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SHORT COMMUNICATION

MULTIPLICITY OF *IN VITRO* GLUCURONIDATION OF 2-HYDROXYESTRIOL

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Summary—*In vitro* glucuronidation of 2-hydroxyestriol has been investigated by means of HPLC with dual-electrode coulometric detection. When incubated with rat or dog liver microsomal preparation in the presence of UDPGA, 2-hydroxyestriol was transformed into the 2-glucuronide together with a small amount of 16- and/or 17-glucuronides. In contrast, incubation of 2-hydroxyestriol with guinea-pig liver microsomal preparation yielded the 3-glucuronide and a trace amount of the 2-glucuronide, but no ring D glucuronides. Upon pretreatment with 3-methylcholanthrene male rat liver exhibited a marked increase in both 2- and 3-glucuronidation activities, whereas female rat liver showed an elevation only in 2-glucuronidation. On the other hand, in male and female rats pretreatment with phenobarbital caused a relatively small increase in the glucuronidation activity of the liver. In the male guinea-pig, glucuronidation was not affected by pretreatment with either of the two compounds. The present result demonstrates the multiplicity of hepatic 2-hydroxyestriol UDP-glucuronyl-transferase in the rat, guinea-pig and dog.

INTRODUCTION

In recent years, considerable attention has been focused on the physiological significance of the formation of catechol estrogens in living animals [1, 2]. The conjugation of catechol estrogens has been much investigated [3, 4]. The multiplicity of hepatic UDP-glucuronyltransferase has previously been demonstrated with steroid hormones [5–7]. Our previous studies revealed that 2- and 4-hydroxyestrones are susceptible to *in vitro* glucuronidation in rat liver [8] and the glucuronides are excreted in rat bile as principal conjugates, following oral administration of 4-hydroxyestriol [9].

The present paper deals with studies on the multiplicity of *in vitro* glucuronidation of 2-hydroxyestriol and the effects of pretreatment with 3-methylcholanthrene (3-MC) and phenobarbital (PB) on the UDP-glucuronyltransferase activity by means of HPLC with electrochemical detection (HPLC/ECD).

EXPERIMENTAL

Chemicals and reagents

2-Hydroxyestriol, its monoglucuronides and 6β-hydroxyestradiol 17-glucuronide were prepared in these laboratories [10]. 3-MC and PB were purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Other chemicals used were commercially available.

HPLC

The apparatus used was a Waters Model 510 chromatograph (Millipore-Waters, Milford, Mass, U.S.A.) equipped with a Coulochem Model 5100A electrochemical detector (Environmental Sciences Assoc. Inc, Bedford, Mass, U.S.A.). The potentials of the detector were set at +0.3 V (1st electrode) and +0.9 V (2nd electrode) vs a palladium electrode. A test sample was introduced using a Rheodyne Model 7125 injector (Rheodyne Inc., Cotati, Calif., U.S.A.) with an effective volume of 100 μl. HPLC was carried out on a Develosil ODS-5 column (5 μm; 15 × 0.4 cm i.d.) (Nomura Chemicals, Seto, Japan) at ambient temperature using 0.5% sodium acetate (pH 3.0)/acetonitrile (6:1, v/v) as a mobile phase. The pH of the mobile phase was adjusted with phosphoric acid.

Pretreatment of animals

Male and female Wistar rats weighing 200–300 g and male Hartley strain guinea-pigs weighing 300–400 g were used. 3-MC dissolved in corn oil was administered intraperitoneally once at a dose of 20 mg/kg body wt. Animals were sacrificed 48 h after dosing. PB dissolved in saline was administered intraperitoneally once a day at a dose of 75 mg/kg body wt for 4 days. Animals were sacrificed 48 h after the last injection.

Enzyme preparation

Male and female Wistar rats, male Hartley strain guinea-pigs and male mongrel dogs (body wt 7–8 kg) were used. Each fresh liver was homogenized in ice-cooled 0.25 M sucrose solution to bring a final concentration to 20% (w/v). The homogenate was centrifuged at 10,000 *g* for 15 min, and the supernatant was further centrifuged at 105,000 *g* for 45 min. The 105,000 *g* fraction was resuspended in 0.25 M sucrose solution and centrifuged at 105,000 *g* for 45 min. Microsomal pellets were used for the enzymic glucuronidation study. Protein was determined by the method of Lowry *et al.* [11].

Assay procedure

The assay medium (1.3 ml) consisted of the substrate (14 nmol in 10 μl methanol), UDPGA (400 nmol), D-glucaro-1,4-lactone (200 nmol), ascorbic acid (280 nmol), and enzyme preparation (105,000 *g* fraction: 2.5 mg protein) in 0.1 M phosphate buffer (pH 7.4) containing 5 mM $MgCl_2$. The mixture was incubated in air at 37°C for 2 h. After

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addition of the internal standard (IS: 6β-hydroxyestradiol 17-glucuronide), the incubation mixture was diluted with 0.3 M acetate buffer (pH 2.0) (4 ml) and immediately cooled at 0°C. The diluted incubation mixture was percolated through a Bond Elut C_{18} cartridge. After washing with water (5 ml), the desired fraction was eluted with methanol (2.5 ml) and dried with a N_2 gas stream. The residue was redissolved in aqueous methanol and subjected to HPLC/ECD on a Develosil ODS-5 column (5 μm, 15 × 0.4 cm i.d.) using 0.5% sodium acetate (pH 3.0, adjusted with phosphoric acid)/acetonitrile (6:1, v/v) as mobile phase [12]. A calibration graph was constructed by plotting the peak height ratio of 2-hydroxyestriol monoglucuronide to 6β-hydroxyestradiol 17-glucuronide (IS) against the amount of the former where the injected amount of IS was 50 ng.

RESULTS AND DISCUSSION

In a previous paper, we reported the separation of positional isomers of 2-hydroxyestriol monoglucuronides by HPLC/ECD on a reversed phase column utilizing their electrochemical characteristics [12]. Four isomeric monoglucuronides of 2-hydroxyestriol and IS could be distinctly separated on the chromatogram. The detection limits obtainable with biological samples under the assay condition were 1 ng and 500 pg for ring A and ring D monoglucuronides, respectively.

Effort was directed to establishing a clean-up procedure for HPLC. The incubation mixture was diluted with acetate buffer (pH 2.0) at 0°C and applied to a Bond Elut C_{18} cartridge. After washing with water, the desired fraction was eluted with methanol and then subjected to HPLC/ECD. This procedure proved to be simple, completed within 1 h and suitable for the routine assay. A linear calibration graph was obtained in the range 0–100 ng/injection of each 2-hydroxyestriol monoglucuronide. Known amounts of four isomeric 2-hydroxyestriol monoglucuronides were added to the incubation mixture, and their recovery rates were determined according to the proposed method. Each monoglucuronide was recovered at the rate over 78% with SD 4.5–6.0 % ($n = 7$). The proposed method was found satisfactory with respect to accuracy and precision.

It appeared to be of particular interest to clarify where *in vitro* glucuronidation would take place among four different kinds of hydroxyl groups, namely the two aromatic and aliphatic hydroxyl functions, in 2-hydroxyestriol. The incubation study was carried out with rat, guinea-pig and dog liver 105,000 *g* fractions in the presence of UDPGA. The results obtained are listed in Table 1. No metabolism of the substrate to substances other than glucuronides was observed.

In the rat 2-hydroxyestriol 2-glucuronide was formed as a principal conjugate. A small amount of the 16-glucuronide was formed in male but not in female liver. It is of interest that sex difference was distinctly observed on glucuronidation of 2-hydroxyestriol in the rat. This result seems to be compatible with the previous finding that sex difference in glucuronidation of 2-hydroxyestrone exists in humans [8]. In sharp contrast, glucuronidation occurred almost exclusively towards the hydroxyl group at C-3 in male guinea-pig liver. The amount of the monoglucuronides formed in male guinea-pig liver was 10–20 times more than that in rat liver. In the male dog, 2-hydroxyestriol 2-glucuronide and 17-glucuronide were formed in a ratio of 13:1, and the 16-glucuronide was produced to a much lesser extent. Our previous study revealed that estriol 17-glucuronide and 16-glucuronide were excreted in bile at a ratio of 2:1 in rats given estriol [13]. In the present case, the yielded amount of 2-hydroxyestriol 17-glucuronide was approx. four times more than that of the 16-glucuronide.

The rat liver microsomal glucuronyltransferase activities have been classified as groups on the basis of their inducibility by two prototype xenobiotic inducers, 3-MC and PB [14]. It was observed that the PB pretreatment increased estrone glucuronyltransferase activity to 4.9-fold of the control [15]. These findings prompted us to investigate the effects of pretreatment with typical inducers on glucuronidation of 2-hydroxyestriol which has four different hydroxyl groups.

The glucuronidation activity in rats pretreated with 3-MC was raised 2–3 times more than that of the control. To the best of our knowledge this is the first report that 3-MC pretreatment exerts a significant increase in glucuronidation in the male rat but not in the female. The male rat seemed to be more responsive than the female rat to the 3-MC pretreatment. The liver of pretreated male rats was more capable of catalyzing the transfer of a glucuronyl moiety to ring A hydroxyl groups. The enzymic activity for glucuronidation of ring D hydroxyl groups was not affected by the 3-MC pretreatment. On the other hand, the PB pretreatment provided no substantial change in the glucuronidation activity in both male and female rats. These results indicate the multiplicity of UDP-glucuronyltransferase involved in conjugation of ring A and ring D hydroxyl groups. In the male guinea-pig, pretreatment with 3-MC or PB exerted no marked influences on glucuronidation of the hydroxyl group at C-3 or on other hydroxyl functions in 2-hydroxyestriol.

It should be emphasized that the availability of a reliable method using HPLC/ECD for the simultaneous assay of monoglucuronides formed from estrogen substrates having various hydroxyl functions is useful for investigating the multiplicity of UDP-glucuronyltransferase with respect to species and sex differences.

Table 1. *In vitro* formation of isomeric monoglucuronides from 2-hydroxyestriol with liver microsomes

Animals	Sex	Pretreated	3-G	2-G	16-G	17-G
			(pmol/mg protein/h)			
Rat	M	None	Trace	12.5	1.8	Trace
	F	None	—	5.2	Trace	—
	M	3-MC	5.2	40.8	1.0	Trace
	F	3-MC	Trace	13.6	Trace	—
	M	PB	1.3	15.4	1.1	Trace
	F	PB	—	5.8	Trace	—
Guinea-pig	M	None	91.2	Trace	—	—
	M	3-MC	92.0	Trace	—	—
	M	PB	96.2	Trace	—	—
Dog	M	None	0.1	77.9	1.4	5.8

$n = 2$

—: not detectable, M: male, F: female, G: glucuronide.

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