An Improved Assay Method for UDP-Glucuronyltransferase Activity towards 5-Hydroxytryptamine and the Characteristic Properties of the Enzyme

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ABSTRACT. A simple and reproducible assay method for UDP-glucuronyltransferase (GT) towards 5-hydroxytryptamine (5-HT) was developed. It consists of the removal of unconjugated 5-HT by 0.6 N NH_4OH -saturated *n*-amyl alcohol and the colorimetric estimation of 5-HT glucuronide. Using this assay method, some properties of the enzyme activity in rat liver microsomes were studied. Simple Michaelis-Menten kinetics was followed with respect to 5-HT and the apparent *K_m* value for 5-HT was 0.1 mM. However, the deviation from this kinetics was observed with respect to UDP-glucuronic acid (UDPGA). The apparent *K_m* values for UDPGA were 0.6 mM and 5 mM. The enzyme activity was stimulated by divalent cations. For Mg^{2+} , the enzyme did not obey this kinetics, and the apparent *K_m* values for Mg^{2+} were 1 mM and 10 mM. In the presence of Mg^{2+} , the apparent *K_m* value for 5-HT did not change but the *V_{max}* value increased. On the other hand, the addition of a low concentration of Mg^{2+} decreased the apparent *K_m* value for UDPGA and increased the *V_{max}* value. The addition of a high concentration of Mg^{2+} did not change the apparent *K_m* value for UDPGA but increased the *V_{max}* value. These results indicate that the enzyme activity is stimulated by the formation of Mg^{2+} -UDPGA complex which showed higher affinity for the enzyme than UDPGA at the low concentration of Mg^{2+} and further stimulated by the formation of Mg^{2+} -enzyme complex at the higher concentration of Mg^{2+} .—KEY WORDS: assay method, enzyme kinetics, magnesium ion, UDP-glucuronyltransferase, 5-hydroxytryptamine.

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Dutton and Storey [6] discovered an enzyme called UDP-glucuronyltransferase (GT) [acceptor unspecific, EC 2.4.1.17] in liver microsomes. This enzyme localizes mainly in hepatic endoplasmic reticulum, and is involved in metabolic conversion of endogenous (bilirubin, steroids) and exogenous (drugs, carcinogens) compounds, which are active and/or water-insoluble forms, into inactive and excretable forms.

5-Hydroxytryptamine (5-HT), one of the biogenic amines, is principally concerning with the peristalsis of intestines [13] and the neural transmission in limbic system of central nervous system [12]. It is mainly metabolized to 5-hydroxyindolacetic acid by monoamine oxidase, and excreted in urine [14]. However, Weissbach *et al.* [15] first reported that a conjugation process is involved in the metabolic breakdown of 5-HT as an alternate route. McIsaac and Page [9] also identified 5-HT glucuronide in a rabbit and rat urine. Recently, Aleksidze *et al.* [3] detected 5-HT glucuronide in a rabbit brain and glial cells.

Although metabolic evidences for 5-HT glucuronidation have accumulated, little is known about

the biochemical properties of the enzyme involved. At present, radioactive [2] and non-radioactive [8] methods are available for the assay of GT activity towards 5-HT. The radioactive method [2] requires the radioisotopic facility and is composed of many steps which are the acetone precipitation, paper chromatography and radioactive scanning of the reaction products. In non-radioactive method [8], 5-HT glucuronide is not selectively precipitated by acetone. It was hoped that a better development of the assay method for the enzyme activity towards 5-HT would help to purify 5-HT GT and to characterize its molecular and catalytic properties [1].

Therefore, the present study was designed to develop a simple and reproducible assay method for the GT activity towards 5-HT. Then, the kinetic properties of this enzyme activity in rat liver microsomes were described.

MATERIALS AND METHODS

Materials: 5-HT (creatinine sulphate complex) was purchased from Merck, Darmstadt, and UDPGA (sodium salt) was from Nakarai Chemicals, Ltd., Kyoto.

Animals and preparation of microsomes: Male,

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Tokyo strain rats were fasted for 24 hr and then killed. The livers were quickly taken out and perfused with cold, isotonic KCl solution. Liver microsomes were prepared and washed as described by Yuasa [17].

Assay of GT: GT activity towards 5-HT was determined by the following method. An enzyme preparation was incubated with 0.1 M Tris-HCl, pH 7.8, 2 mM 5-HT and 10 mM UDPGA in a total volume of 200 μ l at 37°C. The reaction was stopped by adding 5 ml of an extract solvent consisting of *n*-amyl alcohol and 0.6 N NH_4OH (10:1, V/V, organic layer was used). After shaking and centrifugation, the upper layer was removed. The extraction was repeated once. 5-HT glucuronide in the aqueous phase was treated with 0.9 ml of *p*-dimethylaminobenzaldehyde [11.1% (W/V), in 8.3 M HCl]. After centrifugation, the absorbance of the resulting supernatant was measured at 550 nm, with 11.1% *p*-dimethylaminobenzaldehyde in 8.3 M HCl as reference. The activity was calculated by using

$\epsilon_{550} = 1.55 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ for *p*-dimethylaminobenzaldehyde complex of the glucuronide as reported by Leakey [8]. In order to compare, the activity was also assayed by the method of Leakey [8]. Protein concentration was determined by the method of Bradford [4]. As the standard, bovine serum albumin was used.

RESULTS

Improved assay method for 5-HT GT: A better solvent was tried to select for the extraction of unconjugated 5-HT, and *n*-amyl alcohol saturated with 0.6 N NH_4OH brought to a selective extraction. Namely, through the double extraction described in MATERIALS AND METHODS, over 99% of 5-HT present in control tubes was removed, and over 97% of 5-HT glucuronide added to control tubes was recovered.

Figure 1 shows the effect of the amount of microsomal protein on GT activity towards 5-HT

Fig. 1. Effect of the amount of microsomal protein on UDP-glucuronyltransferase activity towards 5-hydroxytryptamine determined by the Leakey's (A) and present (B) methods. The assay was performed with 1 mM 5-hydroxytryptamine and 6 mM UDP-glucuronic acid. The mixtures were incubated for 0 min (●), 10 min (○), 20 min (▲), and 30 min (△). Prior to incubation, microsomes were treated with 0.25% cholate in a volume of 50 μ l at 0°C for 30 min.

assayed by the improved and Leakey's [8] methods under the same substrate concentration. These improvements lowered control A_{550} values from about 0.04 by the Leakey's method (Fig. 1A) to zero by the improved method (Fig. 1B). Moreover, the control values by the improved method were not affected by the incubation time and the concentration of 5-HT and UDPGA (data were not shown). Simultaneously, the improvements heightened the obtained GT activity, and enhanced the linear dependency of the activity upon the amount of an enzyme protein and upon an incubation time. The improvements also resulted in a simplification of the assay method.

Treatment of microsomes with cholate: Sodium cholate was used for the perturbant to determine the enzymatic full activity. The treatment of microsomes with cholate before enzymic incubation activated the glucuronidation of 5-HT (Fig. 2). The same results were obtained from the experiments with two different concentrations of microsomal protein. This activation was maximal at the cholate concentration of 0.25% (0.0625% in incubation mixture). A slight inhibition occurred when the concentration of cholate was more than 0.25%. The

cholate concentration of 0.25% was, therefore, chosen for determination of the activated microsomal 5-HT GT activity.

Effect of pH: Reaction rates at different pH values ranged from 7.0 to 9.5 were determined when the incubation mixtures were buffered with 0.1 M Tris-HCl solution. The enzyme activity in the intact microsomes was maximal at pH 7.8. The behavior in

Fig. 2. Effect of cholate on UDP-glucuronyltransferase activity towards 5-hydroxytryptamine. Incubation mixtures contained 20 μg (●) and 100 μg (○) of microsomal protein. Prior to incubation, microsomes were treated with cholate (abscissa) in a volume of 50 μl at 0°C for 30 min.

Fig. 3. Double-reciprocal plots of UDP-glucuronyltransferase activity towards 5-hydroxytryptamine against the concentration of 5-hydroxytryptamine (A), UDP-glucuronic acid (B), and MgCl_2 (C). Prior to incubation, microsomes (40 μg of protein) were either treated with 0.25% cholate (○) in a volume of 50 μl at 0°C for 30 min, or not treated (●). The results are shown as double-reciprocal plots.

the cholate-activated microsomes was similar.

Effect of 5-HT concentration: With a saturating concentration of UDPGA (10 mM), the effect of the concentration of 5-HT on the activity was studied. The enzyme obeyed simple Michaelis-Menten kinetics in the intact microsomes, and the kinetic behavior did not change upon the cholate-activation (Fig. 3A). The apparent K_m value for 5-HT was 0.1 mM. A slight substrate inhibition was observed at the relatively high concentration.

Effect of UDPGA concentration: With a saturating concentration of 5-HT (2 mM), the effect of the concentration of UDPGA was also studied. In this case, the enzyme in the intact microsomes did not obey simple Michaelis-Menten kinetics (Fig. 3B). Double-reciprocal plots of the enzyme activity against the concentration of UDPGA gave two apparent K_m values. The kinetic behavior did not change upon the cholate-activation, and the apparent K_m values for UDPGA were 0.6 and 5 mM.

Table 1. Effects of divalent cations and EDTA on UDP-glucuronyltransferase activity towards 5-hydroxytryptamine. Each tube contained 40 μ g of microsomal protein

	Specific activity (nmol/min/mg of protein)	
	2 mM	5 mM
None		9.76
MgCl ₂	13.31	15.44
CaCl ₂	12.42	15.08
EDTA	9.41	9.76

Substrate inhibition did not occur in two preparations at relatively high substrate concentration.

Effect of divalent cations: The GT activity towards 5-HT in the intact microsomes was also stimulated by Mg^{2+} and Ca^{2+} as in the case of other substrate used (Table 1). However, EDTA did not affect on this activity. This indicates that these divalent cations are dispensable for the enzyme activity. The activity in cholate-activated microsomes were further stimulated by the addition of Mg^{2+} . Double-reciprocal plots of corrected activity (total activity minus activity found in the absence of the added Mg^{2+}) against the concentration of Mg^{2+} did not obey simple Michaelis-Menten kinetics (Fig. 3C), and gave two apparent K_m values as obtained from those plots of the activity against the concentration of UDPGA (Fig. 3B). The kinetic behavior did not change upon the cholate-activation. The two apparent K_m values for Mg^{2+} were 1 and 10 mM.

Relationship between GT activity and added Mg^{2+} : Figure 4 shows the effect of the added Mg^{2+} on the apparent K_m values for 5-HT and UDPGA and the V_{max} value. When the concentration of 5-HT was varied with a saturating concentration of UDPGA (10 mM) (Fig. 4A), the addition of Mg^{2+} to the cholate-activated microsomes did not change the apparent K_m value for 5-HT, but increased the V_{max} value. However, when the concentration of UDPGA was varied with a saturating concentration of 5-HT (2 mM) (Fig. 4B), the addition of Mg^{2+} changed both the apparent K_m value for UDPGA and the V_{max} value. Namely, when Mg^{2+} was added to give a concentration of 0 to 2 mM, the apparent

Fig. 4. Double-reciprocal plots of UDP-glucuronyltransferase activity towards 5-hydroxytryptamine against the concentration of 5-hydroxytryptamine (A) and UDP-glucuronic acid (B) as the function of $MgCl_2$. Prior to incubation, microsomes (40 μ g) were treated with 0.25% cholate in a volume of 50 μ l at 0°C for 30 min. The final concentrations of $MgCl_2$ were 0 mM (●), 0.2 mM (○), 0.5 mM (▲), 2.0 mM (△), 5.0 mM (■), and 10.0 mM (□).

K_m value for UDPGA changed from 0.6 mM to 0.3 mM and the V_{max} value increased. The further addition of Mg^{2+} over 2 mM fixed the apparent K_m value for UDPGA to 0.3 mM, and changed only the V_{max} value.

DISCUSSION

As the non-radioactive method to measuring a 5-HT glucuronidation, the method of Leakey [8] has been introduced. In this method, UDPGA has caused an increase in the control A_{550} value, which also increased with the reaction time and the concentration of 5-HT [8]. The present improvements overcame this disadvantages. In the present method, the control value was zero and was not affected by those assay conditions (Fig. 1). In addition, the present method showed higher dependency of the activity upon the amount of an enzyme protein and upon an incubation time than the Leakey's method. Thus, the new method enhanced the accuracy, did not confine the assay condition and made it possible to study the kinetic properties of this enzyme. The obtained K_m values for 5-HT, UDPGA and Mg^{2+} were similar to the values in the highly purified preparation [1], except for the higher K_m value for UDPGA.

Because GT is tightly bound to hepatic microsomes and shows partial latency, the full activity can be measured only after the perturbation of the membrane by various treatments such as snake venom digestion [7], sonication [10] and treatment with detergents. In these experiments, sodium cholate was used. The optimum concentration of cholate (0.25%) was similar to the results using 4-nitrophenol as substrate in a rabbit liver microsomes [17].

Divalent metal ions stimulate the GT activities towards many acceptor substrates *in vitro* [5]. Mg^{2+} also stimulated the GT activity towards 5-HT. Double-reciprocal plots of the GT activity against the concentration of 5-HT were fit in Michaelis-Menten kinetics (Fig. 3A). On the other hand, the same plots of the activity against the concentration of UDPGA deviated from this kinetics (Fig. 3B). Similar deviation was observed in this plots of the corrected activity against the concentration of Mg^{2+} (Fig. 3C). This suggests that the relationship might exist among the enzyme, UDPGA and Mg^{2+} .

With regard to 5-HT, the addition of Mg^{2+} did not change the apparent K_m value for 5-HT, and

changed only the V_{max} value (Fig. 4A). This indicates that Mg^{2+} is non-competitive with 5-HT, and do not affect on the 5-HT binding to the enzyme.

In contrast with 5-HT, both the apparent K_m value for UDPGA and the V_{max} value were changed by the addition of the low concentration of Mg^{2+} (below 2 mM, Fig. 4B). Lines on double-reciprocal plots of the enzyme activity against the concentration of UDPGA with different concentrations of the added Mg^{2+} intersected on the second quadrant (Fig. 4B). This type of activation by metal ion has been described by following reactions [11]:

E, 5-HT GT; S, UDPGA; M, Mg^{2+} ;
P, 5-HT glucuronide

Thus, UDPGA is able to bind to the enzyme in the presence or the absence of Mg^{2+} , but Mg^{2+} does not bind directly to the enzyme at the lower concentration of Mg^{2+} (does not form Mg^{2+} -enzyme complex). In other words, UDPGA- Mg^{2+} complex is able to bind to the enzyme, and Mg^{2+} is also able to bind to the UDPGA moiety of UDPGA-enzyme complex. Additionally, the UDPGA- Mg^{2+} complex showed higher affinity for the enzyme (the apparent K_m value was 0.3 mM) than UDPGA (the apparent K_m value was 0.6 mM). Thus, it seems that this results in the increase of the V_{max} value. At the higher concentration of Mg^{2+} (over 2 mM), no-change of the apparent K_m value for UDPGA and the increase of the V_{max} value were observed (Fig. 4B). Because Mg^{2+} is non-competitive against UDPGA at the higher concentration of Mg^{2+} , it seems that Mg^{2+} binds directly to a domain of the enzyme which is different from the active site(s) of UDPGA and 5-HT.

In liver, UDPGA level is 15 to 20 μ mole per 100 g of wet weight [16]. This level is less than one third of the apparent K_m value for UDPGA in the absence of Mg^{2+} . The decrease of this value for UDPGA in the biological Mg^{2+} concentration may be effective for glucuronidation under the biological condition.

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