Systematic Approach to Preparation and Identification of Glucuronic Acid Conjugates

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A method was developed for identifying glucuronide conjugates in complex mixtures. Glucuronides labeled with C^{14} in either the aglycone or glucuronic acid moiety were synthesized using guinea pig liver microsomes, uridine diphosphoglucuronic acid, and an aglycone. The glucuronides were cleaned up on a diethylaminoethyl cellulose column and converted to the free acid form by passage through a cation exchange resin in the hydrogen form. The acids were then freeze-dried on Celite, methylated with diazomethane, and silylated with a trimethylsilylating reagent or acetylated with acetic anhydride methanesulfonic acid and methylated with diazomethane. Gas chromatography was performed on a SE-30 column using a gas chromatograph equipped with a C^{14} detector. The prepared derivatives chromatographed as sharp symmetrical peaks.

Investigations into the metabolism of carbaryl (Knaak *et al.*, 1965), bisphenol A (Knaak and Sullivan, 1966), and 2-ethylhexylsulfate (Knaak *et al.*, 1966), showed that new methods are needed for the separation and identification of glucuronides. Classical biochemical and chemical hydrolytic procedures frequently fail to give reliable results when complex mixtures of glucuronides, sulfates, and hydroxy carboxylic acids are present. Isolation techniques involving the formation and crystallization of suitable derivatives are equally difficult. The problem was initially solved by chromatographing the urinary products on DEAE-cellulose and comparing their retention volumes with known glucuronides. Difficulties were later encountered in completely resolving closely related glucuronides on DEAE-cellulose and in distinguishing them from glycine conjugates (Knaak, unpublished data) chromatographing in the same region. To solve this problem, a gas chromatographic procedure coupled with the above reference methods was developed for separating and identifying microgram quantities of various acyl or aryl glucuronides.

Methods

Chemicals. 1-Naphthyl-β-D-glucuronide and *p*-nitrophenyl-β-D-glucuronide were purchased from Pierce Chemical Co., Rockford, Ill. Bornyl-β-D-glucuronic acid was purchased from Bios Laboratories. Inc., New York, N.Y., and methyl-*p*-nitrophenyl-2,3,4-tri-*O*-acetyl-β-D-glucuronate was purchased from Sigma Chemical Co., St. Louis, Mo.

Uridine diphosphoglucuronide was purchased from Calbiochem, Los Angeles, Calif., and uniformly labeled uridine diphosphoglucuronic-C^{14} acid (0.001 μc. per mmole) was purchased from New England Nuclear Corp., Boston, Mass.

Nonlabeled 1-naphthol was supplied by Union Carbide Olefins Division, South Charleston, W. Va., while *p*-nitrophenol and borneol were purchased from Fisher Scientific, Pittsburgh, Pa.

1-Naphthol-1-C^{14} (2.32 mc. per mmole), phenol-C^{14} (U.L.) (11.2 mc. per mmole), and sodium glucuronate-6-C^{14} (1.13 mc. per mmole) were purchased from Nuclear-Chicago Corp., Chicago, Ill.

Biosynthesis. 1-Naphthyl-1-C^{14}-β-D-glucuronide, 1-naphthyl-β-D-glucuronide-C^{14} (U.L.), bornyl-β-D-glucuronide-C^{14} (U.L.), *p*-nitrophenyl-β-D-glucuronide-C^{14} (U.L.), and phenyl-C^{14}-β-D-glucuronide (U.L.) were prepared according to the method of Knaak *et al.* (1965) using guinea pig liver microsomes, uridine diphosphoglucuronic acid, and the appropriate aglycone. After incubation, the unconjugated aglycone was removed by extraction with diethyl ether (3 × 5 ml.), and the protein was removed from the reaction mixture by precipitation with ethanol. The ethanol-water solution was then concentrated to a volume of 2 to 4 ml. by rotary evaporation.

Ion Exchange Chromatography. The glucuronides in solution were chromatographed on a 1.5 × 24 cm. DEAE-cellulose column (Knaak *et al.*, 1965), and the fractions were analyzed for C^{14} by liquid scintillation counting. The fractions containing the glucuronide (salt form) were pooled, and the glucuronide was converted to the free acid by passage through a 2.5 × 13 cm. column of CM-cellulose (Whatman CM23, fibrous) in the hydrogen form. Distilled water was used to elute the acyl or aryl glucuronide. Conversion of the glucuronide to its acid was omitted when the glucuronide was to be acetylated prior to methylation. The acid and water (100 ml. of solution) were absorbed into 20 grams of Celite (acid washed 30- to 60-mesh Chromosorb W) and freeze-dried (Campbell and Pressman, 1944). The dried Celite was poured into a 2.5 × 24 cm. column, and the acid was eluted using 50 ml. of methanol. The acid was then concentrated to a volume of 0.5 ml. by vacuum distillation at 150 mm.

Methyl Ester-tri-trimethylsilyl Derivatives. 1-Naphthyl-C^{14}-β-D-glucuronic acid, phenyl-C^{14}-β-D-glucuronic acid, and bornyl-β-D-glucuronic-C^{14} acid dissolved in separate 0.5-ml. volumes of methanol were

Mellon Institute, Pittsburgh, Pa.

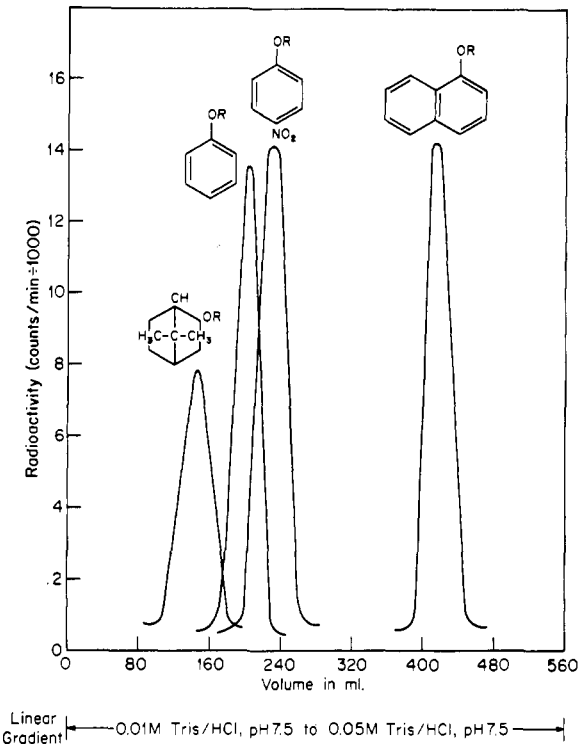

Figure 1. Composite chromatogram of bornyl-β-D-glucuronide-C^{14}, phenyl-C^{14}-β-D-glucuronide, *p*-nitrophenyl-β-D-glucuronide-C^{14}, 1-naphthyl-1-C^{14}-β-D-glucuronide, and 1-naphthyl-β-D-glucuronide-C^{14} on DEAE-cellulose

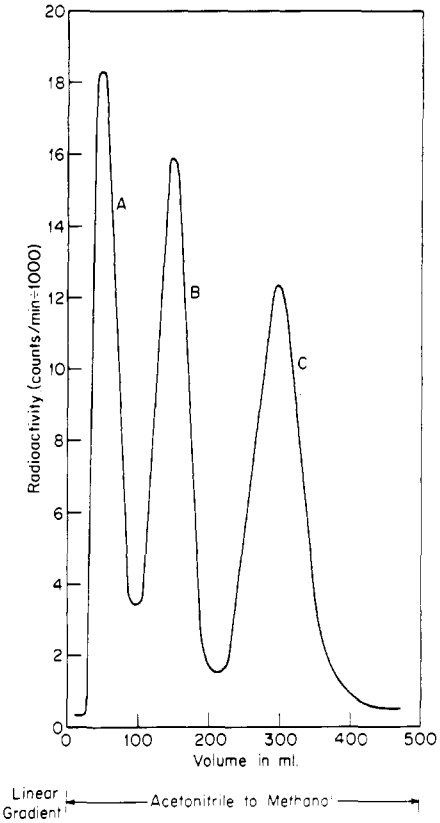

Figure 2. Chromatogram of the reaction products of 1-naphthyl-1-C^{14}-β-D-glucuronic acid, and diazomethane on a silica gel column

A. Mixed methyl ethers of methyl-1-naphthyl-1-C^{14}-β-D-glucuronate
B. Methyl-1-naphthyl-1-C^{14}-β-D-glucuronate
C. 1-Naphthyl-1-C^{14}-β-D-glucuronic acid or salt

individually treated with 5.0 ml. of a freshly prepared ethereal solution of diazomethane and allowed to react 15 minutes at room temperature. The diethyl ether and unreacted diazomethane were then distilled off at 150 mm., and the esters were individually chromatographed on a 1.5 × 24 cm. column of silica gel using an acetonitrile-methanol gradient. Acetonitrile, 250 ml., was used in the mixing chamber, and 250 ml. of methanol were used in the reservoir of the gradient device (Knaak *et al.*, 1965). The methyl ester of the acid appearing in peak *B* (Figure 2) was concentrated to 5.0 ml. by distillation at 150 mm. and then dried by rotary evaporation at room temperature. The ester was treated with 1.0 to 2.0 ml. of a commercial trimethylsilylating reagent (Applied Science Laboratories, State College, Pa.) containing three parts hexamethyldisilazane (HMDS), one part trimethylchlorosilane (TMCS), and nine parts dry pyridine. The silylating reagent was allowed to react for 1 hour. The sample was then ready for gas chromatography.

Methyl Ester-tri-*O*-acetyl Derivatives. Sodium glucuronate-6-C^{14}, *p*-nitrophenyl-β-D-glucuronide-C^{14}, phenyl-C^{14}-β-D-glucuronide, 1-naphthyl-C^{14}-β-D-glucuronide, 1-naphthyl-β-D-glucuronide-C^{14}, and bornyl-β-D-glucuronide-C^{14} were separately dissolved in 1.0 ml. of cold acetic anhydride and 50 μl. of methanesulfonic acid. The sample was allowed to stand at room temperature for 24 hours. The reaction mixture was then poured into ice water and extracted with chloroform. The chloroform was dried using sodium sulfate and the acetylated glucuronide concentrated by vacuum distillation. The glucuronic acid conjugate was then methylated with ethereal diazomethane. Diazomethane and ether were removed by vacuum distillation, and the fully acetylated-methylated product, dissolved in a small volume of chloroform, was ready for gas chromatography.

Gas Chromatography. The methylester-tri-trimethylsilyl and methylester-tri-*O*-acetyl derivatives of the various glucuronides were chromatographed on a Barber-Colman 5000 gas chromatograph equipped with a radioactive monitor and hydrogen flame detector using a 10-to-1 split in column effluent. The chromatograph was fitted with a glass column (8.0 feet × 5.0-mm. i.d.), packed with 2.0% or 5.0% SE-30 on Applied Science Gas Chrom Q, 80- to 100-mesh. Operating conditions were: injection port 320° C., oven temperature as indicated in Figures 3, 4, 5, and 6, and a flow rate of helium at 120 ml. per minute.

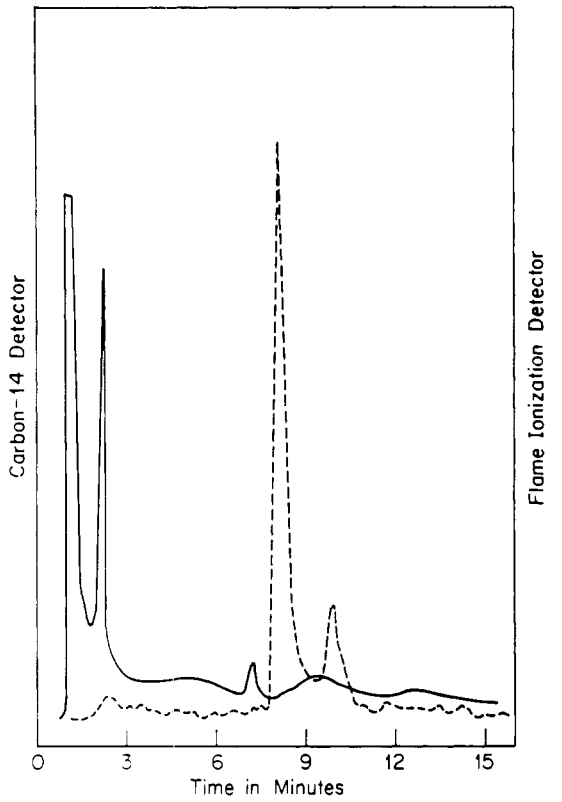

Figure 3. Gas chromatogram of methyl-1-naphthyl-1-C^{14}-2,3,4-tri-trimethylsilyl-β-D-glucuronate

Inst., Barber-Colman 5000, RAM
Column, 8 ft. × 5-mm. i.d.
Support, Gas Chrom Q, 80/100 mesh
Liquid phase, 5% SE-30
Flow rate, 120 ml./min.
Carrier gas, helium
Injection port temp., 320° C
Column temp., 300° C.
– – –C^{14}
——Mass
A double pen recorder was used. The mass peak appears 1.0 min. prior to the C^{14} peak

Results

Figure 1 shows a composite chromatogram of bornyl-β-D-glucuronide-C^{14}, phenyl-C^{14}-β-D-glucuronide, *p*-nitrophenyl-β-D-glucuronide-C^{14}, 1-naphthyl-C^{14}-β-D-glucuronide, and 1-naphthyl-β-D-glucuronide-C^{14} on DEAE-cellulose. Glucuronic-C^{14} acid has the same retention volume as the borneol conjugate, while the phenol and *p*-nitrophenol conjugates are not fully resolved as shown in Figure 1. The free aglycones, borneol, phenol, and 1-naphthol are eluted from DEAE-cellulose in one void volume (40 ml.), while *p*-nitrophenol chromatographs as a yellow band beyond the region of 1-naphthyl-β-D-glucuronide. *p*-Nitrophenol has an absorption maximum at 400 microns which disappears on conjugation with glucuronic acid.

Figure 2 gives the chromatogram obtained with the methylated products of 1-naphthyl-C^{14}-β-D-glucuronic acid on silica gel. The component(s) in peak *C* (~30%) chromatograph similarly to unreacted 1-naphthyl-C^{14}-β-D-glucuronic acid or its salt. The salt and free acid are eluted together from silica gel with approximately 1 to 1 methanol-acetonitrile. The free acid tends to tail, while the salt is eluted in a more symmetrical manner. The extent to which 1-naphthyl-C^{14}-β-D-glucuronide is converted to the free acid form by CM-cellulose (hydrogen form) is at present unknown.

The component appearing in peak *B* (~30%) was trimethylsilylated after the solvent was removed, and it gas chromatographed as indicated in Figure 3 for 1-naphthyl-C^{14}-β-D-glucuronide. A single radioactive peak was obtained followed by a small impurity peak. Peak *B* (Figure 2) was tentatively identified as the methyl-ester of 1-naphthyl-C^{14}-β-D-glucuronic acid. The component(s) in peak *A* (~30%) were believed to be the methylester-(mono-, di-, tri-)methyl ether derivatives of 1-naphthyl-C^{14}-β-D-glucuronic acid. The component(s) in peak *A* were concentrated by removal of ethereal diazomethane under reduced pressure (150

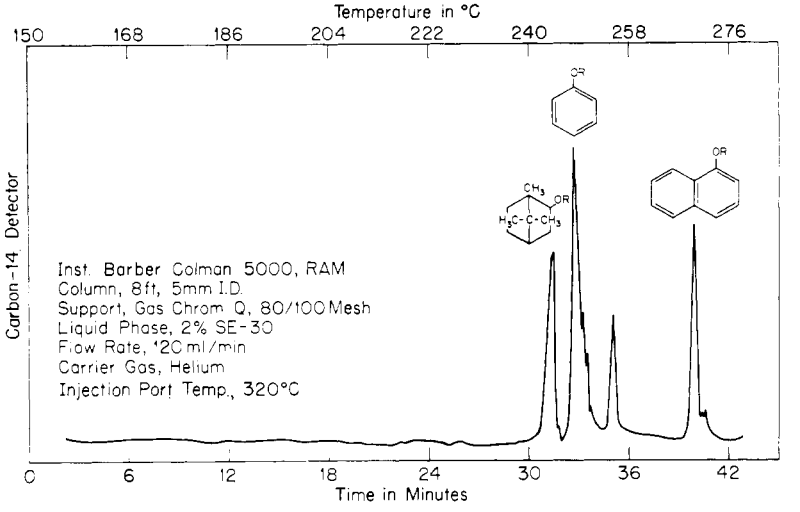

Figure 4. Composite gas chromatogram of methyl-bornyl-2,3,4-tri-trimethylsilyl-β-D-glucuronate-C^{14}, methyl-phenyl-C^{14}-2, 3, 4-tri-trimethylsilyl-β-D-glucuronate, and methyl-1-naphthyl-1-C^{14}-2,3,4-tri-trimethylsilyl-β-D-glucuronate

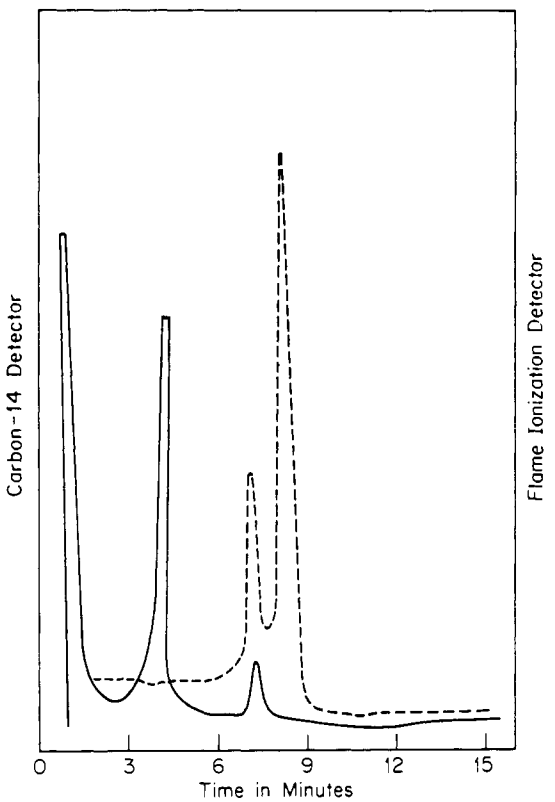

Figure 5. Gas chromatogram of methyl-1-naphthyl-1-C^{14}-2,3,4-tri-*O*-acetyl-β-D-glucuronate

Inst., Barber-Colman 5000, RAM
Column, 8 ft. × 5-mm. i.d.
Support, Gas Chrom Q, 80/100 mesh
Liquid phase, 5% SE-30
Flow rate, 120 ml./min.
Carrier gas, helium
Injection port temp., 320° C.
Column temp., 280° C.
- - -C^{14}
——Mass
The glucuronic acid–labeled derivative cochromatographed with the naphthyl-C^{14} derivative A double pen recorder was used. The mass peak appears 1.0 min. prior to the C^{14} peak

mm.) and gas chromatographed under the same conditions indicated in Figure 3. A series of products chromatographed, thus indicating that partial methylation of the hydroxyl groups of glucuronic acid had occurred. Shorter exposure times to diazomethane (1 to 5 minutes) resulted in the loss of peak *A* and an increase in peak *B*. Chromatograms similar to Figure 2 were obtained with bornyl-β-D-glucuronic-C^{14} acid and phenyl-C^{14}-β-D-glucuronic acid.

Naphthyl-C^{14}-β-D-glucuronic acid, extracted from water at pH 3.0 with diethyl ether, reacts rapidly with diazomethane to form products which chromatograph in the position of peak *A*, Figure 2. These products were separated into three components on a silica gel column using a stepwise gradient of hexane to ethylacetate and ethylacetate to acetonitrile. The chromatogram substantiated the previous finding that small quantities of the various glucuronides in the presence of excess diazomethane are partially or fully methylated.

Figure 4 is a composite gas chromatogram of a temperature-programmed run of the methylester-tri-trimethylsilyl ethers of bornyl-β-D-glucuronic-C^{14} acid, phenyl-C^{14}-β-D-glucuronic acid, and 1-naphthyl-C^{14}-β-D-glucuronic acid. The glucuronides chromatographed as nice sharp symmetrical peaks. Phenyl-β-D-glucuronide was followed by a small impurity peak, as was 1-naphthyl-β-D-glucuronide in Figure 3.

Figure 5 gives the gas chromatographic results of an isothermal run obtained with the methylester-tri-*O*-acetyl derivatives of 1-naphthyl-C^{14}-β-D-glucuronic acid and 1-naphthyl-β-D-glucuronic-C^{14} acid. 1-Naphthyl-β-D-glucuronide chromatographed as a sharp symmetrical peak with a small impurity peak appearing in front of the glucuronide peak.

Figure 6 is a composite gas chromatogram of a temperature-programmed run of the methylester-tetra-*O*-acetyl derivative of glucuronic-6-C^{14} acid and methylester-tri-*O*-acetyl derivatives of phenyl-C^{14}-β-D-glucuronide, bornyl-β-D-glucuronide, *p*-nitrophenyl-β-D-glucuronide, and 1-naphthyl-β-D-glucuronide. The nonlabeled commercial glucuronides of borneol, *p*-nitrophenol, and 1-naphthol were chromatographically identical to the C^{14}-labeled glucuronides produced by the liver microsomes. The commercial sample of methyl-*p*-nitrophenyl-2,3,4-tri-*O*-acetyl-β-D-glucuronate was chromatographically identical to the synthesized *p*-nitrophenyl derivative. The major difference between the chromatograms of the acetyl derivatives and the trimethylsilyl derivatives was the order of elution of phenyl and bornyl glucuronide. The acetyl derivative of phenyl glucuronide chromatographed prior to that of bornyl glucuronide while the reverse was true with the trimethylsilyl derivatives.

Discussion

The tri-trimethylsilyl or tri-*O*-acetyl methylesters of the glucuronides readily gas chromatograph on the columns tested. In addition to these derivatives, the trimethylsilyl ethers and trimethylsilyl esters of crystallized glucuronides are also easily prepared by dissolving them in pyridine and treating them with hexamethyldisilazane and trimethylchlorosilane at room temperature (Dalgliesh *et al.*, 1966). This one-step procedure is attractive in view of the difficulty of obtaining only the methyl esters with the use of excess diazomethane. Dalgliesh *et al.* (1966) have worked up a procedure in which they react 1.0 mg. of the glucuronide dissolved in methanol with precisely enough diazomethane to give them the methyl ester. This procedure works well when the desired glucuronide can be obtained in a pure state. In the preparation of the C^{14}-labeled glucuronides, contaminants are added to the glucuronide in quantities larger than the glucuronides themselves. These contaminants are derived from the liver preparation, cellulose ion exchangers, buffers, water, and the solvents used. Because of these contaminants, direct silylation or silylation after methylation of these preparations is often not quantitative, especially with glucuronides.

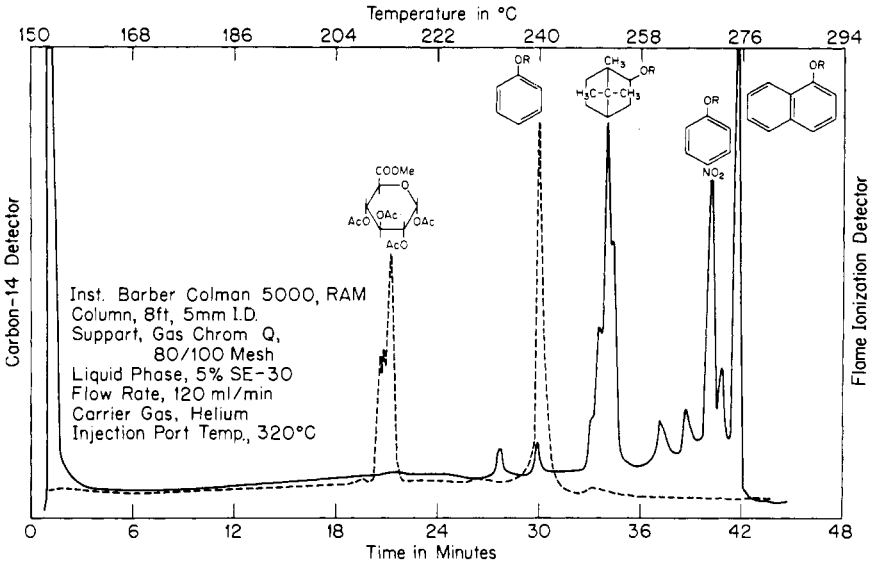

Figure 6. Composite gas chromatogram of methyl-1,2,3,4-tetra-*O*-acetyl-D-glucuronate-6-C^{14}, methyl-phenyl-C^{14}-2,3,4-tri-*O*-acetyl-β-D-glucuronate, methyl-bornyl-2,3,4-tri-*O*-acetyl-β-D-glucuronate, methyl-*p*-nitrophenyl-2,3,4-tri-*O*-acetyl-β-D-glucuronate, and methyl-1-naphthyl-2,3,4-tri-*O*-acetyl-β-D-glucuronate

– – –C^{14}
——Mass

Conclusions

In view of the isolation and contamination problems, the authors prefer the acetylation-methylation procedure as described in the text. The glucuronides as their salts or free acids were quantitatively acetylated using acetic anhydride and methanesulfonic acid. The acetylated glucuronides could then be extracted from water with chloroform in the acid form and be methylated quantitatively with diazomethane.

The gas chromatographic procedure can be used to separate and identify unknown materials suspected of being glucuronic acid conjugates when suitable standards are commercially available or can be made synthetically.

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Received for review November 18, 1966. Accepted May 11, 1967. Division of Agricultural and Food Chemistry, 152nd Meeting, ACS, New York, N.Y., September 1966.