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Note

Simple and rapid ion-pair high-performance liquid chromatographic assay for *p*-nitrophenyl glucuronide in rat-liver microsomal incubations

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Glucuronidation catalysed by liver microsomal UDP-glucuronyltransferase (UDPGT, E.C. 2.4.1.17) is quantitatively the most important phase-2 reaction of drug metabolism. The enzyme activity has often been assayed by using *p*-nitrophenol (PNP) as a substrate which, in the presence of uridine-5′-diphosphoglucuronic acid (UDPGA), is converted into *p*-nitrophenyl-β-D-glucuronide (PNPG) by microsomal UDPGT preparations *in vitro*[1]. To determine PNPG in microsomal incubations, colorimetry[2] and recently high-performance liquid chromatography (HPLC)[3,4] have been applied. The colorimetric assay[2] involves repeated extraction processes, and is, therefore, laborious and quite time-consuming. These problems can be solved by HPLC methods; however, the reported HPLC methods[3,4] are unsuitable for *in vitro* glucuronidation studies, because a simultaneous separation of PNP, PNPG and UDPGA in microsomal incubations cannot be achieved.

In the present paper we describe a simple and rapid HPLC method for the quantitative analysis of PNPG in microsomal incubations. The method enables a chromatographic separation of PNP, PNPG and UDPGA in a single run by injecting aliquots of deproteinized microsomal incubations directly onto a reversed-phase HPLC column eluted with a mobile phase containing an ion-pairing reagent in aqueous methanol.

EXPERIMENTAL

Reagents and standards

UDPGA and PNPG were purchased from Sigma (St. Louis, MO, U.S.A.), PNP, tetrabutylammonium bromide (TBAB), Triton X-100 and 3-methylcholanthrene (MC) from Wako (Osaka, Japan), β-glucuronidase (from bovine liver) from P-L Biochemicals (Milwaukee, WI, U.S.A.) and phenobarbital (PB) sodium from Iwaki (Tokyo, Japan). All other reagents used were of analytical grade. A standard solution of PNPG for HPLC analyses was prepared in 5% trichloroacetic acid (TCA) (1 mg/ml) and diluted as necessary.

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Microsomal incubation

Male Wistar rats (230–270 g) were given daily i.p. doses of either PB (100 mg/kg, in 0.9% NaCl) for 4 days or MC (20 mg/kg, in corn oil) for 2 days. The rats were used 24 h after the last injection of the compounds or of the respective vehicles. Hepatic microsomes were prepared by the Ca^{2+}-aggregation method of Kamath and Rubin[5]. The microsomal incubations, which contained 5 m*M* PNP, 10 m*M* UDPGA, 0.05% Triton X-100 and 20 μl microsomal suspension (equivalent to *ca.* 0.1–0.2 mg protein[6]), were made up to a final volume of 200 μl with 0.25 *M* Tris–HCl buffer (pH 7.4). After being incubated for 15 min at 37°C, aliquots were analyzed for PNPG by HPLC and colorimetric methods, as described below.

HPLC assay of PNPG

The HPLC system consisted of a 6000A pump, a U6K injection valve, a reversed-phase column (100 × 8 mm I.D., Radial-Pak C_{18}), a 440 UV absorption detector operating at 280 nm and a 730 data module (all from Waters Assoc., Milford, MA, U.S.A.). The microsomal incubation (200 μl) was deproteinized with 10% TCA (200 μl) and filtered through a 0.45-μm Millipore filter (Millipore, Bedford, MA, U.S.A.). Aliquots (5 μl) of the filtrates were injected onto the column and eluted at a rate of 1.5 ml/min with 0.05 *M* sodium phosphate buffer (pH 7.1)–methanol (66:34, v/v) containing 5 m*M* TBAB as counter-ion. Chromatography was carried out at ambient temperature. The compounds of interest were monitored using the absorption detector connected to the data module, and peak areas were measured. Calibration curves were constructed by plotting peak areas against concentrations of authentic PNPG. A linear relationship existed in the range 25–250 μg/ml, with a correlation coefficient of >0.999.

Colorimetric assay of PNPG[2]

The microsomal incubation (200 μl) was extracted with ethyl acetate (2.5 volumes, three times) to remove free PNP. From the remaining aqueous phase 150 μl were removed and incubated with 50 μl β-glucuronidase solution (1250 Fishman units) for 30 min at 37°C to hydrolyze PNPG to PNP. PNP was then extracted from the incubation mixture with ethyl acetate (2.5 volumes, three times). The solvent extracts were pooled and evaporated to dryness, and the residue was redissolved in 4 ml 1 *M* NaOH for measurement of the yellow colour of PNP at 400 nm with a Model 101 spectrophotometer (Hitachi, Tokyo, Japan).

RESULTS AND DISCUSSION

Fig. 1 depicts a typical chromatogram of the rat-liver microsomal incubation showing a separation of UDPGA, PNPG and PNP with retention times of 2.1, 4.1 and 13.3 min, respectively in a single run. No interfering peaks due to endogenous substances in the incubation mixture were observed. Complete (baseline) resolution of PNPG from PNP and UDPGA is prerequisite for a good assay and was achieved by this method. The recovery and precision (reproducibility) were determined for 50, 250 and 500 μg/ml PNPG in microsomal incubations by six analyses at each concentration. The results (Table I) showed that for the concentration range tested the recovery was 97.4–100.7% and the precision, expressed as the coefficient of variation

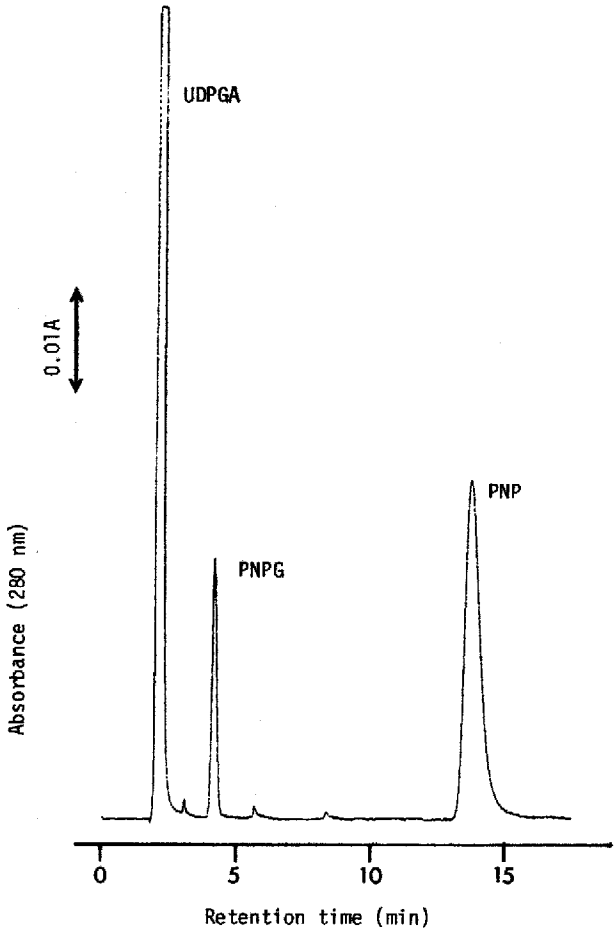

Fig. 1. Chromatogram of rat-liver microsomal incubation showing separation of PNPG from PNP and UDPGA. Column: Radial-Pak C_{18}. Mobile phase: 0.05 *M* sodium phosphate buffer–methanol (66:34) containing 5 m*M* TBAB.

TABLE I

RECOVERY AND PRECISION (C.V.) OF THE DETERMINATION OF PNPG IN RAT-LIVER MICROSOMAL INCUBATIONS

PNPG spiked (μg/ml)	*Recovery* (%)*	*C.V. (%)*
50	98.8 ± 1.3	1.3
250	100.7 ± 1.4	1.4
500	97.4 ± 1.9	1.9

* Mean ± S.D. of six analyses at each concentration.

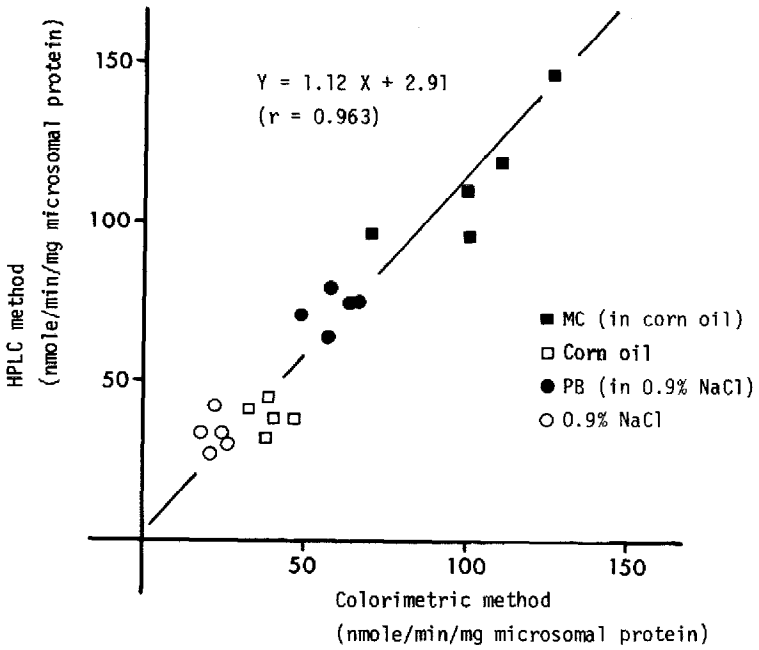

Fig. 2. Comparison of HPLC and colorimetric assays for hepatic UDPGT activities by quantitating PNPG in rat-liver microsomal incubations. Values for individual rats, pretreated with PB, MC or vehicles (see text), are the means of duplicate determinations.

(C.V.), was 1.9% or better. The limit of detection defined as four times the noise was found to be 4.0 μg/ml.

The time needed for analysis was 5 min for sample preparation and 18 min for chromatographic analysis. Once the method has been set up in the automated mode, three samples can be analyzed per hour.

The assay was applied to microsomal incubations obtained from rats pretreated with either PB or MC. HPLC analysis of PNPG in the incubation mixtures indicated that both PB and MC, as previously reported[1], increased UDPGT activity for PNP about two- and three-fold, respectively (Fig. 2). In addition, the results of HPLC analysis of samples from individual rats coincided with those of the colorimetric method, with a correlation coefficient of 0.963.

In conclusion, an ion-pair reversed-phase HPLC assay for PNPG in microsomal incubations has been developed which is simple, rapid and accurate, and is suitable for measuring hepatic UDPGT activity *in vitro*.

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