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APPLICATION OF AMBERLITE XAD-2 RESIN FOR GENERAL TOXICOLOGICAL ANALYSIS*

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SUMMARY

The use of Amberlite XAD-2 resin for the extraction of drugs has been extended to the processing of either direct aliquots of blood and stomach contents or aqueous dialysates of the latter, as well as to that of aqueous Stas–Otto deproteinated extracts of tissue. A two-step elution provides the subsequent recovery of standard acid, neutral, basic compounds, and the more polar, water-soluble drugs and drug metabolites. Inorganics may be recovered from the eluate resulting from passage of the original solution through the resin. Extraction with columns of 1–2 g of Amberlite XAD-2 resin is shown to provide the best general approach for separations in pure form of nearly all toxic compounds from up to 500 g of tissue as well as from biological fluids and stomach contents.

INTRODUCTION

Amberlite XAD-2 resin has been successfully applied to the extraction of drugs from urine[1–4] because of the high recoveries obtainable, the elimination of emulsion problems, and the need for much smaller volumes of solvents than are required with the traditional solvent extraction methods, thereby requiring less time for the evaporation of the final extract. Furthermore, water-soluble drugs (quaternary ammonium compounds, glycosides) and metabolites (ecgonine, glucuronides of morphine and other drugs) which are not extractable by the usual organic solvents, and hence not detected routinely, are partially adsorbed[1,2] by the resin and may be recovered in part by the elution of the column with methanol[1] following the standard elution[3,4].

The application of XAD-2 resin as a general method for the extraction of drugs from other biological materials requires a preliminary purification step involving transfer of the drugs into an aqueous solution suitable for passage through the resin columns. This solution must be of sufficient dilution so that it possesses the

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proper flow characteristics, and in addition it should contain only small amounts of added reagents in order that it be suitable for a general analysis of both the organic compounds adsorbed and the organic and inorganic compounds which pass unadsorbed through the column.

Recently Pranitis *et al.*[5] described the use of XAD-2 resin for the recovery of drugs from biological fluids and tissue extracts. However, their method for the preliminary workup of tissue entails a partial thermal transformation of propoxyphene, and in addition there is no concern for the feasibility of recovery of more polar water-soluble compounds and pesticides. Furthermore, their technique is limited to the processing of 20-g samples using very large columns (12–20 g of resin) and also has the drawback of requiring a high-speed (20,000 rpm) refrigerated centrifuge.

A critical review of various general toxicological methods was undertaken in order to determine which was most suitable for application to the present problem. The traditional Stas–Otto procedure has been recognized as being the most general and suitable for the extraction of large amounts of viscera (100–500 g). We have found it to be the basic method of choice for development of a general procedure, extending the applicability of extraction, using 1.2–2 g of XAD-2 resin, to the general analysis of liver, brain, kidney and lung as an alternative to the traditional solvent extraction procedures.

Utilizing the well established efficiency of the alcohol precipitation of proteins and the efficiency of XAD-2 resin as compared with solvent extraction[3], we evaluated any practical limitations on the use of the XAD-2 resin which might result from a lack of capacity of the 1.2–2 g of this material to adsorb the relatively large amounts of drugs present in the concentrated aqueous extracts obtained from 500 g of tissue.

For the particular case involving analysis of stomach contents, further work resulted in the incorporation of a dialysis procedure, which has been found to be the best general toxicological technique for use in conjunction with extraction on XAD-2 resin, as well as in development of a procedure involving direct contact of viscera with the resin. Dialysis has long been recognized as an excellent separation procedure. The potential for recovery of drugs and pesticides from very dilute aqueous solutions using XAD-2[6] or XAD-4[7] resins thereby makes feasible the toxicological application of free dialysis rather than electrodialysis. This latter technique was often tested in the past[8–12] but was never widely used because of the incurred losses of morphine, cocaine and the ergot alkaloids as a result of oxidation or hydrolysis[9]. The optimal dialysis conditions and the total recoveries of representative drugs extracted from stomach contents by the combined dialysis–XAD-2 column method are also presented in this paper along with a description of the general application to tissues and biological fluids as outlined above.

EXPERIMENTAL

Materials

A Roller-Agitator was supplied by Wheaton Scientific (Millville, N.J., U.S.A.). Plastic containers of 1-l capacity and with wide-mouth screw-cap top were used. 1.75-in. Dialysis tubing, size No. 36, was obtained from VWR Scientific (Brisbane, Calif., U.S.A.). Amberlite XAD-2 resin (Rohm & Haas, Philadelphia, Pa., U.S.A.) was suspended in distilled water prior to use and packed as a slurry.

Drug screen columns of 2.75 × 0.375 in., a filter cartridge, phase separation paper and the evaporation system were obtained from Brinkmann (Westbury, N.Y. U.S.A.).

Pre-coated silica gel 60 TLC plates (E. Merck, Darmstadt, G.F.R.), 250 nm, without fluorescent indicator, were used.

For acid drugs the solvent system chloroform–methanol–ammonium hydroxide (90:10:1) and for basic drugs the system ethyl acetate–methanol–ammonium hydroxide (85:10:3) were used. It has been found that the best chromatographic resolution is obtained with the latter system when two blank plates are "chromatographed" in succession to a height of 18 cm each before the plate(s) containing the samples for analysis is developed.

Preparation of columns and chromatographic procedure

The bottom of each column is fitted with a small cotton plug or porous circular disc. The resin slurry is poured in, leaving about 1/4-in. space on top for insertion of a protective plug of glass wool. The column should be fitted with the reservoir vessel into which may be poured the solution to be extracted. A specially modified filter cartridge (Fig. 1) is attached to the opening of a 500-ml separatory funnel, inserted upside down, which is used as an extended reservoir allowing automatic control of flow through the column. Alternately the solution may be fed onto the column by means of a flow pump equipped with multiple channels for the simultaneous processing of series of samples[2]. The eluate obtained after elution is filtered through phase separation paper to remove all water, and then evaporated to dryness in the evaporation system as described below.

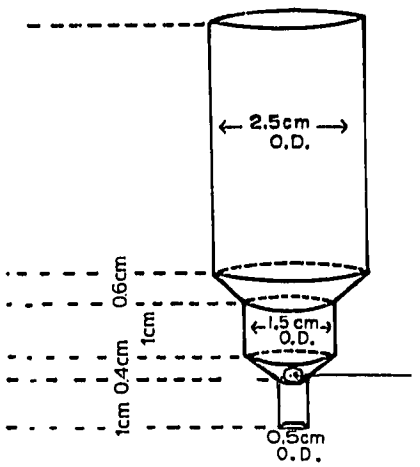

Fig. 1. Adaptation of the filter cartridge (Brinkmann) for control flow delivery. An opening of 4–5 mm in diameter is cut at the point indicated by the arrow. The large upper part of the cartridge is attached to the top of a 500-ml separatory funnel inserted in an inverted position with the stop-cock closed. The lower part of the cartridge is dipped into the reservoir of the routinely assembled XAD-2 column. When the level of aqueous solution is above the opening indicated, the flow is interrupted until enough solution passes through the column to allow air to enter through the aperture, thus allowing the resumption of the flow.

General procedure for analysis of large quantities of liver and brain

500 g of tissue are homogenized with 500 ml water in a Waring blender, the resulting slurry is transferred to a 2-l flask, acidified with 3 ml of a saturated solution of tartaric acid, and then steam distilled. The distillate is put aside for analysis for volatile poisons. The remaining slurry is filtered through Whatman No. 1 fluted filter paper.

The solid fraction retained in the filter paper (as well as any fraction remaining in the distillation flask) is washed with 500 ml of acetone and the extracts are combined, filtered and evaporated to a volume of approximately 100 ml. The aqueous acetone solution is cooled for 15 min, filtered and transferred to a 500-ml separatory funnel to which are added with mixing 100 ml of distilled water and 40 ml of a saturated aqueous solution of sodium sulfate. This solution is then extracted twice with 100 ml of hexane. The aqueous acetone solution is discarded and the hexane extract transferred to an evaporation flask and evaporated to dryness on a water-bath. The residue is further purified by partition of the hexane solution with acetonitrile[13] or by column chromatography using alumina[14] or silica gel[15,16]. The final purified extract may be chromatographed on silica gel plates in the system hexane–acetone (9:1) for the analysis of pesticides.

The filtrate obtained from the distillation residue is evaporated to about 100 ml in an evaporation dish*. Proteins are precipitated from the concentrated aqueous extract by the addition of 95% ethanol in the ratio of six volumes of alcohol for each volume of the concentrate. The alcohol–water solution is cooled for at least 1 h, filtered, vacuum evaporated to remove excess alcohol, and taken up with 100 ml of hot water. The pH is adjusted to 8.5–9.0 using concentrated ammonium hydroxide, the column is now diluted to 250 ml with water, and passed through the column of XAD-2 resin and discharged.

The first step in the general desorption process of XAD-2 resin with eluents is the passage through the column twice with 10 ml of a solution of chloroform–isopropanol (3:1). The eluate is passed through PS-1 phase separation paper and evaporated to dryness after the addition of 0.1 ml of a solution of 10% HCl in alcohol. An additional elution with 10 ml of methanol is recommended whenever compounds of high polarity, *e.g.*, glycosides, glucuronides, quaternary ammonium salts, etc., are thought to be present or are to be included in the analysis.

Direct procedure for the analysis of stomach contents and blood

Approximately one half of the available quantity of the stomach contents or 10 ml of blood are diluted to about twice their volume with distilled water. The pH is determined (pH paper) and if necessary the solution is acidified using 10% tartaric acid solution. The acidic solution is warmed on a steam-bath for 15 min, allowed to cool and filtered through Whatman No. 1 filter paper.

Where filtration is rapid, the residue in the filter paper is washed with 200 ml or less of distilled water, depending upon the viscosity of the stomach contents. The total filtrate is now adjusted to pH 8.5–9.0 and then passed through the XAD-2 column. Elution is as described above.

* When glycosides or other thermolabile compounds are to be determined, the use of a rotary film evaporator under reduced pressure is suggested.

Dialysis of stomach contents

When the filtration is found to be slow, or where it is observed that the diluted stomach contents contain large amounts of solid food, mucosa, or other portions of stomach wall, the contents are transferred to a dialysis bag 1 3/4-in. wide and 12-in. long previously soaked in distilled water. The bag is firmly secured and placed in a wide-mouth plastic container with 250 ml of distilled water. The container is placed on a roller agitator set at 8 rpm and allowed to equilibrate for 1.5 h, at the end of which time the dialysate is recovered and the dialysis procedure repeated as before. The combined dialysates are set aside, and the contents of the bag adjusted to pH 8.5–9.0 by injecting 1 or 2 ml ammonium hydroxide into the dialysis bag below the knob. The bag is firmly re-tied below the injection point and then redialyzed twice as described above. The acidic and basic dialysates are combined, adjusted to pH 8.5–9.0, and then passed through the XAD-2 column. The eluate from this step may be transferred to an evaporation dish, concentrated to a small volume on a hot plate and analyzed for organic acids, fluorides, borates, and other inorganic compounds. The resin is now eluted as described previously.

Evaluation of the XAD-2 resin extraction method

In order to compare the efficiency of XAD-2 resin extraction with the established Stas–Otto procedure, the following case material, all found to be drug positive when processed by the Stas–Otto type technique, was now analyzed by the XAD-2 resin column method described above but without dialysis: brain, fourteen samples; liver, six samples; stomach content, twelve samples. In addition, three 500-g liver samples, to which propoxyphene was added to a concentration of 3 mg % per sample, were analyzed by each method.

RESULTS AND DISCUSSION

A comparison of the results of the toxicological analyses on the indicated biological material by both the Stas–Otto procedure and direct XAD-2 resin extraction is presented in Table I. Based upon estimations derived from thin-layer chromatography (TLC) it was found that the resin was not overloaded nor was the extraction process hindered by the concentration of components present in the extract obtained from 500 g of tissue. Hence there was no need for the use of more than 2 g of XAD-2 resin, larger quantities of which would require special columns and larger elution volumes. All non-volatile organic drugs detected by the Stas–Otto procedure were also detected by the XAD-2 resin extraction described above.

The average recovery of 15 mg of propoxyphene from 500 g of liver was the same (69%) using either XAD-2 resin or a Stas–Otto type procedure which involves solvent extraction after steam distillation of an acidic tissue homogenate (acidification with tartaric acid) and alcohol deproteinization. These results demonstrate the benefit of the milder, non-destructive extraction for labile compounds as compared to other analytic methods which involve heating in a hydrochloric acid medium. The latter process is known to cause thermal degradation of propoxyphene, thereby interfering with the TLC analysis of this drug[2,5].

Toxicological analysis of large (500-g) quantities of biological material has become less common wherever sophisticated analytical instrumentation is available

TABLE I

RESULTS OF TOXICOLOGICAL ANALYSES ON STAS-OTTO POSITIVE TISSUE USING DIRECT XAD-2 RESIN EXTRACTION WITHOUT DIALYSIS

Drug	*Brain*	*Liver*	*Stomach contents*	*Total*
Amitriptyline			1	1
Amobarbital	3	1		4
Caffeine			1	1
Chlorpromazine	1			1
Codeine			1	1
Diazepam		1	2	3
Imipramine	3	1	1	5
Methadone	6	2	2	10
Methaqualone			1	1
Phenacetin			1	1
Phenobarbital	1	1		2
Propoxyphene	1	1	2	4
Secobarbital	2			2
Total	17	7	12	36

for the identification of drugs in the microgram range, but it is the practice all around the world and also in our laboratories to analyze a certain number of special cases using such oversized samples in order to allow more efficient purification and the use of microcrystal tests and chromophoric chemical reactions for the elucidation of doubts arising during the process of identification.

A similar experiment was carried out with stomach contents which were analyzed both by the alcohol precipitation method and directly dialyzed following adsorption of drugs on XAD-2 resin.

Secobarbital, phenobarbital, amobarbital, diazepam, flurazepam, meprobamate, phenacetin, caffeine, propoxyphene, methaqualone, amitriptyline, codeine, imipramine, methadone, thioridazine and quinine were detected by both methods. Ethchlorvynol, which is removed by the steam distillation step of the classical procedure, is however readily detected after dialysis of stomach contents and adsorption by XAD-2 resin.

In general, material present in stomach contents does not require special grinding or extensive drug extraction procedures because the extent of the occlusion of drugs to any organic matter that may be present is not as strong as that which occurs with other tissues and fluids, *e.g.*, liver and blood*. Separation of the free drugs from inert food material can be achieved essentially by physical methods.

Solid and viscous material in suspension that might eventually interfere with the flow-rate of the liquid phase through the column may be removed by prior centrifugation[5] or filtration, by adding a filter paper to the column[17], by shaking the suspension directly with the resin as is recommended in rapid, direct procedures for the

* Exceptions do, however, occur particularly with regard to drugs or toxins consumed in close conjunction with large amounts of food and consumption of certain food products, *e.g.* oils or dairy products, that may bind or occlude the drugs or otherwise impede their ready diffusion.

analysis of blood and stomach content[18,19] whenever the time of analysis is a major factor, *e.g.*, in hospitals for the chemical diagnosis of intoxications. However, when the physical state of the suspension of stomach contents is such as to make the utilization of this direct method impractical, the entire contents should be subjected to the dialysis procedure previously described. In the routine work of the Toxicological Laboratories of the Office of Chief Medical Examiner, dialysis has been used routinely regardless of the physical state of suspension.

Slurries of stomach contents containing different amounts of representative drugs were subjected to dialysis and the combined dialysis fractions extracted with XAD-2 resin.

The recovery of drugs following dilution and dialysis of the stomach contents is given in Table II, where the amount of drug recovered after each step of the procedure is indicated.

TABLE II

RECOVERY OF REPRESENTATIVE DRUGS FROM STOMACH CONTENTS AFTER DIALYSIS AND EXTRACTION WITH XAD-2 RESIN

Drug	*Amount (mg) added to 60 ml of stomach contents*	*Method of analysis*	*Typical per cent recoveries in different dialysis fractions obtained on one sample*				*Average per cent recoveries and standard deviations on three samples*
			Acid (1st)	*Dialysis (2nd)*	*Basic (3rd)*	*Dialysis (4th)*	
Amitriptyline	2.50	UV[20]	40.4	33.2	2.2	1.6	73 ± 9
Diphenylhydantoin	1.00	UV[21]	24.0	14.6	12.0	12.0	63 ± 3
Imipramine	0.25	Fluorescence[22]	20.0	18.1	17.2	10.0	64 ± 2
Morphine	0.05	Fluorescence[23]	40.0	12.0	8.0	6.0	63 ± 11
Propoxyphene	1.00	UV[24]	37.2	23.0	10.7	5.1	76 ± 5
Quinine	1.00	Fluorescence	52.5	11.7	1.5	1.7	64 ± 11
Secobarbital	1.00	UV[25]	26.0	19.0	10.1	6.7	60 ± 5

Most of the drugs, with the exception of diphenylhydantoin and secobarbital, are obtained in the first two acid dialysates. Based upon the data, it can be seen that even where a preliminary dilution of the contents is not done, the single direct dialysis against 250 ml of acidified water will nevertheless result in at least a 25% recovery of all drugs. With the subsequent use of analytical techniques possessing sensitivity in the microgram range, the threshold of analytical detection of nearly all toxic compounds is considerably below the actual levels present in stomach contents following acute poisoning by oral ingestion.

The XAD-2 column is eluted with chloroform–isopropanol (3:1) for desorption of those drugs normally encountered in a general screening procedure. In this fraction there will also be recovered small amounts of such compounds as organophosphorous and chlorine pesticides, which, though only slightly water soluble, can thus be found in the aqueous fraction. In general, the bulk of these compounds remain in the residue obtained from filtration of the acid steam-distilled tissue homogenate. They

are recovered by subjecting this material to the acetone wash–hexane partition procedure as described in Experimental.

It should also be noted that pesticides of the water-soluble quaternary ammonium type, *e.g.*, paraquat and diquat, are also retained in the aqueous fraction and recovered as indicated below.

The corresponding chloroform–isopropanol eluate contains neutral, acid and basic drugs, These should be separated by TLC, which has been found to be preferable to gas chromatography for the analysis of resin extracts, prior to further identification. The recommended chromatographic development system is ethyl acetate–methanol–ammonium hydroxide (85:10:3) and chromatography is carried out after prior conditioning of the development solvent as described in *Materials*. The results are presented in Table III. The necessity for the conditioning step resides in the fact that the observed chromatographic differentiation of drugs with high R_F values will not occur in the absence of this operation. It may be noted that chloroform–methanol (90:10) has also been found very useful as a general solvent system. After examination of the plates under UV light for the detection of fluorescence, optimum chromophoric visualization may be achieved by application of the following sequence of spray reagents: (a) ninhydrin–methanol, (b) mercuric sulfate, (c) diphenyl carbazone and (d) iodoplatinate.

Highly polar compounds, partially retained on the column, such as glucuronide

TABLE III

INFLUENCE OF SOLVENT CONDITIONING ON THE DEVELOPMENT OF TLC PLATES IN THE SYSTEM ETHYL ACETATE–METHANOL–AMMONIUM HYDROXIDE (85:10:3)

Drug	R_F *value*	
	Fresh development solution	*Conditioned development solution**
Morphine	17	14
Codeine	39	27
Quinine	51	38
Amphetamine	55	37
Quinidine	60	48
Nicotine	80	62
Meperidine	81	67
Oxycodone	82	77
Imipramine	87	69
Chlorpromazine	90	76
Pentazocine	91	85
Amitriptyline	92	78
Flurazepam	92	85
Diazepam	94	92
Methaqualone	95	94
Cocaine	95	91
Methadone	96	88
Lidocaine	96	96
Propoxyphene	97	94

* The solution was conditioned by running two blank plates to a height of 18 cm and removing from the tank just prior to development of sample(s) plate.

derivatives and quaternary ammonium compounds, *e.g.* pavulon, diquat, paraquat, etc., are eluted with 10 ml of methanol following the elution with chloroform–isopropanol.

A significant advantage of the XAD-2 resin extraction, as compared to solvent extraction, for the purpose of general screening is the possibility of detecting such compounds which would otherwise not be isolated owing to their lack of solubility in the organic solvents normally used in such extractions. Colchicine has been recovered from tissue with a 96% yield[5] and our trial experiments with urine obtained from heroin addicts, known to contain morphine glucuronide, revealed that about 10% of this morphine metabolite could be so recovered by the XAD-2 resin extraction. Other qualitative trials revealed that when pavulon, a quaternary ammonium compound, was added in a 0.5-mg quantity to 50 ml of stomach contents, it could be detected by TLC following dialysis, adsorption on XAD-2 resin and differential elution as described above, the pavulon being eluted with the methanol.

The eluate recovered from the initial adsorption of the sample may be concentrated and analyzed directly for inorganic compounds by such means as spot tests, ion-sensitive electrodes, or atomic absorption spectroscopy.

CONCLUSION

Stomach contents are generally the material of first choice in the forensic toxicological screening for the detection of acute drug intoxication. For this purpose simple dilution/filtration of the contents, or if necessary extraction of drugs by dialysis, provides a solution suitable for passage through an XAD-2 resin column. Successive elution of the column with chloroform–isopropanol (3:1) and methanol will effect desorption of both pesticides together with drugs and the polar compound fractions. The effluent from the initial column adsorption step may be concentrated for subsequent analysis for salicylic acid and a variety of inorganics (borates, fluorides, etc.).

Whenever large quantities of organic tissue material, *e.g.* liver and brain, are used for the isolation of drugs, an initial Stas–Otto type extraction of the tissue, followed by protein precipitation with alcohols, is required for the ultimate production of the appropriate aqueous solution.

Because of its affinity for a wide variety of organic compounds and the simplicity and efficiency of application, the use of XAD-2 resin columns has become an important new tool for both routine toxicological screening as well as for special extraction purposes. In addition, the overall retention characteristics of the resin may be applied to a general scheme of analysis for unknown compounds.

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