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# EVALUATION OF AMBERLITE XAD-4 FOR RECOVERY OF GLUCURONIDES AND SULFATES FROM AQUEOUS SOLUTION

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## SUMMARY

Amberlite XAD-4 was shown to adsorb *p*-nitrophenyl glucuronide more strongly than XAD-2 does. A 10-g XAD-4 column quantitatively retained over 100 μg of *p*-nitrophenyl glucuronide at flow-rates up to 6 ml/min. Acetone–water (1:1) was the most suitable solvent system for elution of the glucuronides and sulfates tested on XAD-4.

## INTRODUCTION

Amberlite XAD-2 resin has been used to extract steroid conjugates from bile[1], urine[2–4] and milk[5], and conjugates of morphine[6] and hexachlorophene[7] from urine. Amberlite XAD-4, a chemically similar resin with a smaller pore size and larger surface area, has been used to recover non-steroidal conjugates from milk[8,9]. The XAD-4 resin was used in preference to XAD-2 because of its stronger adsorption of organic compounds from milk[10]. Similar observations were recently made by others[11,12] on the adsorption of various non-conjugated compounds from aqueous solutions.

Methanol is the most commonly utilized solvent for elution of conjugates from XAD-2 and XAD-4 resins[1–9], although ethanol and tetrahydrofuran were found to be equally satisfactory for certain steroid conjugates[4]. When XAD-2 resin is used for extraction and methanol for elution, some[1,4] but not all[5] steroid conjugates are recovered quantitatively. No data for recovery of conjugated compounds with XAD-4 resin have been published.

Since XAD-4 is reported to adsorb organic compounds more strongly than does XAD-2, it could prove to be more efficient for recovering conjugated compounds from aqueous samples. The purpose of this research was to study the ability of XAD-4 to adsorb conjugated compounds from aqueous solutions and to find a solvent which would satisfactorily elute the adsorbed conjugates from the resin. Non-steroidal conjugates were of particular interest because quantitative data for their recovery from any resin are lacking.

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## EXPERIMENTAL

### *Materials*

Amberlite XAD-2 and XAD-4 resins were obtained from Rohm & Haas*, Philadelphia, PA, U.S.A. Conjugated compounds were obtained from Sigma, St. Louis, MO, U.S.A.; precoated, 250-$\mu$m silica gel G plates were from Analtech, Newark, DE, U.S.A. Solvents were ACS grade or better and were used as received.

### *Methods*

The resin was rinsed in 10-g amounts with two successive 100-ml portions of methanol, acetone, acetone–water (1:1) and methanol. The resin and each portion of solvent were allowed to stand for at least 5 min, with occasional stirring, before the solvent was decanted. An additional portion of methanol was used to transfer the resin to a 50-ml buret containing a glass wool plug just above the stopcock. The bed volume of 10 g of wet XAD-4 was *ca.* 15 ml. Methanol was then rinsed from the resin with *ca.* 250 ml of distilled water and the column was ready for use.

Aliquots of a standard solution of each conjugate were used to obtain quantitative data. One aliquot, analysed directly, served as the control. Other aliquots were diluted with distilled water to simulate a sample. These samples and a 5-ml rinse of the beaker were passed over the resin, and the eluate was collected as the wash-on fraction. The resin was then rinsed with a portion of distilled water (eluate designated as rinse fraction) before being eluted with a solvent (solvent fraction). The solvent fraction was concentrated under nitrogen on a warm hot-plate or by a rotary evaporator with a 50°C water bath and water aspirator vacuum. Residual water in the solvent fraction and the water in the wash-on and rinse fractions were removed by evaporation on a hot-plate. No hydrolysis of standard conjugates occurred during concentration under neutral conditions.

Enough heat was generated when the eluting solvent first passed through the resin to cause the formation of a large number of air bubbles in the column. These bubbles were removed by back-flushing with *ca.* 10 ml of solvent with a rubber tubing and a 50-ml glass syringe. The resin was allowed to resettle in the column before the solvent flow was continued.

All quantitative measurements were made on a Cary Model 14 spectrophotometer. Absorbances of control and sample fractions were compared to determine percent recoveries.

Indoxyl sulfate (IS) and *p*-nitrocatechol sulfate (pNC-S) were quantitated as the intact conjugate. Androsterone glucuronide (AG) was determined by the procedure of Wardi *et al.*[13]. The *p*-nitrophenyl sulfate (pNP-S) and *p*-nitrophenyl glururonide (pNP-G) samples were hydrolysed, and the free *p*-nitrophenol was measured under basic conditions. About 20 ml of the pNP-S sample was heated with 0.5 ml of concentrated hydrochloric acid for 20 min on a hot-plate to effect hydrolysis. Sufficient sodium hydroxide pellets were then added to make the solution basic and develop the *p*-nitrophenol color. The pNP-G samples were hydrolyzed by adding one sodium hydroxide pellet to *ca.* 20 ml of aqueous sample and heating for at least

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20 min on the hot-plate. For fractions containing 20 ml or more of water, hydrolysis was conveniently performed during evaporation of water from the sample.

Androsterone sulfate (AS) and phenolphthalein glucuronide (PG) were determined qualitatively by thin-layer chromatography (TLC). The plates were developed in chloroform–methanol (2:1) or in chloroform–isopropanol–formic acid (5:3:1)[2]. The compounds were visualized by spraying the plates with 50% sulfuric acid and heating.

Experiments conducted included a comparison of XAD-2 and XAD-4 for adsorption of pNP-G from water, retention of pNP-G by XAD-4 at various flow-rates, and an estimation of XAD-4 capacity for pNP-G. The recovery of standard conjugates with various mixtures of acetone and water was measured. The mixture of acetone and water that gave good recovery of all compounds tested was then compared with methanol for elution of standard conjugates from XAD-4 resin.

## RESULTS AND DISCUSSION

The abilities of XAD-2 and XAD-4 resins to adsorb pNP-G from an aqueous solution are compared in Table I. Retention is nearly quantitative with XAD-4, but over 40% of the sample is lost from the XAD-2 effluent.

TABLE I

RETENTION OF *p*-NITROPHENYL GLUCURONIDE BY XAD-2 AND XAD-4 RESINS*

| *Fraction (ml)* | *Percent lost in effluent*** | |
|---|---|---|
| | *XAD-2* | *XAD-4* |
| Wash-on (25) | 31 ± 5 | 1.2 ± 0.1 |
| Rinse (25) | 13 ± 5 | 0.8 ± 0.3 |

* 200 μg through 10-g columns at ≦5 ml/min.
** Mean ± standard deviation.

Adsorption and subsequent elution of pNP-G on XAD-4 resin were similar for flow-rates up to 6 ml/min (see Table II). Accordingly, in all subsequent experiments, flow-rates were kept at or below 5 ml/min. Table II also shows that pNP-G is adsorbed strongly onto the XAD-4 resin. When 100 μg of conjugate is placed on the

TABLE II

EFFECT OF FLOW-RATE ON *p*-NITROPHENYL GLUCURONIDE RETENTION AND ELUTION USING 10 g OF XAD-4

Wash-on, 100 μg/100 ml; rinse: 1, 50 ml; 2, 100 ml; acetone–water (1:1), 100 ml each fraction.

| *Flow-rate (ml/min)* | *Percent found in fraction** | | | | | |
|---|---|---|---|---|---|---|
| | *Wash-on* | *Rinse* | | *Acetone–water* | | |
| | | *1* | *2* | *1* | *2* | *3* |
| 1.6–3.1 | 0 | 0 | 0 | 60 ± 0.5 | 22 ± 0.5 | 10 ± 0.5 |
| 5.0–6.0 | 0 | 0 | 0 | 63 ± 1 | 19 ± 0 | 8 ± 0 |

* Mean ± standard deviation.

resin in 100 ml of water, no pNP-G is eluted when the column is rinsed with an additional 150 ml of water.

The capacity of the 10-g XAD-4 column for pNP-G was studied by adding 200 and 500 $\mu$g amounts of conjugate in 100-$\mu$g portions and collecting the eluate from each addition. The first 100 $\mu$g was quantitatively retained (Table III). However, 3–4% of the second 100-$\mu$g portion was eluted during passage of the sample over the column, and an additional 5% of the total added was lost in the rinse fraction. As amounts in excess of 200 $\mu$g were added, losses steadily increased. However, even at the 500-$\mu$g level of addition, the point was not reached where all of the sample being added was lost in the eluate. It appears that 10 g of XAD-4 will quantitatively adsorb between 100 and 200 $\mu$g of pNP-G. For samples containing more than 100 $\mu$g, the use of proportionately larger columns is necessary.

TABLE III

CAPACITY OF 10-g XAD-4 COLUMNS FOR *p*-NITROPHENYL GLUCURONIDE

| *Fraction** | *Amount eluted ($\mu$g)*** | |
|---|---|---|
| | *200 $\mu$g conjugate* | *500 $\mu$g conjugate* |
| Wash-on 1 | 0 | 0 |
| 2 | 3.4 ± 0.2 | 3.0 ± 0.6 |
| 3 | — | 10.4 ± 0.4 |
| 4 | — | 18.0 ± 0.4 |
| 5 | — | 28.0 ± 0.4 |
| Rinse | 9.6 ± 0.4 | 30.7 ± 1.2 |
| Acetone–water 1 | 135 ± 1.0 | 294 ± 0.4 |
| 2 | 33.8 ± 0.4 | 63.2 ± 6.1 |
| 3 | 12.5 ± 1.6 | 26.5 ± 1.6 |
| Total recovered | 194.3 | 473.8 |

* Each wash-on fraction contained 100 $\mu$g/75 ml water; rinse, 80 ml; each acetone–water (1:1) fraction was 100 ml.

** Mean ± standard deviation.

Although methanol is commonly used to elute adsorbed conjugates from XAD-2 and XAD-4 resins[1–9], it afforded poor recovery of the standard conjugates evaluated in this study (Table IV). Several solvent systems were tested for their ability to elute adsorbed pNP-G from XAD-4 resin. These included various combinations of acetone, butanone, methanol, ethanol, propanol, benzene and water. Certain combinations gave better recovery than could be obtained with methanol alone, but the most suitable solvent system was acetone–water (A–W).

Different A–W mixtures were tested for their ability to elute pNP-G, pNP-S, and AG from XAD-4 resin (Fig. 1). A 50% A–W mixture was most suitable for the elution of all conjugates. The data also suggest that certain A–W mixtures ($\leq$20%) could be used to obtain selective elution of non-steroidal glucuronides from XAD-4 resin. Sulfates and steroid glucuronides could then be eluted with a 50% A–W mixture. However, this possibility was not investigated.

Recoveries of standard conjugates from XAD-4 resin with methanol and with A–W are compared in Table IV. Except for *p*-nitrocatechol sulfate, which adsorbed

TABLE IV

ELUTION OF STANDARD CONJUGATES FROM 10-g XAD-4 WITH METHANOL AND ACETONE–WATER

Elution volume, 200 ml; no compounds detected in wash-on and rinse fractions.

| *Compound* | *Percent recovered*[*] | |
|---|---|---|
| | *Methanol* | *Acetone–water (1:1)* |
| *p*-Nitrophenyl sulfate[**] | 2.2 ± 2.6 | 86.8 ± 1.3 |
| *p*-Nitrocatechol sulfate[***] | 0 | 0 |
| Indoxyl sulfate[***] | 0 | 98.5 ± 5.5 |
| *p*-Nitrophenyl glucuronide[**] | 29.2 ± 6.8 | 91.0 ± 3.7 |
| Androsterone glucuronide[§] | 9.5 ± 3.5 | 78.7 ± 12.9 |

[*] Mean ± standard deviation.
[**] Wash-on, 100 μg/80 ml; rinse 80 ml.
[***] Wash-on, 200 μg/50 ml; rinse, 25 ml.
[§] Wash-on, 200 μg/50 ml; rinse, 80 ml; methanol elution volume, 150 ml.

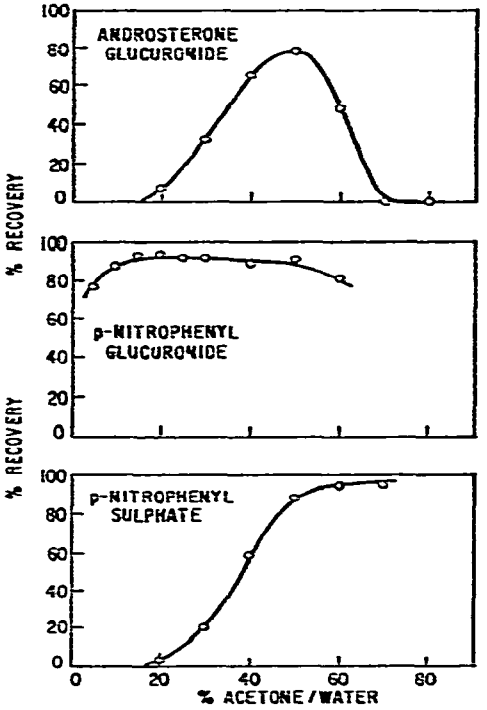


Fig. 1. Recovery of standard conjugates from 10-g XAD-4 with various acetone–water mixtures.

so strongly on the XAD-4 resin that neither solvent eluted it, good recovery of standard conjugates was obtained with A–W. With methanol, only small amounts of glucuronide and essentially no sulfate conjugate were recovered.

AS and PG were qualitatively evaluated by TLC after elution from XAD-4 columns with methanol and with A–W. The fractions were carefully evaporated to

dryness and equal aliquots were spotted. AS was recovered in barely detectable amounts with methanol, but an intense spot was obtained from the sample eluted with A–W. Likewise, some PG was recovered with methanol, but a much more intense spot was observed when A–W was used to elute the resin.

The increased adsorptivity of XAD-4 *versus* XAD-2 could be useful in recovery of steroid diconjugates. Quantitative recovery of these compounds with XAD-2 resin is possible only if rinse volumes are kept at an absolute minimum[1]. Use of XAD-4 resin could allow more complete rinsing of inorganic salts from the column without loss of the steroid disconjugates.

We conclude that Amberlite XAD-4 resin is a better adsorbent than XAD-2 for the conjugated compounds studies and that aqueous acetone is far superior to methanol as a means of desorbing them from the resin.

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