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Note

Measurement of the formation of a thioglucuronide, a metabolite of malotilate, in rat hepatic microsomes by high-performance liquid chromatography

MINORU NAKAOKA* and HIDEO HAKUSUI

Research Institute, Daiichi Pharmaceutical Co , Ltd , 16-13 Kitakasai 1-chome, Edogawa-ku, Tokyo 134 (Japan)

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Uridine diphosphate glucuronosyltransferase (UDPGT) (EC 2.4.1.17) catalyses the glucuronidation of endogenous and foreign substances [1]. The analysis of UDPGT activity has been extensively reported. Previous assays involve the measurement of the disappearance of substrate or production of glucuronide by spectrophotometric, fluorometric or radiometric methods [2–4]. Recently the glucuronides formed in in vitro systems have been directly analysed by high-performance liquid chromatography (HPLC) [5,6].

Malotilate is a hepatotrophic agent used for the treatment of patients with compensated liver cirrhosis [7]. A major biliary metabolite was isolated and its structure was determined to be a thioglucuronide (Fig. 1) [8]. Also a dithiol metabolite, an intermediate to the thioglucuronide, was detected in rat liver, after derivatization with a trapping reagent [9]. We have already confirmed

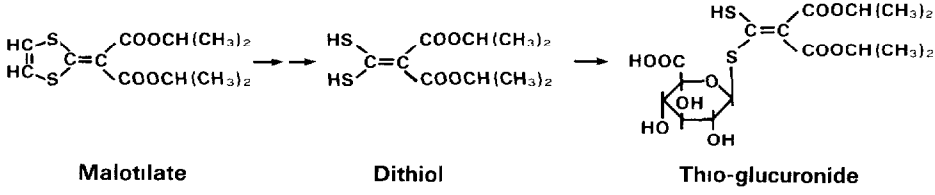

Fig. 1. Metabolism of malotilate to the thioglucuronide.

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that the dithiol is converted into the corresponding thioglucuronide in vitro and in vivo [9]. To obtain further evidence for this metabolic pathway, kinetics of the glucuronidation reaction were investigated in vitro.

This paper describes the development of an assay method for the evaluation of glucuronide conjugation of the dithiol in rat hepatic microsomes. The enzyme activity was assayed by direct determination of the thioglucuronide formed after separation by high-performance liquid chromatography (HPLC). This method has been successfully used for the measurement of apparent kinetic constants of UDPGT activity towards the dithiol.

EXPERIMENTAL

Chemicals

2,2-Di(isopropoxycarbonyl)ethylene-1,1-dithiol was synthesized as the disodium salt by the method of Tanaka and Kurono [10] and kept at −20°C before use as the substrate.

6-(3-Thienyl)[1,3,4]thiadiazolo[3,2-*a*]-1,2,3-triazolo[4,5-*d*]pyrimidin-9-(1*H*)-one was synthesized in our laboratory as the internal standard [11]. The chromatographically pure thioglucuronide obtained from isolation of the biliary metabolite was used as the standard sample [8]. UDP-glucuronic acid (UDPGA) was obtained from Sigma (St. Louis, MO, U.S.A.). Acetonitrile, from Kanto (Tokyo, Japan), was of HPLC grade. Other chemicals and solvents were of reagent grade.

HPLC conditions

A Hitachi Model 635S HPLC system was used with a 150 mm × 4.6 mm I.D. stainless-steel column packed with 5 μm Ultrasphere ODS packing material (Tosoh, Tokyo, Japan). A Shimadzu SPD-6A spectrophotometric detector (Kyoto, Japan) was set at 336 nm, which was the optimal wavelength for detection of the thioglucuronide. The mobile phase was acetonitrile–0.1 *M* phosphate buffer, pH 7.0 (9:41, v/v). The flow-rate was 1.0 ml/min.

Incubation conditions

Male Sprague–Dawley rats aged seven weeks were obtained from Shizuoka Agricultural (Shizuoka, Japan). The microsomes were independently prepared from the livers of three rats by a modification of the method of Lucier et al. [12], and their protein content was measured by the technique of Lowry et al. [13]. For the assay of UDP-glucuronosyltransferases the following mixture was used: 100 m*M* phosphate buffer (pH 6.0), 3 m*M* UDPGA, ca. 0.5 mg of microsomal protein, 0.06% Triton X-100 and 5 m*M* magnesium chloride, in a total volume of 1.0 ml. After preincubation at 25°C for 2 min, the reaction was started by addition of the dithiol dissolved in 0.1 ml of water (12.5–100 μ*M* final concentration). After incubation in a shaking water-bath at 25°C for up

to 10 min, the reaction was stopped by addition of 0.2 ml of ice-cold methanol containing the internal standard (6.6 μg). Then the mixed solution was frozen in dry ice–ethanol for 5 min to precipitate the protein. The protein was pelleted by centrifugation at 1200 *g* for 10 min. The resulting supernatant was diluted with four volumes of water.

Enzyme activity

An aliquot (50 μl) of the diluted supernatant was injected into the HPLC system. Blanks were prepared using boiled microsomes. Calibration curves were obtained by plotting the peak-height ratio (thioglucuronide/internal standard) against the concentration of the thioglucuronide. Incubations were conducted in duplicate at 25 °C. The initial rate of the glucuronidation was calculated on the basis of the amounts of the thioglucuronide formed.

RESULTS

Quantification of the thioglucuronide

Following incubation of the dithiol as the substrate in rat hepatic microsomes, the enzyme reaction was stopped by addition of methanol containing the internal standard, and the resulting supernatant was subjected to reversed-phase HPLC. As shown in Fig. 2, the thioglucuronide that was formed and the dithiol were completely separated, and the retention times were 4.1 and 34.3 min, respectively. Blank tubes with boiled liver microsomes showed no detectable change. Under the same conditions, the internal standard was eluted after 7.1 min. For quantitation of the thioglucuronide, the peak-height ratio of the

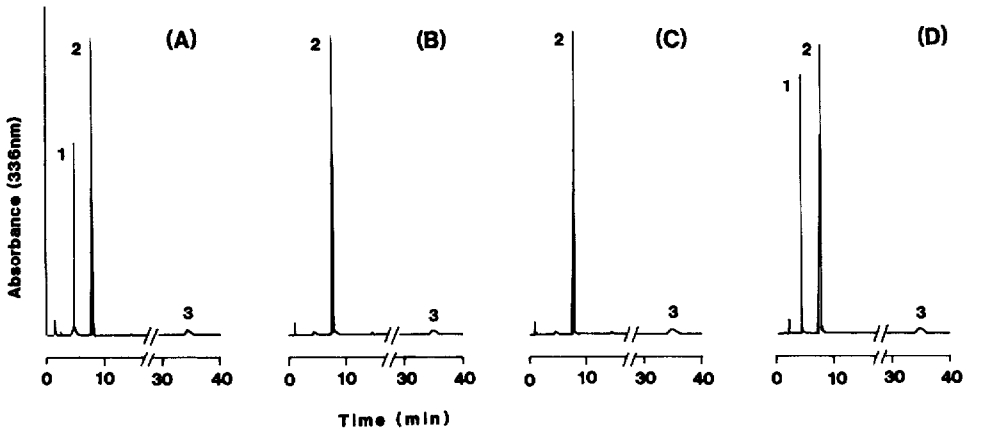

Fig. 2. Typical HPLC profiles of the thioglucuronide after incubation of rat hepatic microsomal enzymes with the dithiol and UDP-glucuronic acid at 25 °C for 5 min. (A) Complete system; (B) heat-inactivated microsomes; (C) complete system without the dithiol; (D) standard mixture. Peaks: 1 = thioglucuronide; 2 = internal standard; 3 = dithiol.

thioglucuronide to the internal standard was calculated. The calibration curve was linear up to 280 μM with a correlation coefficient of 0.998. The detection limit was 1.4 μM. For assessment of the coefficient of variation (C.V.) of the enzyme activity, six samples with the dithiol substrate (100 μM final concentration) were incubated for 5 min at 25°C and injected into the HPLC system. The C.V. was found to be 6.5%.

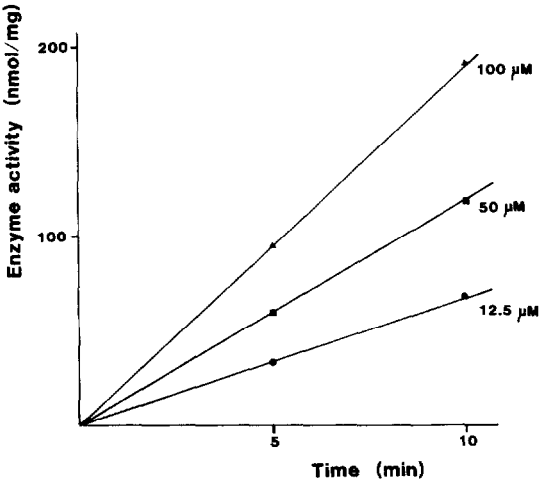

Fig. 3 Effect of incubation time on rat hepatic UDP-glucuronosyltransferase activity towards the dithiol Each line is labelled with the initial concentration of the dithiol

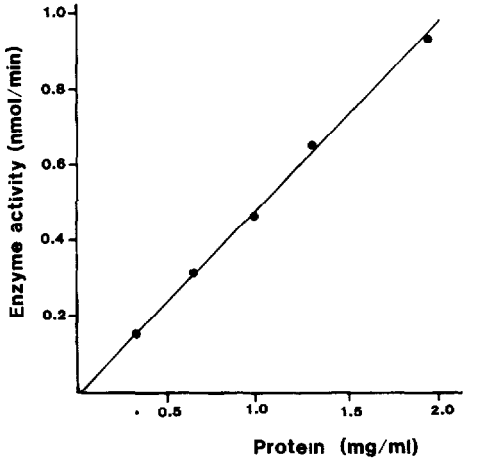

Fig. 4 Effect of the amount of microsomal protein on rat hepatic UDP-glucuronosyltransferase activity towards the dithiol

Enzyme activity

Fig. 3 shows that formation of the thioglucuronide from various concentrations of the dithiol was linear for up to 10 min. As shown in Fig. 4, the enzyme activity increased proportionally with the amount of microsomal protein present in the reaction mixture up to a protein concentration of 1.94 mg/ml. The optimum pH was found to be 6.0 (data not shown). Under the optimal conditions described in Experimental, the initial rates of glucuronidation versus the dithiol concentrations (12.5–100 μM) were measured, and kinetic parameters were determined using a Hanes plot ($[S]/v - [S]$ plot). Apparent Michaelis constants (K_M) for glucuronidation of the dithiol were 36.3 ± 3.3 μM and maximum initial rates (V_{max}) were 7.14 ± 0.61 nmol/min per mg protein (mean $\pm$ S.D., $n=3$). The V_{max}/K_M ratios, which dictate the rate of glucuronidation at low substrate concentrations, were estimated at 199 ± 36 μl/min/mg.

DISCUSSION

Glucuronidation of a great number of endogenous and foreign substances possessing hydroxyl or carboxyl groups is catalysed by families of UDPGT [1]. Recent studies indicate the existence of at least eleven isoenzymes in rats [14]. Occasionally, thioglucuronides have been reported as urinary and biliary metabolites [1]. No detailed study of the enzymes responsible for the biosynthesis of thioglucuronides has been reported [15].

This HPLC method is sensitive and specific, hence permits the measurement of the formation of the thioglucuronide in vitro. The K_M values for glucuronide conjugation of the present dithiol were compared with those of the hydroxyl compounds reported previously [16,17]. The K_M values (36.3 ± 3.3 μM) for the dithiol were smaller than those of 1-naphthol ($K_M = 1250$ μM) and morphine ($K_M = 212 \pm 26$ μM) and in accordance with those of buprenorphine ($K_M = 36 \pm 10$ μM) and naloxone ($K_M = 76 \pm 2$ μM). These results suggested that the dithiol showed relatively high affinity for the catalytic site of the UDPGT.

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