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Extraction of Conjugates from Urine by Nonionic Adsorption

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NOTE

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Summary

Procedures are described for extracting glucuronic acid and sulfuric acid conjugates from animal urine by using a nonionic type of adsorption chromatography. A chromatographic column (Porapak Q coated with trioctylamine) was developed to take advantage of nonspecific adsorption characteristics of these compounds on anion-exchange columns. The separation procedure provided sufficient cleanup of the conjugates to allow for their chromatography by other chromatographic methods.

INTRODUCTION

When organic compounds undergo metabolism in the animal, they are usually excreted in the urine. The parent compound may be capable of conjugation. Hydroxylation may occur by a number of metabolic reactions with the resulting metabolities then conjugated (*1*). The resulting metabolites are in many cases conjugates of glucuronic or sulfuric acid and are extremely water soluble. In some instances these conjugates can be extracted into diethyl ether or ethyl acetate. Generally, they will partition into butanol, as will a very large number of other components of urine. Since urine is a complex mixture, with the amounts of metabolites usually small in proportion to the other compounds present, purification of conjugates from urine becomes a formidable task. At present, no isolation procedures have been developed that offer a unified approach to this purification prob-

lem. Our goal has been to isolate conjugates of organic compounds excreted in animal urine with sufficient purity and in adequate quantity to obtain physical data (infrared, NMR, uv, and mass spectra) for identification. This offers additional advantages, since enough of these compounds are available for chemical reactions, hydrolysis, and enzyme studies. The purpose of this article is to report the use of nonionic adsorption on a liquid anion-exchange column for the extraction of conjugates from animal urine.

EXPERIMENTAL

A liquid anion-exchange column was prepared by coating 50 g of 200- to 325-mesh Porapak Q (Waters Assoc., Inc., Framingham, Mass.) with 10 ml trioctylamine (Eastman Organic Chemicals, Rochester, N.Y.). Porapak Q was poured into a chromatographic column (60×2.5 cm) and a 1-cm cotton plug placed on top of the support. Ten milliliters of trioctylamine was dissolved in 15 ml ethanol and this solution added to the top of the column. After this solution entered the Porapak Q, wetting about one-half the support, 100 ml water was pumped through the column. One hundred milliliters 1 *N* formic acid was then pumped on the column, followed by 150 ml water.

Urine was collected from rats and milk goats dosed orally with Mobam (4-benzothienyl *N*-methylcarbamate; 4,7-^{14}C) supplied by Mobil Chemical Co., Metuchen, N.J.; from milk goats dosed with ring-labeled Propazine (2-chloro-4,6-bis(isopropylamino)-*s*-triazine); and from sheep dosed with dieldrin-^{14}C (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4 endo-5,8-exo-dimethanonaphthalene), Nuclear-Chicago Corp., Des Plaines Ill. Fifty to 400 ml goat urine containing the Mobam or Propazine metabolites acidified to pH 5 with formic acid and pumped through the column at 1 ml per minute. Ether and ethyl acetate extracts were made of the sheep urine containing the dieldrin metabolites. These were vaporatd to dryness, dissolved in water, and placed on the column.

The Porapak-trioctylamine column was washed with 200 ml water, followed by 200 ml 1 *N* NH_4OH. The column was then washed free of NH_4OH with water. Elution of the trioctylamine-metabolite complex was accomplished by washing the column with 200 ml methanol. The methanol eluate was taken to dryness, dissolved in chloroform, and partitioned with 1 *N* NH_4OH.

RESULTS AND DISCUSSION

The Porapak-trioctylamine column was developed for isolation of 4-benzothienyl glucuronide from rat urine (*2*). Preliminary studies with anion exchangers, AG 3 × 4 and AG 1 × 8 (Bio-Rad Lab., Richmond, Calif.), indicated the metabolites could be removed by passing the urine through a column of these resins. However, elution of the metabolites with HCl or NaOH resulted in poor recoveries. This type of nonionic adsorption of steroid conjugates on anion-exchange resins has been reported by Bush (*3*).

An attempt was made to extract these conjugates by the use of liquid anion exchange. Trioctylamine dissolved in choloroform was used to partition untreated urine. Serious emulsion problems developed, but these studies indicated the ^{14}C to partition into the trioctylamine-chloroform layer. The use of liquid anion exchangers has been reviewed by Cerrai (*4*) and their use for extraction of sulfates (*5*) and sulfuric acid (*6*) reported.

A Porapak-trioctylamine column was developed to avoid the emulsion problem of the extraction procedure. Also, without experimental evidence, it was thought that nonionic adsorption would not occur as had been found with the anion-exchange resins. However, the metabolites of Mobam in rat urine, 4-benzothienyl sulfate, and 4-benzothienyl glucuronide could not be removed by NH_4OH. Similar results have been obtained with the water-soluble metabolites of dieldrin metabolism, which have been identified as glucuronide conjugates (*7*). Since these compounds could be retained on the column by nonspecific adsorption until elution with methanol, a significant cleanup step was obtained. Also, 4-benzothienyl sulfate and 4-benzothienyl glucuronide could not be partitioned into diethyl ether or ethyl acetate from acidified urine. Without the liquid anion column, their isolation would have been extremely difficult.

A metabolite in the urine of a goat treated with Mobam, which has been tentatively identified as the sulfuric acid ester of 4-hydroxybenzothiophene-1-sulfoxide, has been removed from the Porapak trioctylamine column with NH_4OH. This compound was adsorbed to the column from acidified urine but not retained under basic conditions. Preliminary studies of metabolites of Propazine in goat urine indicate that three fractions exist. When 50 ml goat urine was chromatographed on the adsorption column, 25% bypassed the column and 75% was

retained. Sixty-five percent of the adsorbed radioactivity was removed by 1 N NH_4OH and the remaining metabolites eluted with methanol. Metabolites from triazines (Propazine and Atrazine) have been shown to be amphoteric compounds. Failure to develop a suitable extraction procedure has been one of the problems in their isolation and identification (*8,9*).

The precleanup or "break away" procedures for isolation of conjugates is a very important step. For those conjugates that will not extract into organic solvents, purification becomes extremely difficult. Preliminary evidence suggests that nonionic adsorption, as described, may be useful for the isolation of a number of conjugates. Although the results of this technique for separation are less than ideal, enough cleanup is obtained to allow the use of other types of chromatography (ion-exchange, gel filtration, paper, and gas-liquid chromatography), as previously described for the purification of metabolites of Mobam (*2*). Therefore, this type of nonspecific adsorption chromatography may warrant consideration in certain isolations.

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