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Note

Direct determination of D-[U-^{14}C]glucaric acid in urine by ion-exchange high-performance liquid chromatography

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The measurement of D-glucaric acid, and other metabolites (Fig. 1) of the D-glucuronic acid pathway, in urine has been used [1, 2] to monitor the levels of activity of hepatic microsomal enzymes, which are responsible for the metabolism of both endogenous compounds and environmental xenobiotics in the liver [3]. Whilst earlier studies were conducted using colorimetric methods [4, 5], more recent work has concentrated on the gas—liquid chromatographic analysis of urinary metabolites of D-glucuronic acid, after derivatisation [5—8].

It has been suggested [9] that D-glucaric acid may undergo further metabolism in mammals, as it does in microorganisms [10]. Since D-glucaric acid is present as an endogenous constituent of normal urine, making small variations in excretion difficult to detect by traditional methods, any possible metabolism would best be investigated by the use of ^{14}C-radiolabelling techniques, for which a non-destructive separation of D-glucaric acid and metabolites from urine was required.

Although the literature contains many methods for the measurement of sugars and organic acids [11, 12] no suitable separations of sugar acids could be found. This paper describes the development of a high-performance liquid chromatographic (HPLC) technique for the direct determination of D-glucaric acid and other metabolites of D-glucuronic acid in urine by both radioactivity monitoring and UV absorption.

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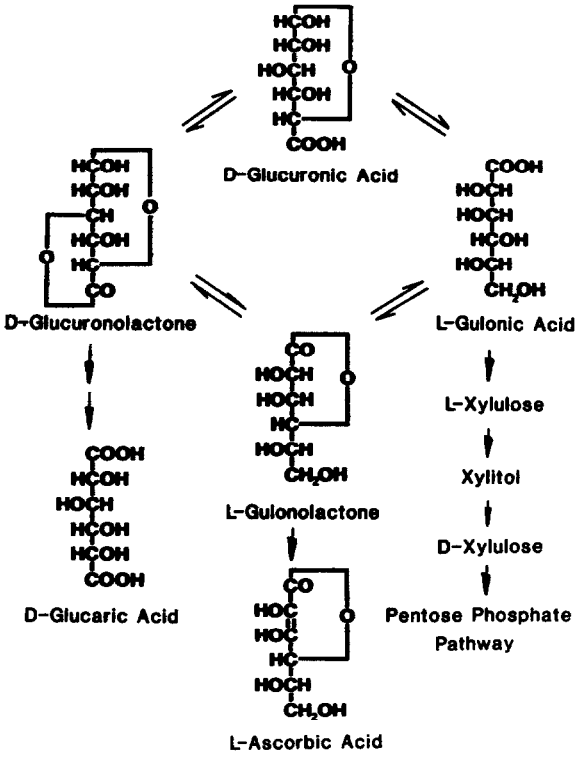


Fig. 1. The D-glucuronic acid pathway.

EXPERIMENTAL

Equipment

An Applied Chromatography Systems (Luton, Great Britain) LC750 high-performance liquid chromatograph was used for all separations. Columns, 250 × 4.6 mm I.D., were slurry-packed using a Haskel MCP-71 pump and packing reservoir (HPLC Technology, Macclesfield, Great Britain). Samples were applied via a Rheodyne 7120 valve and the effluent monitored either by UV absorbance at 200 nm using a CE2012 variable-wavelength monitor (Cecil Instruments, Cambridge, Great Britain) or by liquid scintillation counting [13] of collected fractions [14] in aqueous Tritosol [15] scintillant.

Materials

D-[U-^{14}C]Glucaric acid (1.3 μCi/mg) was prepared from D-[U-^{14}C]glucose (Amersham, Great Britain) by the method of Truchan [16] and purified by rapid recrystallisation from hot water [17].

D-Glucaric acid, D-glucaric acid-1,4-lactone, D-gulonic acid, D-glucuronic acid (Sigma, Poole, Great Britain), D-glucurono-3,6-lactone (Aldrich, Poole, Great Britain), L-γ-gulonolactone (Phase Separations, Queensferry, Great Britain) and L-ascorbic acid (Fisons, Loughborough, Great Britain) were all used as supplied. Ion-exchange stationary phases were Partisil-10 SAX (Whatman Labsales, Maidstone, Great Britain) and Calbiochem BA-X4 (C.P. Labo-

ratories, Bishops Stortford, Great Britain), packed in-house. All other chemicals were reagent grade or better. HPLC grade methanol was obtained from Rathburn Chemicals (Walkerburn, Great Britain) and water used was glass distilled.

Prepared mobile phases were sparged with helium and passed through 2-μm filters before use. Columns were flushed with water each night after use.

Sample treatment

Solutions of chromatographic standards were prepared and injected in the appropriate mobile phases. Urine samples were also directly injected without pre-treatment other than centrifugation to remove particulate matter.

Method development

Initial attempts at separation by reversed-phase chromatography with ion suppression, previously used for the determination of organic acids and esters in urine [18, 19] proved unsuccessful, with the sugar acids all virtually unretained in 1% aqueous sulphuric acid. Ion-pairing with tri-*n*-butylamine, successfully applied to the separation of L-ascorbic acid and its oxidation products [20] failed to resolve glucaric acid from UV-absorbing urinary material and gave consistently poor recoveries of radio-labelled material. Pre-column benzoylation, used for carbohydrate analysis [21] also failed to produce a useful separation in our hands.

Our attention was therefore directed towards anion-exchange chromatography. Partisil-10 SAX, a 10-μm silica based packing with a bonded quaternary amine functionality, was found to give a useful separation of D-glucaric acid from D-glucuronic acid, L-ascorbic acid and γ-gulonolactone (Fig. 2a), using an elution buffer of 0.05 *M* KH_2PO_4, adjusted to pH 6.0 with sodium hydroxide, at a flow-rate of 2.0 ml/min.

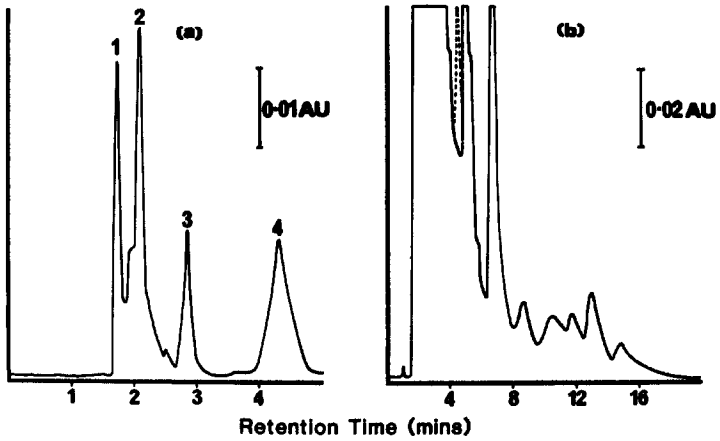


Fig. 2. Separations on Partisil-10 SAX. (a) 1 = γ-gulonolactone (25 μg) + D-glucuronic acid (25 μg), 2 = L-ascorbic acid (25 μg), 3 = unknown, 4 = D-glucaric acid (50 μg). (b) Rat urine: control and spiked with D-glucaric acid (1 mg/ml, shown dotted). See text for conditions.

Urine, spiked with D-glucaric acid showed only a partial resolution of the acid from other UV-absorbing material (Fig. 2b) although ^{14}C-monitoring showed only a single radiolabelled peak, corresponding to D-glucaric acid. Recovery of D-[U-^{14}C]glucaric acid from the column, however, was found to be directly dependent on the amount of unlabelled D-glucaric acid spike present in the sample. Even on saturation of a urine sample with D-glucaric acid, reproducible recoveries of better than 85% could not be achieved.

This problem was attributed to non-specific absorption onto the silica base of the packing, so a resin-based microparticulate anion exchanger was sought. Calbiochem anion-exchange resin BA-X4, a 7—10 μm polystyrene—divinylbenzene resin with 4% crosslinking and quaternary amine functional groups [22], appeared to suit our requirements. Preliminary trials with D-[U-^{14}C]glucaric acid gave 99.8% recoveries of label, independent of spiking. Although the actual column efficiency was relatively low, optimum performance was obtained at a flow-rate of 0.7 ml/min and column temperature of 60°C. The addition of a small amount of methanol to the mobile phase marginally improved peak shape and eliminated the tendency for the pump seals to leak at high buffer concentrations.

Optimum separation of D-glucaric acid from urine was achieved using an eluent of 0.67 *M* KH_2PO_4 in 6.7% methanol—water, adjusted to pH 5.50 with sodium hydroxide (Fig. 3a). Under these conditions, D-glucaric acid was resolved from its lactone with most of the other metabolites eluting much closer together, earlier on (Fig. 3b). L-Ascorbic acid gave a peak which was very sensitive to pH and methanol concentration, presumably through variations in its ionic state, and could not be accurately determined; increasing pH improved the L-ascorbic acid peak shape considerably, but correspondingly destroyed the resolution of D-glucaric acid and its lactone, as well as increasing the absorbance of the mobile phase and thus reducing sensitivity.

The early-eluting metabolites could readily be separated by reducing the buffer concentration to 0.067 *M* KH_2PO_4, keeping pH and methanol con-

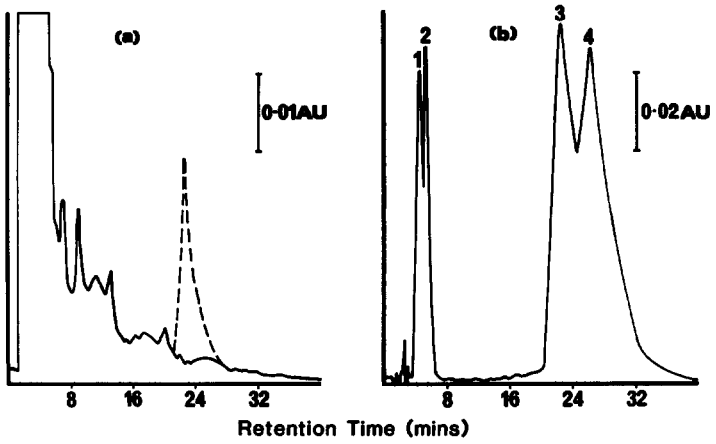
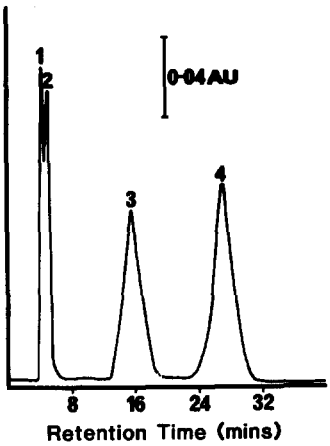


Fig. 3. Separations on BA-X4 resin. (a) Rat urine: control and spiked with D-glucaric acid (1 mg/ml, shown dotted). (b) 1 = D-glucuronolactone (0.10 mg), 2 = D-glucuronic acid (0.06 mg), 3 = D-glucaric acid (0.17 mg), 4 = D-glucaric acid lactone (0.29 mg). See text for conditions.



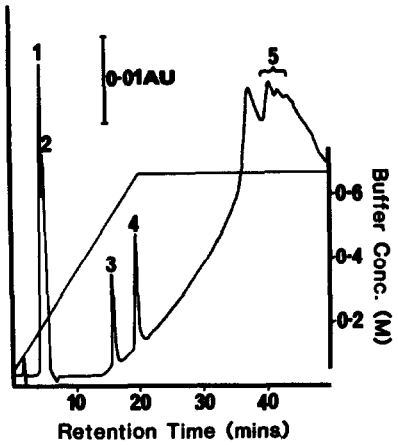


Fig. 4. Separation of early eluting peaks on BA-X4. Peaks: 1 = γ-gulonolactone; 2 = D-glucuronolactone; 3 = D-gluconic acid; 4 = D-glucuronic acid (0.5 mg each). See text for details.

Fig. 5. Gradient elution of D-glucuronic acid metabolites. Peaks: 1 = γ-gulonolactone; 2 = D-glucuronolactone; 3 = D-gluconic acid; 4 = D-glucuronic acid; 5 = L-ascorbic acid + D-glucaric acid + D-glucaric acid lactone (0.5 mg each).

centration the same (Fig. 4). Gradient elution between these two buffer compositions, with detection at 220 nm to overcome baseline intervention, albeit at the cost of sensitivity, gave excellent resolution of the early peaks but ran together L-ascorbic acid, D-glucaric acid and its lactone (Fig. 5). Isocratic elution at intermediate concentrations gave increased sensitivity over the gradient system but resulted in very badly tailed peaks and poor resolution.

RESULTS AND DISCUSSION

Detection by UV monitoring

Determination by UV absorbance at 200 nm is restricted by low sensitivity to the compounds of interest, the detection limit for D-glucaric acid being of the order of 20 μg in the isocratic BA-X4 system in Fig. 3, with a linear response up to around 1 mg. The sensitivity in gradient elution is still lower, due to the effects of baseline drift, though this might be eliminated by the addition of some unretained, UV absorbing compound to the initial mobile phase [23].

Determination by ^{14}C-monitoring

Gradient elution on the BA-X4 column was found to be a useful rapid screening process for the presence of radiolabelled D-glucuronic acid metabolites in urine. Isocratic elution, as in Fig. 3, has been used for the routine analysis of the urine of rats, mice, guinea pigs and marmosets treated with D-[U-^{14}C]glucaric acid. The mean recovery of label from all urine samples analysed was 100.3 ± 0.8% (S.E.M., n = 49) with detection down to 1000 dpm/ml urine, equivalent to 0.3 μg/ml glucaric acid. Greater sensitivity can be achieved by freeze-drying the urine to concentrate the radio-label or by increasing the specific activity of the administered material.

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