric Contents—To a 5-g sample, 25 ml of distilled water were added and the mixture was homogenized for 30 s in a blender. The homogenate was then filtered and the pH adjusted to 7.5 to 8.0.

Tissues—Twenty grams of tissue and 100 ml of water were homogenized in a blender for 30 to 45 s. The homogenate was transferred to a beaker and 2 ml of concentrated HCl were added. The mixture was then placed on a steam table for 30 min. Steam table hydrolysis should be omitted when the presence of heat-labile drugs is suspected. After cooling, the pH of the homogenate was adjusted to 7.5 to 8.0 with 10 percent NaOH. The sample was then centrifuged at 20,000 rpm for 10 min in a refrigerated centrifuge. The supernatant (water extract) was filtered through glass wool to remove any particulate matter. A second extract was prepared by suspending the tissue pellets in 100 ml of water and the suspension shaken vigorously in a 250-ml, stoppered Ehrlenmeyer flask. This was then centrifuged again and the supernatant filtered through glass wool. Both water extracts were then combined.

A timesaving step in the procedure is adjusting the pH of the aqueous tissue extract *before* centrifuging. If the pH is adjusted after centrifugation, particulate matter may become suspended in the aqueous phase, requiring even further centrifugation.

Extraction Procedures

The prepared sample (biofluids or combined tissue water extracts) was poured onto the column and allowed to drain through the column bed. The resin was then washed four times with 20-ml aliquots of distilled water. All the liquid passed through the column to this point was discarded. For desorption of the drug(s) from the resin, 25 ml (for biofluids) or 50 ml (for tissue extracts) of ethyl acetate were passed through the column and the eluate collected in a beaker. The eluate separated into two layers and the bottom water layer was removed and discarded. Twenty-five millilitres (for biofluids) or 50 ml (for tissue extracts) of methanol were then added to the column and collected in a second beaker. Both solvents were combined and mixed with 2 drops of 1 percent HCl in methanol. This mixture was then divided into two equal portions, and each evaporated under a stream of air.

The resin in the column was regenerated after each analysis by washing with 50 ml of 1 percent HCl in methanol followed by 50 to 100 ml of distilled water. Hydrolyzed samples discolored the resin to various degrees, but it was found that the discoloration

did not affect adsorption efficiency. Most urinary pigments and other colored material were removed with the methanolic HCl wash.

Clean-Up Procedure (Optional)

On some occasions, expecially with hydrolyzed bile, the residual extract was moderately colored. Under these circumstances an optional clean-up procedure was performed. The residue was taken up in 3 to 5 ml of water and the pH adjusted to 3 to 4 with 0.5 percent H_2SO_4. This aqueous solution was extracted three times, for one minute each, with 5 ml of chloroform (for acid and neutral drugs). The aqueous layer was then adjusted to pH 9 with 1 percent NaOH and extracted three times with 5-ml aliquots of chloroform:isopropanol (3:1). The combined solvent extracts were filtered through glass wool layered with anhydrous sodium sulfate and the filtrate evaporated under an air stream.

Preliminary Screening

The residue was dissolved in two drops of 95 percent ethanol and approximately one half the residue was spotted on Silica Gel G plates (coated to a thickness of 0.25 mm). As a primary drug screen, the plates were developed 10 to 15 cm in a solvent system composed of ethyl acetate:methanol:ammonium hydroxide (84:10:5) or chloroform:methanol:ammonium hydroxide (90:10:1).

Thin-Layer Chromatography (TLC) Spray Reagents

Ninhydrin—0.4 g of ninhydrin was dissolved in 100 ml of acetone.

Mercurous nitrate—1 g of mercurous nitrate was dissolved in 49 ml of water and 1 ml of concentrated nitric acid. The acid solution was then diluted to 100 ml with water.

Cobaltous thiocyanate reagent—0.8 percent weight per volume of colbaltous thiocyanate was dissolved in 1 percent ortho phosphoric acid solution.

Iodoplatinate reagent—4.5 g of potassium iodide were dissolved in 45 ml of water and then mixed with 5 ml of 5 percent platinic chloride. The mixture was then diluted to 150 ml with water.

Dragendorff's reagent—A stock solution was prepared by dissolving 20 g of potassium iodide in 50 ml of water. To this solution was added 12.5 g of bismuth subnitrate, 25 ml of glacial acetic acid, and 100 ml of water. The spray reagent was prepared from 1 part stock solution, 2 parts glacial acetic acid, and 3 parts water.

Trinder's reagent—4 g of mercuric chloride were dissolved in 85 ml of water. To this solution was added 12 ml of 1 *N* hydrochloric acid and 4 g of ferric nitrate. The solution was then diluted to 100 ml with water.

Furfural—The reagent was distilled prior to use.

TLC Spray Sequence—After drying, the plates were visualized under shortwave and longwave ultraviolet radiation to locate quinine and its metabolites. The plates were then sprayed with ninhydrin reagent, followed by activation with ultraviolet light to visualize amphetamines and phenylpropanolamine. Then the plates were sprayed with mercurous nitrate to detect barbiturates, glutethimide, and diphenylhydantoin. After thorough drying, the plates were sprayed with the cobaltous thiocyanate reagent to visualize methadone, methaqualone, and propoxyphene. The plates were then sprayed lightly with iodoplatinate reagent. A moderately heavy spray of Dragendorff's reagent was then applied. Basic drugs and narcotics appeared in this step. The plates were then oversprayed with iodoplatinate to better visualize morphine and other basic drugs.

Salicylates were visualized next by spraying with Trinder's reagent. Finally, the plates were sprayed with distilled undiluted furfural followed by concentrated HCl to detect meprobamate and other carbamates.

Identification and Confirmation of Drugs—Drugs detected by the preliminary thin-layer chromatography were investigated further and their identification confirmed by other methods, including ultraviolet spectrophotometry, gas-liquid chromatography, and spectrophotofluorometry.

Results

Blood and urine samples were spiked with a number of different drugs to determine and confirm that the XAD-2 extraction system was useful for qualitative detection of these drugs in blood as well as urine. In addition, samples submitted to this laboratory for toxicological analyses were utilized to this end. Samples of biofluids and tissues were obtained from clinical and medical examiners' cases. A variety of acid, basic, and neutral drugs were isolated and detected as shown in Table 1. With our technique, thin-layer chromatographic visualization was sufficiently sensitive to detect 0.5 μg/ml of morphine in a 5-ml sample of blood and urine (Table 2). Detection limits of the other drugs (amobarbital, amphetamine, codeine, methadone, and phenobarbital) ranged from 0.5 to 1.0 μg/ml.

Recovery studies were performed by adding known amounts of the listed drugs to blood (Table 3) and to human and dog tissues (Table 4). Drug concentrations in the blood for recovery studies ranged from 0.400 to 0.529 mg percent. Table 4 depicts the recoveries of some drugs from tissue samples by ultraviolet spectrophotometry. The

TABLE 1—*Drugs detected in clinical and medical examiners' specimens (urine, blood, bile, gastric contents, brain, liver, and kidney) using the XAD-2-TLC screening procedure.*

Acid Drugs	Neutral Drugs	Basic Drugs
Amobarbital	Ethchlorvynol	Aminophylline
Butalbital	Caffeine	Amphetamine
Methohexital	Meprobamate	Chlordiazepoxide
Pentobarbital	Methyprylon	Chlorpromazine
Phenobarbital	Phenacetin	Cocaine
Salicylamide		Codeine
Salicylic acid		Desipramine
Secobarbital		Diazepam
		Ephedrine
		Imipramine
		Isoniazid
		Meperidine
		Methadone
		Methamphetamine
		Methapyrilene
		Methaqualone
		Morphine
		Nicotine
		Perphenazine
		Phencyclidine
		Phenylpropanolamine
		Propoxyphene
		Quinine
		Trifluoperazine
		Trimethobenzamide

drugs shown in the aforementioned tables were chosen as representatives of the acid, basic, and neutral classes of drugs.

Detection of a number of drugs in the urine of hospitalized patients[4] are shown in Table 5. The drugs shown in this table were detected by a thin-layer screening system after extraction of the urine by XAD-2 resin. The analyses of urine samples from hospitalized patients (under 24-h supervision) administered therapeutic doses of a number of drugs were found to be very significant because the dosage of the various drugs was known, and the excretion of the unmetabolized drugs was observed, even after as little as 5-mg doses. We feel that sensitivity of the detection method confirms the feasibility of this procedure for clinical samples.

Comparisons of drug recoveries were made between the XAD-2 procedure and several other extraction methods which have been reported in the literature [5-7]. The tissue samples from medical examiners' cases were used and the results are shown in Table 6.

TABLE 2—*Detectable limits of a number of drugs in blood and urine by XAD-2-TLC screening procedure.*

Drugs	Sample Size, ml	Sensitivity with TLC Visualization, μg/ml	
		Blood	Urine
Amobarbital	5	0.5	0.5
Amphetamine	5	0.5	1.0
Codeine	5	0.5	0.5
Methadone	5	1.0	1.0
Morphine	5	0.5	0.5
Phenobarbital	5	0.5	0.5

TABLE 3—*Percent recovery of added drugs to blood (10 ml).*

Drug	Quantity Added, μg	Recovery, %[a]
Amobarbital	52.9	80.2
Methaqualone	21.1	90.0
Morphine	20.0	76.0
Phenobarbital	44.7	98.7

[a] Result of three or more determinations.

TABLE 4—*Percent recovery of drugs added to human and dog brain, liver, and kidney.*

Drug	Quantity Added, μg	Recovery, %[a]
Amobarbital	52.9	93.0
Caffeine	32.7	88.7
Colchicine	6.37	96.0
Morphine	247.0	92.0
Phenobarbital	44.7	58.6
Thioridazine	15.5	78.6

[a] Result of three or more determinations by ultraviolet spectrophotometry. Drugs were added to 20 g of tissue.

[4] These samples were graciously furnished by Dr. F. Quintana, Connecticut Valley State Hospital, Middletown, Conn.

TABLE 5—*Drugs detected in urine from patients receiving clinical doses of various drugs. (A 5-ml aliquot of urine was analyzed in all cases.)*

Patient	Drugs Detected	Drugs Administered	Dosage, Mg
1	Chlorpromazine and metabolites	Chlorpromazine	100 (a.m.)
2	Imipramine and Desipramine	Imipramine	75 (t.i.d.)
3	Chlorpromazine	Chlorpromazine	100 (a.m.) 400 (h.s.)
4	Perphenazine	Perphenazine	32 (h.s.)
5	Perphenazine and Chlorpromazine	Perphenazine and Chlorpromazine	48 (h.s.) 100 (t.i.d.)
6	Trifluoperazine	Trifluoperazine	5 (b.i.d.)
7	Methyprylon	Methyprylon	300 (h.s.)
8	Meperidine	Meperidine	50 (q.4 h)
9	Chlordiazepoxide	Chlordiazepoxide	50 (q.4 h)

t.i.d. = three times daily b.i.d. = twice daily
h.s. = at bedtime q.4 h = every four hours

TABLE 6—*Comparison of drugs recovered from medical examiners' specimens using various extraction procedures.*

Drug	Tissue	XAD-2,[a] mg/100 g	Kaempe [5], mg/100 g	Acetonitrile Ether [6], mg/100 g	Butyl Chloride [7], mg/100 g
Amitriptyline	brain	1.0		0.5	
	liver	4.0		1.6	
Propoxyphene	brain	0.67	0.4		
	kidney	1.75	0.7		
	liver	4.75	0.8		0
Glutethimide	brain	0.5			0
Thioridazine	liver	2.5	1.1	1.5	
	brain	0.48	0.17	0.32	
Meprobamate	liver	0.7	0.25		
Pentobarbital	brain	1.5	0.79		
Phenobarbital	brain	0.52	0.39		0
	liver	2.39	1.08		0
Barbiturate combination (amobarbital and secobarbital)	brain	3.81	1.01		0
	liver	5.62	5.68		0.18

[a] Twenty grams of tissue were used in the XAD-2 method and 50 to 100 g were used in the other three procedures.

The amount of tissues received in these cases was insufficient for recovery comparisons by all four methods. We found that most recoveries by the XAD-2 procedure were 50 to 493 percent greater than by the other methods. Because the drug recoveries by the XAD-2 procedure are greater than the recoveries of other procedures, we found that 20 g of tissue used in the XAD-2 extraction are ample for drug identification, compared to the larger tissue samples suggested in the other procedures (Table 6).

A mild steam table hydrolysis of the tissue before XAD-2 extraction resulted in higher drug yields varying from 30 to 206 percent. An exception to this is propoxyphene, whose ultraviolet spectrum changes on acid hydrolysis and results in increased absorptivity (Fig. 1). Thin-layer chromatography of the hydrolyzed propoxyphene results in three distinct spots, only one of which has the same R_F as propoxyphene. It is advisable that tissues containing propoxyphene not be hydrolyzed, because a highly erroneous drug concentration will result if ultraviolet spectrophotometry is used as the technique for quantitation.

Discussion

Amberlite® XAD-2 resin is a styrene-divinyl benzene copolymer which adsorbs drugs mainly by van der Waals forces, hydrophobic bonding, and dipole-dipole interactions [*1*]. Amberlite resin has been used for screening urine in drug abuse cases [*1-4*]. We have expanded it to the extraction of drugs from biofluids and tissues with a *preliminary* drug screen using thin-layer chromatography.

The extension of the resin extraction procedure to tissue samples produced noteworthy results which we found, in general, are not matched by the published methods for drug

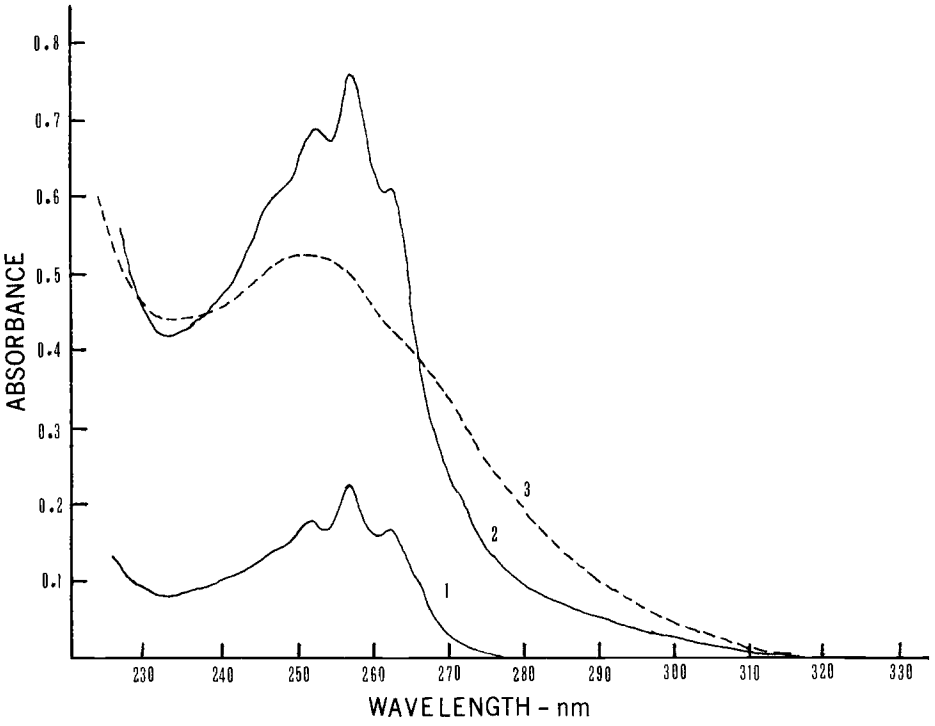

FIG. 1—*Absorption curves for propoxyphene, 0.2 mg/ml in 0.5* N *HCl:* (1) *untreated,* (2) *after standing on steam bath for 60 min, and* (3) *after autoclaving at 15 psi (103 kPa) for 30 min (had to be diluted 1 to 10 with 0.5* N *HCl before measurement).*

extraction of autopsy material. If the analyst is proficient in making a *preliminary* thin-layer identification with the spray sequence described, multiple pH-dependent solvent extractions [*5-9*] are not essential. Emulsions, a major problem in solvent extractions, and the utilization of large volumes of organic solvents are also eliminated.

The isolation and identification of drugs from tissues is the prime aim of any toxicological tissue extraction procedure. The initial determinant for accomplishing this aim is dependent on the amount of tissue used for sufficient drug recovery. In the numerous published extraction procedures the amounts used have been large, as in the instance of the classic Stas-Otto [*10*] procedure in which the suggested quantity of tissue was 200 to 300 g. Subsequent procedures, such as the tungstate method [*11,12*] and the ammonium sulfate method [*13*], utilized 200 g of tissue. In 1967, Stevens [*8*] developed a screening method using 100 g of tissue and utilizing $AlCl_3$ for protein precipitation. After adding 10 to 20 mg of drug his recoveries ranged from 5 to 53 percent. Steven's procedure, as with all protein precipitation methods, results in poor recovery data. Goldbaum [*14*] developed a procedure using 50 g of tissue for drug screening, utilizing a combination of distillation and extraction procedures. Recoveries presented by Goldbaum ranged from 50 to 80 percent. Our results with the XAD-2 procedure show that considerably less tissue is required for drug analysis.

After XAD-2 extraction, the eluants were thoroughly mixed and divided into two equal parts. One fraction was used to qualitatively identify the drug(s) extracted, whereas the other fraction was reserved for quantitation. Quantitative values were obtained by ultraviolet spectrophotometry or spectrophotofluorometry.

The sensitivity of this particular screening method was sufficient to detect concentrations of drugs found in blood or urine or both from individuals suspected of drug abuse. The readily detectable limits of drugs from 5-ml samples of urine or blood were 0.5 μg/ml for free morphine, codeine, amobarbital, and phenobarbital, and down to 1.0 μg/ml for other drugs tested. The limits of detection are dependent on the detection methods used. Although other detection methods such as gas-liquid chromatography or spectrophotofluorometry may be more sensitive for some drugs, the authors believe thin-layer chromatography offers the most rapid method of screening multiple samples effectively.

Table 5 readily demonstrates the sensitivity of the method in detecting drugs excreted in the urine after therapeutic doses were administered. It is noteworthy that trifluoperazine was isolated and identified from a 5-ml aliquot of urine obtained from a patient who was administered only 5 mg of the drug twice a day.

Another salutary point is the quality of the extract. The XAD-2 residue, after cleanup, is sufficiently free of interfering substances for ultraviolet analysis and gas-liquid chromatographic quantitation to be feasible in laboratory practice. Centrifugation of the homogenate for 10 min in the cold (4°C) seems to separate most of the fat from the aqueous phase, thus preventing fat accumulation in the residue. In fact, subsequent extraction of the residue with petroleum ether does not result in any improvement over the quality of the residue.

Table 6 clearly demonstrates comparative recoveries of the XAD-2 procedure and other extraction methods in a number of cases involving a limited amount of tissue submitted by medical examiners. Even though the acetonitrile-ether method [*6*] was developed specifically for basic drugs, more than a 100 percent increase in the recovery of the drugs amitriptyline and thioridazine was obtained by the adsorption method. The Kaempe [*5*] method (extractions using chloroform and chloroform-isopropanol) also proved to be less efficient for drug recovery than the column method and the acetonitrile-ether extraction method. It is a tedious method and requires large amounts of solvents and tissues. The butyl chloride extraction technique [*7*] appears to be useful

for a limited number of drugs, particularly propoxyphene and amphetamine. In contrast to the XAD-2 extraction, no propoxyphene was detected or recovered in the liver by this solvent extraction method (Table 6). The limitations of that method were also demonstrated by the fact that no glutethimide was recovered from the tissue (Table 6). Although the butyl chloride technique was found to be suitable for the extraction of propoxyphene and amphetamine from biological fluids, it is definitely inferior to the XAD-2 extraction procedure when it is extended to tissue analysis.

The low recoveries for phenobarbital (Table 4) in tissue were unexpected under the stated conditions. The probability that an increase in the recovery may be obtained [*15*] if one were to adjust the pH of the water extract to 2 to 4 is presently being studied. Another aspect under consideration is the use of different eluting solvents. In any event, if phenobarbital is detected by the screening procedure described here, any alternative specialized method designed especially for phenobarbital extraction may be employed for more accurate quantitation. From present indications a modification of the XAD-2 extraction procedure may be utilized for better phenobarbital recovery.

Toxicological screening of autopsy tissue specimens has always been a laborious, time-consuming procedure. The XAD-2 thin-layer chromatography screening procedure described here provides an uncomplicated, rapid technique for the detection of all classes of drugs in biological fluids and tissues.

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