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REMOVAL OF SALTS FROM PURINES, PYRIMIDINES AND NUCLEOSIDES USING AN XAD-4 RESIN

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SUMMARY

In the present study, procedures are described for using columns of XAD-4 resin beads, a styrene–divinylbenzene copolymer, as adsorbents for purines, pyrimidines and their nucleosides. Elution of these nitrogenous compounds from the beads is carried out with either water or 19% ethanol, depending upon how tightly the particular compound is bound to the beads. The various salts [*i.e.*, sodium chloride, sodium acetate, sodium phosphates, tris(hydroxymethyl)aminomethane (Tris), etc.] that have been tested are not adsorbed on the columns and appear in the first few fractions of the column effluent. Studies have been carried out with approximately 30 different compounds, including a number of methylated purines and purine nucleosides. Nearly all of the compounds were obtained entirely free of salt with an average recovery from the column of 96%.

INTRODUCTION

In the purification and analysis of purines, pyrimidines and their nucleosides, it is often necessary to subject samples to a second separative procedure for positive identification and quantitation. In many cases the presence of the eluant buffer or acid present in the sample from the first procedure prevents the direct application of a second procedure. This is often true when the second procedure involves ion-exchange chromatography, thin-layer chromatography, gas chromatography or high-performance liquid chromatography. Consequently, a simple means of separating purines, pyrimidines and their nucleosides from various salts would appear to be of considerable usefulness. In addition, this type of separative technique may be of value in the preliminary separation of these compounds from urine or tissue extracts.

XAD-4 resins have been used by other workers for adsorption of various non-polar compounds[1,2]. In most cases, elution has been carried out with non-polar solvents. In our initial studies with XAD-4 resins, it was noted that purine nucleosides could be eluted from the resin with dilute ethanol. Although the column was not very effective in separating the different purines, pyrimidines and nucleosides, it did separate each of them from the buffer or salt that was applied to the column. When the

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resin was prewashed with a higher ethanol concentration than that used for elution, the background absorbance in eluant solutions coming from the column was reduced to a very low level. This indicated that a column of XAD-4 resin could be effectively used for the separation of purines, pyrimidines and nucleosides from various salts or buffers, with the nitrogenous compounds present in a solvent that could readily be removed, if necessary, prior to application of the next separative procedure.

MATERIALS AND METHODS

Chemicals and solutions

XAD-4 resin* (Amberlite non-ionic polymeric adsorbent), 20–50 mesh, was obtained from Mallinkrodt. The sources of most of the purines, pyrimidines and nucleosides used as standards have been given previously[3]. Others were obtained as follows: uridine, P-L Biochemicals; cytosine, S-adenosylhomocysteine, creatinine, 2′-O-methyladenosine, 5′-deoxy-5′-methylthioadenosine and theophylline (1,3-dimethylxanthine), Sigma. All components of buffers were reagent grade, and their pH values refer to pH at 23°C.

Preparation and use of XAD-4 columns

Excessively light or heavy particles in the XAD-4 resin were removed by suspending and resuspending the resin repeatedly in water. After preparation of the columns using a liquid slurry, the resin in the column was prewashed with *ca.* 25 times the column volume of 47.5% (v/v) ethanol to remove ultraviolet absorbing materials. After washing with deaerated water to remove ethanol and air bubbles, the column was ready for use. This prewashing reduced the absorbance at 260 nm of a 19% (v/v) ethanol effluent from the column to 0.03 or lower. This effluent also had negligible fluorescence (280 nm excitation, 320–400 nm emission). Since ultraviolet absorbance readings are routinely used for identification of purines and pyrimidines, adequate prewashing of the resin is absolutely essential and the absorbance of a 19% ethanol column effluent should always be checked prior to column use.

Unless controlled, flow through the columns was rapid due to the large mesh size of the resin. During operation, the flow was limited with a valve to *ca.* 0.8 ml/min for the 30 × 1.3 cm column, and *ca.* 0.25 ml/min for the 24 × 0.6 cm column. The change from water flow through columns to 19% ethanol often caused a release of some gases within the column. Although this did not impair the use of the columns, it did tend to reduce the flow-rate. Gas release in the columns could be minimized by boiling and cooling the water used on the columns. With the small columns, compounds were applied in a solution volume (9 ml) equivalent to 1.3 times the column volume. With the larger columns, the solution volume (26 ml) applied to the column was 0.65 times the column volume. In the latter case, the applied volume was approximately equivalent to the volume of liquid surrounding and within the beads of the column (the breakthrough volume). Small volumes of solutions could be applied to the columns, of course, with some improvement in the separations. In practice, however, this might necessitate the prior concentration of samples, an extra procedural step which it was hoped could be avoided.

* Bio-Beads SM-4 (Bio-Rad Labs.) is a more highly purified and washed preparation of the XAD-4 resin.

Compounds were detected in eluant fractions from the columns by measuring absorbance at the appropriate wavelength in the ultraviolet region of the spectrum. Salts in the column effluent were detected by drying 0.5-ml aliquots in tared beakers and weighing the residues. The elution volume, V_e, for a given compound was measured from the midpoint of the application volume to the peak for that particular component in the elution profile. The breakthrough volume was determined with various small unabsorbed molecules. This volume, using NaCl, NaOH or sodium acetate was found to be 65% of the column volume, or 26 ml for the 1.3 × 30 cm column and 4.4 ml for the 0.6 × 24 cm column. The breakthrough volume would also be the elution volume for unabsorbed molecules.

For the known compounds used in the present study, there was no problem regarding column regeneration. The columns were used repeatedly, with the only precaution being some additional washing prior to reuse to assure the complete removal of the previous compounds. If more complex solutions are applied to the columns (*e.g.*, urine, tissue extracts, etc.), the columns may be regenerated by washing with a higher ethanol concentration (*e.g.*, 47.5% ethanol) than that used for elution. When columns are used with urine, it may be desirable to discard the top centimeter of the resin after each use due to the discoloration caused by strongly adsorbed compounds.

RESULTS

The elution volumes for the various compounds from the XAD-4 columns are summarized in Tables I and II. Pyrimidines, and some purines (*e.g.*, hypoxanthine, xanthine and guanine) are retained only slightly by the XAD-4 resin. For these compounds, a 30 × 1.3 cm column was most satisfactorily used with water as eluant (Fig. 1A). A comparison is made in Table I of the elution volumes for a number of these compounds from both the small and large columns. Some of the compounds

TABLE I

WATER ELUTION OF COMPOUNDS FROM XAD-4 COLUMNS

Resin bed volumes in the large (30 × 1.3 cm) and small (24 × 0.6 cm) columns were 40 and 7 ml, respectively. Compounds were applied to the columns at a pH of 5.5–7 in either acetate buffer or NaCl. The volumes applied were 26 ml (large column) and 9 ml (small column).

Compound	*Large column, V_e (ml)*	*Compound*	*Small column, V_e (ml)*
Hypoxanthine	112	Hypoxanthine	17
Xanthine	130	Adenine	42
Guanine	96	Adenosylhomocysteine	135
Cytosine	63	7-Methylguanine	77
Uracil	84	Cytosine	13
Thymine	316	Uracil	16.5
Cytidine	131	Pseudouridine	14
Uridine	174	Creatinine	14.5
Pseudouridine	72	1-Methylnicotinamide	11.5
		Tyrosine	15

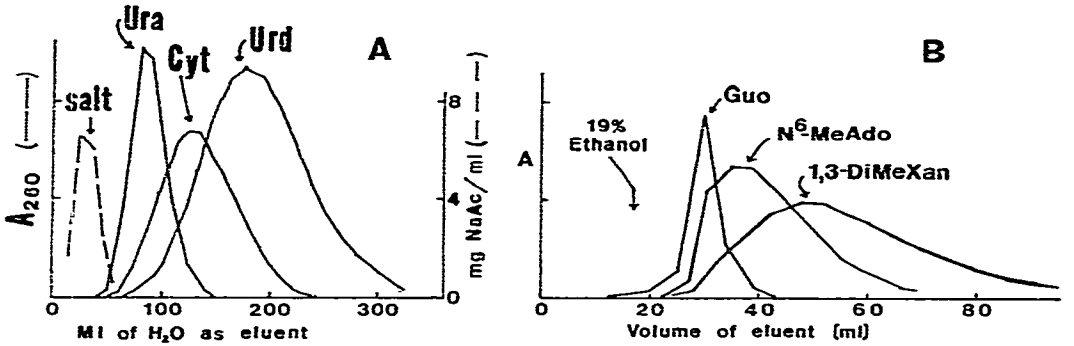

Fig. 1. A, Composite profile showing water elution of weakly retained compounds. The elutions were carried out separately using a 30 × 1.3 cm XAD-4 column. The samples (3–6 μmol) were applied to the column in 26 ml of 0.10 *M* NaCl (cytidine), or 26 ml of 0.077 *M*, pH 5.2, sodium acetate (NaAc) buffer (uracil and uridine). Fraction volumes were 8–12 ml. Abbreviations: Cyt = cytidine; Ura = uracil; and Urd = uridine. B, Composite profile showing elution with 19% ethanol of several more strongly bound compounds from a 24 × 0.6 cm XAD-4 column. The elutions were carried out separately on three columns and absorbance (*A*) measurements were made at 250 nm (guanosine), 260 nm (N^6-methyladenosine) and 267 nm (1,3-dimethylxanthine). The samples (0.5–1.0 μmol) were applied to the columns in 9 ml of 0.06 *M*, pH 5.2 sodium acetate buffer. Columns were washed with 15 ml of water prior to starting 19% ethanol. Fraction volumes were 4–5 ml. Abbreviations: Guo = guanosine; N^6-MeAdo = N^6-methyladenosine; and 1,3-DiMeXan = 1,3-dimethylxanthine (theophylline).

TABLE II

ETHANOL ELUTION OF COMPOUNDS FROM XAD-4 COLUMNS

Compounds were applied to the column (24 × 0.6 cm) in a 9-ml volume. Each column was washed with 15 ml of water prior to starting 19% ethanol.

Compound	V_e*	*Compound*	V_e*
Adenine	13	Guanosine	13
Adenosine	5	N^2,N^2-Dimethylguanosine	15
2′-Deoxyadenosine	15	N^2-Methylguanosine	12
2′-O-Methyladenosine	20	1-Methylguanosine	12
N^6-Methyladenosine	19	Inosine	12
1-Methyladenosine**	9	1,Methylinosine	12
5′-Deoxy-5′-methylthio-adenosine	68	7-Methylinosine***	14
Adenosylhomocysteine	12	1-Methylxanthine	15
7-Methylguanine	13	7-Methylxanthine	13
Tryptophan	11	1,3-Dimethylxanthine	33

* Elution volume in ml after the start of the 19% ethanol.

** This compound was applied to the column in pH 7 phosphate buffer and eluted with 19% ethanol containing 0.01 *N* acetic acid.

*** This compound was partially eluted by the water prior to initiation of the 19% ethanol.

listed in this table for purposes of comparison are more satisfactorily recovered using 19% ethanol elution because of the large volumes required for elution with water. By using the larger column, a nearly complete separation of each compound from the salt was obtained for all of the uncharged purines, pyrimidines and nucleosides which were tested. There was, however, a slight overlap of the salt peak with the component peak when cytosine and pseudouridine were eluted from the XAD-4 resin.

Purine nucleosides and various methyl derivatives of purines were more strongly adsorbed by the XAD-4 resin. These were most satisfactorily separated using a 24 × 0.60 cm column using 19% ethanol for elution. Salts were removed from the column with water prior to beginning the 19% ethanol elution. Examples of this type of separation are shown in Fig. 1B, with elution volumes for a number of compounds listed in Table II.

In addition to purines, pyrimidines and nucleosides, several other ultraviolet absorbing components of urine have been tested for retention on the XAD-4 resin column. Tryptophan is retained and is eluted with 19% ethanol in much the same manner as the various nucleosides (Table II). Tyrosine is retained only slightly and is eluted with water (Table I). Creatinine and 1-methylnicotinamide show only very slight retention and are eluted rapidly with water (Table I).

Recovery of the different compounds from the XAD-4 columns was excellent. With 46 different separations (32 different compounds), the recovery of the added component in the eluent was 96.3% with a standard deviation of 4.5%. Of all the compounds tested, only one (N^6-dimethylallyladenosine) could not be satisfactorily eluted from the column with 19% ethanol. In this instance, 47.5% ethanol was required for elution. It seems likely that other nucleosides with longer aliphatic side chains or with three or more methyl groups may also be too strongly adsorbed by the XAD-4 resin for satisfactory elution.

Effects of salts and pH

Since the primary purpose of this study was to devise a technique for removal of salts from the desired compounds, the behavior of various salts, acids, etc., has been examined. NaOH, HCl, NaCl, Tris, $NaNO_2$ and sodium phosphate buffers all move rapidly through the column and appear in the first few eluant fractions as shown for sodium acetate in Fig. 1A. Acetate buffers are of particular interest since the undissociated acid is retained by the column to a greater extent than the acetate anion. This produces a pH gradient in the elution profile. When a pH 5.2 buffer is used for application to the large column, the effluent pH starts at about 6.5 and decreases to about 3.5 after 50 ml of water have passed through the resin column. If the compound to be eluted has a pK_a between 3.5 and 6.5, two peaks may be noted in the elution profile as a consequence of the pH gradient. This type of behavior is illustrated for uric acid in Fig. 2A, where the first peak in the elution profile is uricate anion and the second peak is undissociated uric acid. When the sample is applied to the column in a phosphate buffer, there is no appreciable pH gradient.

Weakly bound compounds, such as hypoxanthine, xanthine and guanine were not retained on the column when applied in HCl. In these cases, adjustment of the sample pH to *ca.* 6 prior to application was essential for proper retention of these purines by the XAD-4 resin. However, some of the more tightly bound compounds were retained by the resin when applied to the column in 0.10 *N* or 1.0 *N* HCl.

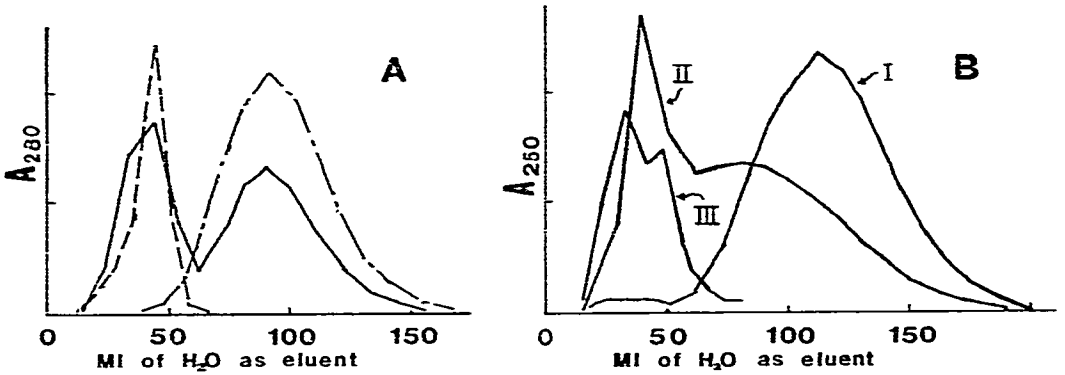

Fig. 2. Composite elution profiles showing the effect of pH. Water was used for elution of 30 × 1.3 cm XAD-4 columns with *ca.* 8–10 ml per fraction. A. Elution of uric acid: ——, application to column in 0.06 *M* acetate buffer, pH 5.2; – · –, application in 0.10 *N* HCl; – – –, application in 0.13 *M* phosphate buffer, pH 7.4. B. Elution of hypoxanthine: I, application to column in 0.10 *M* NaCl; II, application in 0.10 *N* HCl; and III, application in 0.10 *N* NaOH.

Examples of compounds that were retained directly from HCl solutions are N^2,N^2-dimethylguanosine, 1-methylinosine and 1-methylguanosine. These compounds were obtained essentially free of HCl when eluted with 19% ethanol.

DISCUSSION

Although most of the purines, pyrimidines and nucleosides have no charged groups at physiologic pH values, the amino nitrogens or ring nitrogens become cationic in acid solutions while enolic groups become anionic in alkaline solutions. Differences in the pK_a values for these groups on the various purines, pyrimidines and nucleosides have provided the basis for ion exchange separations of these compounds[3–7]. Elution of compounds from ion exchange columns requires the use of solutions of increasing ionic strength (buffers, acids, etc.). When it is desired to rechromatograph a particular component, the presence of the eluant buffer or acid impairs the retention of the compound on the second ion exchange column. The presence of ions in the solution to be applied causes peak spreading or in some cases, actual elution of compounds. Consequently, removal of the previous eluant ions is usually a necessary prerequisite to rechromatography of these nitrogenous compounds. The lack of a good procedure for removal of salts, buffers or acids from the desired compounds has been a major problem in the application of a second separative technique for identification and determination of purines, pyrimidines and nucleosides, and the present technique provides a possible solution to this problem.

One of the primary methods used for peak identification in high-performance liquid chromatography is peak displacement after compound modification by enzymatic or chemical means. Following deamination of purines or pyrimidines with nitrous acid, removal of nitrite is generally necessary prior to use of a separative technique for product identification. An XAD-4 column has been successfully used by the author for nitrite removal following conversion of guanine to xanthine with nitrous acid.

Although most purine or pyrimidine containing compounds are adsorbed from aqueous salt-containing solutions onto charcoal[8], subsequent recovery of these compounds from the charcoal is never quantitative and the release of ultraviolet absorbing materials from the charcoal is always a problem. Adsorption of purines, pyrimidines and nucleosides onto an XAD-4 column with methanol elution was utilized by Mrochek *et al.*[7] as a preliminary purification step in the study of these compounds in urine. However, these authors provided no details of their procedure nor did they carry out any recovery studies using known compounds. Sweetman and Nyhan[9] studied the adsorption of a large number of purines, pyrimidines and nucleosides on Sephadex G-10. Reasonably effective separations were obtained using dilute buffers as eluents with 100 × 1 cm columns. More recently, other workers have utilized the binding of ribonucleosides to phenylboronate affinity gel columns for preliminary separation of nucleosides of urine[10–12]. These affinity columns bind nucleosides with free 2′- and 3′-OH groups. Hence they do not bind either deoxynucleosides, 3′-O-methylnucleosides or free purines and pyrimidines.

Several characteristics of the adsorption of purines, pyrimidines and nucleosides onto the XAD-4 resin are evident as a consequence of the present study. First, the presence of a charged group on the column lessens the binding to the resin. Second, the addition of a methyl group increases the binding, unless the presence of the methyl group puts a charge on the molecule. Third, purine nucleosides are more tightly bound to the resin than purines, and purines more tightly than pyrimidines.

The effect of a charged group on the molecule is evident from the reduced binding of hypoxanthine in HCl, where the molecule is a cation, and of hypoxanthine in NaOH where the purine ring is negatively charged. The hypoxanthine is retained best by the resin at a pH of 4–7 where there is no charge on the molecule (Fig. 2B). 7-Methylinosine has a quaternary nitrogen at N^7 with a corresponding positive charge. It is not bound to the XAD-4 resin as tightly as inosine despite the presence of a methyl group on the former compound. 1-Methyladenosine has a higher pK_a (7.6) than other adenosine compounds and is retained by the resin at pH 5 to a lesser extent than adenosine. At pH 7, however, it is bound quite strongly. Therefore, the presence of the cationic group on 1-methyladenosine at pH 5 has reduced its binding to the resin.

The effect of methyl groups in increasing binding to the resin, when no change in charge is involved, is evident from a comparison of the following: adenosine *vs.* N^6-methyladenosine or 2′-O-methyladenosine; guanine *vs.* 7-methylguanine; uracil *vs.* thymine; and xanthine *vs.* 1-methylxanthine or 7-methylxanthine. The only instance where the presence of methyl groups did not increase binding was in the comparison of guanosine with N^2-methylguanosine and 1-methylguanosine. In this case, since all three compounds were eluted rapidly, the conditions were not ideal for a comparison of elution positions.

Adenosylhomocysteine has a zwitterionic aliphatic group attached to an adenosine moiety. Since the zwitterion is some distance from the purine, it did not have as great an effect on the retention of the molecule by the resin as that noted for charged groups on the purine ring itself. The uncharged methylthio group attached to adenosine (*e.g.*, in 5′-deoxy-5′-methylthioadenosine) markedly increased the binding of this nucleoside to the XAD-4 resin.

Although a fraction collector has been used with XAD-4 resin columns in the

studies described in the present report, separations with manual collection of only a minimal number of fractions could reasonably be devised with the information provided. The products from the column are obtained free of salt with excellent recoveries, and may be used, either directly or after concentration, for a subsequent separative procedure. These characteristics should make this technique of considerable value to others involved in the study of purines, pyrimidines and nucleosides in biological materials.

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