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Note

Measurement of the formation of paracetamol and *p*-nitrophenol glucuronides *in vitro*, by ion-pair high-performance liquid chromatography

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Uridine diphosphate glucuronyltransferase (UDPGT) (E.C. 2.4.1.17) catalyses the glucuronidation of endogenous and foreign substances[1], which, in most mammalian species, is the most important of the synthetic metabolic reactions. In the course of investigating whether or not certain drugs and their metabolites are conjugated by liver microsomal UDPGT preparations *in vitro*, it was necessary to ascertain the activity of the enzyme preparation under identical incubation conditions. For this purpose, assays by high-performance liquid chromatography (HPLC) were developed to measure the formation of glucuronides of two known substrates of UDPGT (*p*-nitrophenol and paracetamol) by injecting aliquots of incubation mixtures directly on to a reversed-phase HPLC column; elution was with a mobile phase containing an ion-pairing reagent in aqueous methanol.

EXPERIMENTAL

Chemicals

p-Nitrophenol (PNP), *p*-nitrophenyl-β-D-glucuronide (PNP-G), uridine diphosphoglucuronic acid (UDPGA), uridine monophosphate (UMP) and uridine were obtained from Sigma (Poole, Great Britain). Paracetamol and tetrabutylammonium hydroxide (TBAH) were obtained from BDH (Poole, Great Britain). Paracetamol-β-D-glucuronide (P-G) was a gift. Tetrabutylammonium phosphate (TBAP) was prepared by titrating TBAH with 10% phosphoric acid to pH 7.5.

HPLC apparatus and conditions

A Spectra-Physics liquid chromatograph (Model 3500B), with ultraviolet detection at 254 and 280 nm, in conjunction with a Leeds-Northrup dual-pen recorder was used. Samples were injected on to a column (10 × 0.50 cm) slurry-packed with ODS-Spherisorb (5 μm mean particle size). The mobile phase was 5 m*M* TBAP in either methanol–water (5:95, v/v) for P-G or methanol–water (12:88, v/v) for PNP-G; it was delivered at a flow-rate of 0.8 ml min^{-1}.

Incubation conditions

A guinea pig-liver microsomal preparation (1 ml) prepared with 154 m*M* KCl according to the method of Graham and Wood[2] and containing either 2 or 5 mg

of protein per ml suspended in 154 mM KCl, was added to a solution (0.4 ml) of UDPGA and either PNP or paracetamol in 0.1 M KH_2PO_4–KOH buffer solution of pH 7.1. The resulting solution was incubated at 37°. The final concentrations of substrate (PNP or paracetamol) and of UDPGA were 0.6 mM and 4.0 mM, respectively. Aliquots (100 μl) of the incubation mixture were removed at zero time and at appropriate intervals up to 2 h, then centrifuged in tapered 10-ml test-tubes to sediment microsomal protein; aliquots (3 μl) of the supernatant solutions were injected on to the HPLC column. Control incubations contained heat-denatured (5 min at 100°) protein.

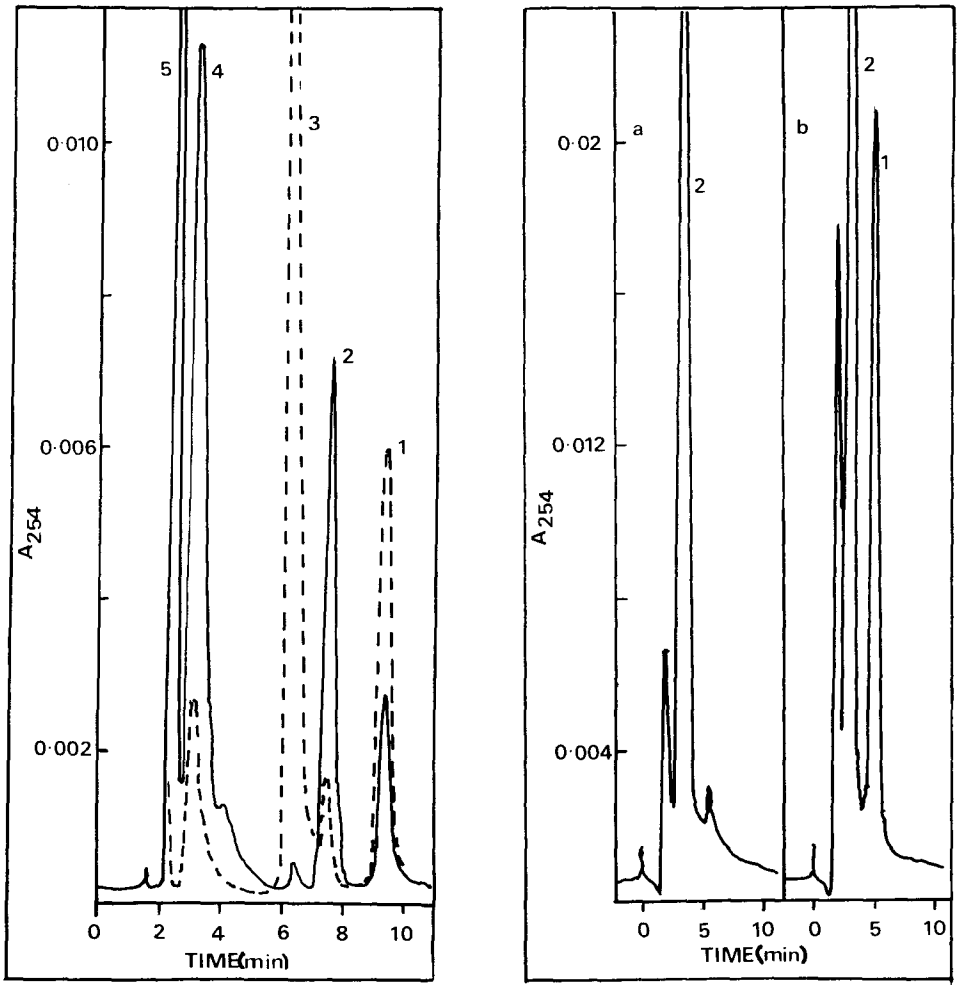

Fig. 1. Chromatograms recorded during the *in vitro* glucuronidation of paracetamol at 2 min (broken line) and 30 min (solid line) from the start of incubation. Peaks: 1 = paracetamol; 2 = P-G; 3 = UDPGA; 4 = UMP; 5 = uridine.

Fig. 2. Chromatograms recorded during the *in vitro* glucuronidation of PNP at 2 min (a) and 20 min (b) from the start of incubation. Peaks: 1 = PNP-G; 2 = UDPGA. Unconjugated PNP is retained on the column.

RESULTS AND DISCUSSION

A recent method[3] for the determination of PNP-G in urine by reversed-phase HPLC was unsuitable in our hands for *in vitro* glucuronidation studies, as PNP-G could not be separated from UDPGA.

In the work reported here, the methanol–water ratio of the mobile phase was adjusted to separate the components of the incubation media. With paracetamol as substrate, both the formation of the glucuronide (P-G) and the decrease in the paracetamol concentration can be monitored simultaneously (see Fig. 1). However, with PNP as substrate, PNP is retained on the column under the conditions described, whereas PNP-G has a retention time of 5 min (see Fig. 2).

The method described would also be suitable for studying another enzyme present in rat-liver microsomal preparations, UDPGA pyrophosphatase[4], which hydrolyses UDPGA and hence might interfere with glucuronidation.

In general, the need for an ion-pairing reagent in the mobile phases arises because most glucuronides are insufficiently lipophilic to be retained on a reversed-phase column. This method is applicable to a variety of substrates, as the retention times of their glucuronides can be adjusted by altering the proportion of methanol in the mobile phase. Therefore, studies on UDPGT may be performed without being limited to the few substrates for which published assays are available.

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