Acta Chemica Scandinavica B 30 (1976) 685–690

Short Communications

Biosynthetic Production of ^{14}C-Labeled Reference Glucuronides

E. PUHAKAINEN,[a] M. LANG,[b] A. ILVONEN[b] and O. HÄNNINEN[b]

[a] Department of Clinical Chemistry, University Hospital of Kuopio, SF-70210 Kuopio 21, Finland and [b] Department of Physiology, University of Kuopio, SF-70100 Kuopio 10, Finland

The conjugation of various aglycones with α-D-glucopyranosiduronic acid (α-D-glucuronic acid), a residue of UDPglucuronic acid, is catalyzed by microsomal UDPglucuronosyltransferase (EC 2.4.1.17).[1] The synthesized *R*-β-D-glucopyranosiduronic acids (glucuronides) are important detoxication products in mammalian tissues.[2] The glucuronides are excreted in the bile or urine. In studies of the glucuronide biosynthesis and excretion, labeled conjugates would be of great help. Because these compounds are not available or they are very expensive, we have developed a simple method for the production of ^{14}C-labeled reference glucuronides and used 4-methylumbelliferone, 4-nitrophenol, and ethylmorphine as model substrates. These aglycones are often used in studies of UDPglucuronosyltransferase.[1]

Materials and methods. Radioactive UDPglucuronic acid (^{14}C in glucuronic acid residue specific activity > 200 mCi/mmol) was purchased from NEN Chemicals GmbH (Frankfurt am Main, Federal Republic of Germany). 4-Nitrophenol, K_2EDTA and digitonin were obtained from E. Merck AG (Darmstadt, Federal Republic of Germany) and D-glucaro-1,4-lactone was purchased from Calbiochem (Los Angeles, USA). Ethylmorphine chloride (Ph.Nord.) was obtained from Lääketukku OY (Turku, Finland) and 4-methylumbelliferone, 3-methylcholanthrene, trypsin (type III), trypsin inhibitor (type II-o), α-D-glucuronic acid 1-phosphate, D-glucuronic acid sodium salt (grade II) and UDPglucuronic acid trisodium salt from Sigma Chemical Co. (St. Louis, Mo., USA). Olive oil was purchased from Fisher Scientific Company (Fairlawan, N.J., USA). 4-Methylumbelliferyl-β-D-glucuronide trihydrate and 4-nitrophenyl-β-D-glucuronide were obtained from Koch-Light Laboratories Ltd (Bucks, England).

Male rats (*Rattus norvegicus*) of Wistar Af/Han/Mol/(Han 67) strain aged 2–2.5 months, outbred by rotational mating system in the Laboratory Animal Centre of Kuopio University, were used as experimental animals. 3-Methylcholanthrene dissolved in olive oil was administered to rats intraperitoneally (20 mg/kg) as a single daily dose for five days to induce UDPglucuronosyltransferase. Microsomes and partly purified UDPglucuronosyltransferase were prepared as described earlier.[3]

In the preparation of ^{14}C-labeled glucuronides, ^{14}C-labeled UDPglucuronic acid (1 μCi) dissolved in 60 % ethanol was evaporated to dryness in an ice bath under a nitrogen stream. To the tube, 50 μl of 0.3 M potassium phosphate buffer (pH 7.0) containing 0.5 mM of aglycone,

Table 1. Biosynthesis of ^{14}C-labeled glucuronides and some other metabolites in 0.3 M potassium phosphate buffer pH 7.0 containing 0.5 mM aglycone, 80 mM K_2EDTA, 10 mM D-glucaro-1,4-lactone. The ratio of labeled intermediates was analyzed from the incubation mixture and the yield and purity from a lyophilized eluent after the chromatographic purification.

Aglycone	Ratio of labeled glucuronide: UDPGlcUA: GlcUA-1-P:GlcUA [a]	Yield/%	Purity/%
4-Methyl-umbelliferone	96:1:2:1	93	99
4-Nitrophenol	93:3:3:1	90	99
Ethylmorphine	50:12:22:16	45	95

[a] Abbreviations: UDPGlcUA = UDPglucuronic acid, GlcUA-1-P = α-D-glucuronic acid 1-phosphate and GlcUA = D-glucuronic acid.

80 mM of K_2EDTA and 10 mM of D-glucaro-1,4-lactone was added. The reaction was started by adding 10 µl of partly purified UDPglucuronosyltransferase and immersing the tube into a 38 °C water bath. After a 2 h incubation period the reaction was stopped by adding 100 µl of absolute ethanol and denatured protein was spun down. The supernatant was applied as a band on Whatman No. 1 paper and the paper was developed for 20 h with ethyl acetate:acetic acid:water (6:3:4) as solvent using a descending chromatographic technique. Glucuronides and other metabolites of UDP-glucuronic acid were localized on the paper with aid of reference samples as described earlier.[4] The strips containing conjugates were eluted by 5 ml of distilled water and dried by lyophilisation.

From every step small aliquots were taken and analyzed by the same chromatographic method. The amounts of different intermediates of UDPglucuronic acid metabolism in samples were analyzed by a Wallac LSC 8100 liquid scintillation counter as earlier described.[4]

During development of the method, 0.3 M Tris-HCl and 0.3 M potassium citrate buffers as incubation medium, and native rat liver microsomes as enzyme source were also used. In addition 2-aminophenol was tested as aglycone.

Results and discussion. After an incubation of radioactive UDPglucuronic acid, aglycone and partly purified UDPglucuronosyltransferase in potassium phosphate buffer pH 7.0 containing K_2EDTA and D-glucaro-1,4-lactone, labeled 4-nitrophenyl-β-D-glucuronide and 4-methylumbelliferone-β-D-glucuronide contained more than 90 % of the radioactivity of the reaction mixture. If ethylmorphine was used as aglycone the yield was decreased to the half. By the method described we failed to produce 2-aminophenyl-β-D-glucuronide. No labeled spot was found, but the radioactivity was spread over a large area. The tailing may be due to the lability of *N*-glucuronides of aromatic amines.[5] If 4-nitrophenol or 4-methylumbelliferone were used as aglycone, the glucuronide yield after the chromatographic separation and elution contained more than 99 % of the radioactivity of the elution mixture. The purity of ethylmorphine glucuronide by the same procedure was found to be about 95 % (Table 1). When native microsomes were used as UDPglucuronosyltransferase source a lower glucuronide yield was obtained. This was probably due to the lower specific activity of UDPglucuronosyltransferase in native microsomes and partly due to the sedimentation of radioactive material with denatured microsomes.[4]

EDTA and D-glucaro-1,4-lactone were added to the reaction mixture to optimize the production of glucuronides. EDTA is a powerful inhibitor of UDPglucuronic acid pyrophosphatase [6] which shares the substrate UDPglucuronic acid with UDPglucuronosyltransferase. D-Glucaro-1,4-lactone is a specific inhibitor of β-glucuronidase.[7] This enzyme hydrolyses β-glucuronides to free aglycone and D-glucuronic acid. The use of these inhibitors in the reaction mixture containing native microsomes as enzyme source greatly improves the yield of glucuronide synthesis. If partly purified UDP-glucuronosyltransferase is used as an enzyme source, the effect of these inhibitors on the yield is small, because the preparation contains only traces of β-glucuronidase and UDP-glucuronic acid pyrophosphatase.

If the incubation was carried out in 0.3 M potassium citrate buffer pH 7.0 containing 10 mM of D-glucaro-1,4-lactone, the radioactive metabolites, 4-nitrophenyl-β-D-glucuronide, UDPglucuronic acid, α-D-glucuronic acid 1-phosphate and free D-glucuronic acid were found in the ratio of 60:1:12:17 in the reaction mixture. K_2EDTA could not be used in this mixture, because it caused a sediment to form, but citrate itself inhibits UDPglucuronic acid pyrophosphatase. When 0.3 M Tris-HCl buffer pH 7.0 containing 80 mM of K_2EDTA and 10 mM of D-glucaro-1,4-lactone was used, labeled metabolites could be found in the ratio of 92:4:2:2, respectively. In every buffer the final purity of 4-nitrophenyl-β-D-glucuronide after chromatographic purification was better than 99 %, but if citrate or Tris-HCl buffers were used the yield was poorer than in phosphate buffer.

The method described is reproducible, easy to perform and can also be applied for preparation of labeled glucuronides from unlabeled UDPglucuronic acid and a labeled aglycone.

Acknowledgements. This work was supported by a grant from Orion Research Council (Finland), which is gratefully acknowledged.

1. Dutton, G. J. In Dutton, G. J., Ed., *Glucuronic Acid Free and Combined*, Academic, New York and London 1966, p. 186.
2. Mandel, H. G. In La Du, B. N., Mandel, H. G. and Way, E. L., Eds., *Fundamentals of Drug Metabolism and Drug Disposition*. The Williams & Wilkins, Baltimore 1971, p. 155.
3. Hänninen, O., Lang, M. and Koivusaari, U. In *IUPHAR Satellite Symposium on Active Intermediates: Formation, Toxicity and Inactivation*, Turku 1975, p. 23.
4. Puhakainen, E. and Hänninen, O. *Eur. J. Biochem. 61* (1976) 165.
5. Parke, D. V. In Parke, D. V., Ed., *The Biochemistry of Foreign Compounds*, Pergamon, Oxford 1974, p. 76.
6. Miettinen, T. A. and Leskinen, E. *Biochem. Pharmacol. 12* (1963) 565.
7. Levvy, G. A. *Biochem. J. 52* (1952) 464.

Received March 5, 1976.