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REMOVAL OF SALTS FROM AROMATIC AMINO ACIDS, THEIR METABOLITES AND RELATED COMPOUNDS USING AN XAD-4 RESIN

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SUMMARY

Previous studies using XAD-4 columns for desalting purines, pyrimidines and nucleosides have been extended to other compounds. In the present study, procedures are described for using these columns as adsorbents for aromatic amino acids, their metabolites and other related compounds. Elution of these compounds from the column is carried out with either water or aqueous ethanol. Since salts are not retained by the XAD-4 resin, these column procedures provide an effective means of desalting solutions of the different compounds. In the present report, studies of approximately thirty compounds are described to provide a total of about sixty compounds studied in the two reports. Recoveries have been uniformly high (98%).

INTRODUCTION

In a previous study from this laboratory, XAD-4 resin columns have been effectively utilized for desalting purines, pyrimidines and nucleosides prior to the application of additional separative procedures[1]. In that study, it was shown that the most commonly used eluant buffers were not retained on the XAD-4 resin, and that the nucleosides could be recovered salt-free with excellent yields using either water or 19% (v/v) ethanol as the column eluent. This desalting technique has subsequently been applied in the purification and quantitative determination of a number of modified bases and nucleosides found in human urine[2–5]. In the present study, the usefulness of this desalting technique has been extended to other aromatic compounds, with special emphasis on urinary metabolites of tyrosine and tryptophan, and on other structurally related compounds. Using columns of two different lengths, it has been shown that the XAD-4 resin could be effectively used for desalting a wide variety of these compounds.

MATERIALS AND METHODS

Chemicals

XAD-4 resin (Amberlite non-ionic adsorbent), 20–50 mesh, was obtained from

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Mallinkrodt. The sources of chemicals were as follows: 3,4-dihydroxymandelic acid and ferulic acid, General Biochemicals; 3-methoxy-4-hydroxymandelic acid and homovanillic acid, Regis Chemicals; L-kynurenine, 3-hydroxy-DL-kynurenine, kynurenic acid, xanthurenic acid, *p*-aminohippuric acid, indole-3-acetic acid, 3-methoxy-4-hydroxyphenylglycol, xanthopterin, creatinine, 1-methylnicotinamide, anthranilic acid and nicotinamide, Sigma; L-histidine, L-leucine, L-isoleucine, L-methionine, L-phenylalanine, L-proline, L-tryptophan, L-tyrosine and L-valine, Mann Research Lab.

Preparation and use of XAD-4 columns

The XAD-4 resin was prewashed as described previously to remove ultra-violet absorbing materials[1]. The extensive washing of the resin reduced blank absorbance to 0.010 or lower at 220–330 nm when eluting with 19% (v/v) ethanol, and to 0.015 or lower at 260 nm when eluting with 30% (v/v) ethanol. Use of ethanol concentrations above 30% (v/v) for elution resulted in higher blank absorbance values. In a modification from the previously described procedure, the resin columns have been washed in some instances just prior to use with two or more bed volumes of 50% (v/v) ethanol containing 0.01 *M* ammonium hydroxide, then with water. This treatment assures that the resin will have no bound hydrogen ions. Although the XAD-4 resin is essentially non-ionic, there are trace amounts of hydrogen ion binding groups on the resin (probably aromatic hydroxyl groups). On the unused resin, there were 0.0015 meq. of H^+ binding groups per ml of resin bed. After prolonged use of the resin, this value had increased to 0.005 meq. The binding of H^+ by the resin may modify the pH on the column and affect the retention on the column of compounds whose retention is pH dependent. Also compounds that are cationic at pH 6 or above may be retained on the column by ion exchange rather than by adsorption. For compounds that are applied to the column in an acid solution (*e.g.*, sodium chloride–hydrochloric acid), the pretreatment with ethanol–ammonium hydroxide is not necessary.

Since there is considerable variability of retention of compounds on XAD-4 resins, columns of two different sizes have been utilized in the present study. A very short column (6.5 × 1.0 cm I.D.) has been used for compounds that are strongly retained and a longer column (30 × 1.3 cm I.D.) for those compounds less strongly retained. These two sizes of columns were adequate for the separation of nearly all of the compounds tested in the present study. As noted previously[1], some compounds may be eluted with water, but elution is much more rapid and the elution volume is smaller when 19% (v/v) ethanol is used as eluent. For tightly retained compounds, 30% (v/v) ethanol has been used as eluent with the short column to assure rapid elution of these compounds and their recovery in a small volume of eluent.

The procedures utilized for separations on the two different columns were as follows. The sample (usually 0.5–3.0 mmol) was applied in each case in a 10-ml volume in either (a) 0.20 *M* sodium chloride (neutral) or (b), 0.20 *M* sodium chloride–0.10 *M* hydrochloric acid (acid). In cases where the addition of the compound under study might appreciably alter the pH of the 0.20 *M* sodium chloride, the pH was adjusted back to 7 prior to addition to the column. The compound was washed onto the resin with 3 ml of water. With the long column (30 × 1.3 cm I.D.), elution was begun with water (ca. 40 ml) followed by 19% ethanol (v/v). The flow-rate was *ca.* 0.8 ml/min with collection of 7–9 ml fractions. With the short column

(6.5 × 1.0 cm I.D.), elution was begun with water (*ca.* 25 ml) followed by 30% (v/v) ethanol. The flow-rate was *ca.* 0.6 ml/min with collection of 4–6-ml fractions. Detection of components in the various fractions was carried out in most cases by measurement of ultraviolet absorbance, with the wavelength utilized depending upon the absorption maxima for the particular compound. For amino acids with no ultraviolet absorbance, the ninhydrin procedure[6] was used for assaying the fractions. Salt in the eluant fractions was measured by drying a small aliquot and weighing the residue.

RESULTS

The composite profiles in Fig. 1 illustrate the elution of three different compounds from the long XAD-4 resin column, while the composite profiles in Fig. 2 illustrate elution of compounds from the short XAD-4 resin column. The separations on XAD-4 columns of a wide variety of compounds are summarized in Tables I–III. Recovery of compounds from these columns was excellent (98.2 ± 6.3%; mean ± standard deviation for $n = 29$). Several factors are of importance in evaluating separations: (a) whether the salt is completely eluted from the resin prior to the beginning of the elution of the desired compound, and (b) whether the compound is eluted sharply and in a small enough volume to make the separation practical. In order to summarize the data for the tables, the elution position of each compound is indicated by V_e. For compounds eluted with water, V_e is the volume in ml from the midpoint of the application volume to the midpoint of the peak for that particular compound in the elution profile. For compounds eluted with aqueous ethanol, V_e is the volume in ml from the start of the ethanol to the midpoint of the peak. In order to provide an indication of the breadth of peaks in the elution profiles, the volume of eluent containing 92.5% of the recovered compound is shown. This value omits the leading and trailing edges of each peak and provides a reasonable means of comparing peak spreading for the different separations.

The compounds listed in Table I include tyrosine, various urinary metabolites of tyrosine, epinephrine or norepinephrine, and compounds that are structurally related to these metabolites. Tyrosine is zwitterionic, while most of the metabolites and

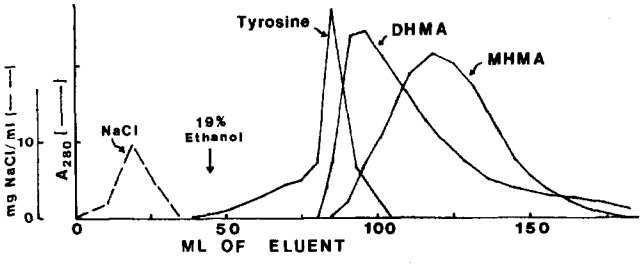

Fig. 1. Composite profiles from three separate columns (30 × 1.3 cm I.D.) illustrating the elution of compounds from the XAD-4 resin. The tyrosine was applied in 0.20 *M* sodium chloride (pH 6.5) and the DHMA and MHMA samples were applied in 0.20 *M* sodium chloride–0.10 *M* hydrochloric acid. Elution was with water initially, then with 19% (v/v) ethanol as indicated by the arrow. For additional details, see Materials and methods. Abbreviations: DHMA = 3,4-dihydroxymandelic acid; MHMA = 3-methoxy-4-hydroxymandelic acid.

TABLE I

ELUTION OF TYROSINE, TYROSINE METABOLITES AND STRUCTUALLY RELATED ORGANIC ACIDS FROM XAD-4 RESIN COLUMNS

Abbreviations: MHMA = 3-Methoxy-4-hydroxymandelic acid; DHMA = 3,4-dihydroxymandelic acid; MHPG = 3-methoxy-4-hydroxyphenylglycol.

Compound	*Application solution pH*	*Column size (cm)*	*V_e water (ml)*	*V_e 19% (v/v) ethanol (ml)*	*V_e 30% (v/v) ethanol (ml)*	*Volume for elution of 92.5% of peak (ml)*	*Separation* from salt*
L-Tyrosine	Acid	30 × 1.3 I.D.		41		29	+
L-Tyrosine	Neutral	30 × 1.3 I.D.		41		35	+
MHMA	Acid	30 × 1.3 I.D.		73		56	+
MHMA	Neutral	30 × 1.3 I.D.	43				−
MHMA	Acid	6.5 × 1.0 I.D.			7.8	27	+
Homovanillic acid	Neutral	30 × 1.3 I.D.	46				−
Homovanillic acid	Acid	30 × 1.3 I.D.			110	126	0
Homovanillic acid	Acid	6.5 × 1.0 I.D.			15	33	+
DHMA	Acid	30 × 1.3 I.D.		53		75	+
DHMA	Acid	6.5 × 1.0 I.D.	20				−
Ferulic acid	Neutral	30 × 1.3 I.D.		30		53	+
Ferulic acid	Acid	6.5 × 1.0 I.D.			16	57	+
MHPG	Neutral	30 × 1.3 I.D.		62		42	+
MHPG	Neutral	6.5 × 1.0 I.D.			9.5	11	+

* Key: +, good separation; −, not adequately separated; 0, elution volume too large for a useful separation.

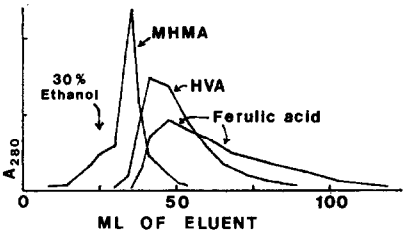

Fig. 2. Composite profiles from three separate columns (6.5 × 1.0 cm I.D.) illustrating elution of compounds from the XAD-4 resin. The compounds were applied in 0.20 *M* sodium chloride–0.10 *M* hydrochloric acid. Elution was with water initially, then with 30% (v/v) ethanol as indicated by the arrow. For additional details, see Materials and methods. Abbreviation: HVA = homovanillic acid. For other abbreviations, see the legend to Fig. 1.

structurally related compounds are organic acids. However, one metabolite, 3-methyl-4-hydroxyphenylglycol, is uncharged at pH 7 or below. Conditions are described for the successful separation of each of these compounds from salt. The compounds listed in Table II include tryptophan, a number of tryptophan metabolites, and several structurally related compounds. Many of these compounds have one or more amino groups and a carboxyl group, while others (*e.g.*, xanthurenic acid and kynurenic acid) have a carboxyl group and a ring nitrogen that may be protonated in acid solutions. Conditions are described for the successful separation of each of these compounds from salt, although the separation of indole-3-acetic acid was only marginal.

Since only three naturally occurring amino acids have aromatic groups in the side-chain, it was of interest to determine whether amino acids with aliphatic side chains were retained by the XAD resin. These studies are summarized in Table III. Only leucine, isoleucine and methionine showed sufficient retention to permit separation from salt. Histidine, proline and valine showed virtually no retention by the XAD resin. Studies on a few other compounds with heterocyclic rings are also shown in Table III.

DISCUSSION

In the previous study, several structural characteristics were noted that played a role in the binding of purines, pyrimidines and nucleosides to the XAD-4 resin[1]. In most cases, these same factors are involved in binding of the compounds described in the present communication. First, the presence of one or more charged groups on the compound lessens the binding to the resin. Second, the addition of a methyl group increases the binding. Consequently, the various organic acids studied in Table I are all retained much more strongly from an acid solution when they have no charge than they are from a neutral solution when they are negatively charged. For zwitterionic compounds (*e.g.*, tyrosine), retention was nearly the same in an acid solution, where the charge would be plus one, as it was in the neutral solution. The effect of additional methyl groups on binding may be seen in the comparison of several compounds shown in Table I. 3-Methoxy-4-hydroxymandelic acid is retained more strongly than 3,4-dihydroxymandelic acid. Ferulic acid, homovanillic acid and 3-

TABLE II

ELUTION OF TRYPTOPHAN AND TRYPTOPHAN METABOLITES FROM XAD-4 RESIN COLUMNS

Compound	*Application solution pH*	*Column size (cm)*	*V_e water (ml)*	*V_e 19% (v/v) ethanol (ml)*	*V_e 30% (v/v) ethanol (ml)*	*Volume for elution of 92.5% of peak (ml)*	*Separation* from salt*
L-Tryptophan	Neutral	30 × 1.3 I.D.		92		79	+
L-Tryptophan	Neutral	6.5 × 1.0 I.D.			9.5	10.7	+
L-Kynurenine	7.0	30 × 1.3 I.D.		46		32	+
3-Hydroxy-DL-kynurenine	Neutral	30 × 1.3 I.D.		49		25	+
5-Hydroxytryptophan	Neutral	30 × 1.3 I.D.		42		20	+
Anthranilic acid	Acid	6.5 × 1.0 I.D.			39	66	+
Xanthurenic acid	Neutral	30 × 1.3 I.D.		21		53	±
Xanthurenic acid	Acid	6.5 × 1.0 I.D.			12	26	+
Kynurenic acid	Neutral	30 × 1.3 I.D.		25		41	±
Kynurenic acid	Acid	6.5 × 1.0 I.D.			13	35	+
Indole-3-acetic acid	8.3	30 × 1.3 I.D.	44	31		47	±
p-Aminohippuric acid	Acid	30 × 1.3 I.D.		69		89	+

* Key: +, good separation; ±, barely separated.

TABLE III

ELUTION OF OTHER AMINO ACIDS AND MISCELLANEOUS COMPOUNDS FROM AN XAD-4 RESIN COLUMN (30 × 1.3 cm I.D.)

Compound	*Application solution pH*	*V_e water (ml)*	*V_e 19% (v/v) ethanol (ml)*	*Volume for elution of 92.5% of peak (ml)*	*Separation* from salt*
L-Phenylalanine	Neutral		45	31	+
L-Histidine	Neutral	28			−
L-Leucine	Neutral	74		48	±
L-Methionine	Neutral	65		45	±
L-Isoleucine	Neutral	70		49	±
L-Proline	Neutral	33			−
L-Valine	Neutral	38			−
Nicotinamide	Neutral		63	49	+
1-Methylnicotinamide	Acid	41		34	±
Xanthopterin	Neutral	39		39	±
Creatinine	Neutral		44	26	+

* Key: +, good separation; −, not adequately separated; ±, barely separated.

methoxy-4-hydroxymandelic acid differ only in the nature of the aliphatic side chain on the aromatic ring, with –CH=CH–COOH, $-CH_2-COOH$ and –CHOH–COOH side chains, respectively. They are retained on the XAD-4 resin in the order given above with ferulic acid being retained most strongly. Consequently, the differing portions of these side chains affect retention in the following order: –CH=CH– > $-CH_2-$ > –CHOH–.

For compounds with an amino group on the aromatic ring and a carboxyl group (*e.g.*, anthranilic acid and *p*-aminohippuric acid), retention on the XAD resin from an acid solution was sufficiently strong to permit a good separation from salt. However, retention of these compounds from a neutral solution was much weaker and the compounds were not separated from salt. Kynurenine and hydroxykynurenine, each with two amino groups and one carboxyl group, were readily separated from salt when applied to the longer column at a neutral pH. The two quinoline derivatives with a carboxyl side chain, kynurenic acid and xanthurenic acid, were poorly retained by the resin at a neutral pH, but could be separated readily from salt at an acid pH.

The separative studies with amino acids indicated that compounds with an indole ring (tryptophan) or a benzene ring (tyrosine and phenylalanine) were retained more strongly by the resin than amino acids with long aliphatic side chains (leucine or isoleucine). The retention of methionine is undoubtedly somewhat greater due to the methylthio group, a group previously shown to increase retention of nucleosides on XAD (*e.g.*, methylthioadenosine[3]). Neither the imidazole ring of histidine nor the heterocyclic ring of proline caused any retention on the resin of these amino acids. Also, amino acids with aliphatic side chains shorter than those of leucine or isoleucine were not retained appreciably.

Elution of compounds from columns usually requires an acid or buffer as eluent. When it is desired to re-chromatograph a particular compound, it is often

necessary to remove the eluent acid or buffer. In the previous study[1] it was shown that a number of different salts and buffers (*e.g.*, sodium chloride, sodium nitrite and Tris acetate and phosphate buffers) showed virtually no retention by the XAD-4 resin. Consequently, the column separations described in the present report should provide a means of providing salt-free solutions of aromatic amino acids and their metabolites prior to application of any additional chromatographic procedures.

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