Separation and Purification of Uridine Diphosphate–Glucuronosyltransferases by Chromatofocusing on a High-Performance Liquid Chromatograph

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A rapid method for the separation and purification of uridine diphosphate–glucuronosyltransferases (GT) was developed with the use of chromatofocusing on a high-performance liquid chromatograph. GT isoenzymes solubilized from hepatic microsomes of Wistar rats were separated on a Mono P column, a pre-packed column for chromatofocusing. Using 4-nitrophenol, testosterone and androsterone as substrates, four fractions with different GT activities were separated in a pH gradient from 9.5 to 7.0. Two isoenzymes, testosterone GT and androsterone GT were purified to apparent homogeneity. They were eluted at pH 8.9 and 8.0 and had subunit molecular weight values of 50000 and 52000, respectively. Approximately 10 mg of solubilized microsomal proteins was applied and the elution was completed within 2 h. Addition of *N*-nitrodiethylamine, an *in vitro* activator of GT activity, enhanced the GT activity toward 4-nitrophenol in the three fractions. This chromatographic analysis confirmed the absence of androsterone GT isoenzyme in LA Wistar rats, a mutant strain in terms of androsterone glucuronidation.

Keywords high-performance liquid chromatography; chromatofocusing; rat liver; uridine diphosphate–glucuronosyltransferase; isoenzyme deficiency; *N*-nitrodiethylamine

Introduction

Uridine diphosphate (UDP)–glucuronosyltransferase (GT) catalyzes glucuronidation of xenobiotics as well as endogenous compounds.[1] Recently chromatographic separation and purification of GT isoenzymes have revealed the heterogeneity of GT activities.[2,3] Different forms of rat hepatic GT isoenzymes with partially overlapping substrate specificities were successfully separated according to their charge (isoelectric point) heterogeneity.[4] From rat liver microsomes, six GT isoforms have so far been purified by using chromatofocusing and UDP–hexanolamine affinity chromatography.[4-8] These are androsterone (3α-hydroxysteroid) GT,[4] testosterone (17β-hydroxysteroid) GT,[4] bilirubin GT,[5] 4-nitrophenol GT,[4,6] morphine GT[7] and digitoxigenin monodigitoxoside GT.[8]

Previously we reported a genetic variation of androsterone GT activity in Wistar rat colonies,[9] which could be divided into two groups on the basis of hepatic androsterone GT activity: one with high activity (HA) and the other with low activity (LA). Purification of GT isoenzymes from HA and LA Wistar rats by chromatofocusing and UDP-hexanolamine affinity chromatography has demonstrated that androsterone GT isoform is deficient in LA Wistar rats,[10] probably due to the deletion of androsterone GT gene.[11]

In this study, we report a rapid chromatofocusing method on a high-performance liquid chromatograph (HPLC) for the separation and purification of rat hepatic GT isoenzymes. The hepatic GT activities of HA and LA Wistar rats were analyzed and compared by this method. The effect of *N*-nitrodiethylamine (NEA), which is an *in vitro* activator of GT activity,[12] was also examined on each GT isoform separated by this method.

Materials and Methods

Materials [9,11-^{3}H]Androsterone (53.3 Ci/mmol) and [1,2-^{3}H]testosterone (49.0 Ci/mmol) were purchased from New England Nuclear Corp., Boston, MA, U.S.A. 4-Nitro[1-^{14}C]phenol (56 mCi/mmol) was obtained from Amersham International, Buckinghamshire, England. Androsterone, testosterone and phosphatidylcholine (egg yolk; type IIIE) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. The standard proteins used in gel electrophoresis were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. 4-Nitrophenol and Emulgen 911 were obtained from Wako Chemical Ind. Ltd., Tokyo, Japan, respectively. UDP-Glucuronic acid disodium salt and UDP–glucuronic acid trisodium salt were obtained from Boehringer, Mannhein, Germany and Yamasa Syoyu Co., Ltd., Tokyo, Japan and Kao Atlas Ltd., Tokyo, Japan, respectively. Polybuffer 96 and Pharmalyte 8—10.5 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. NEA was prepared as described previously.[12] All other reagents were of analytical grade.

Animals and Preparation of Microsomal Fractions Wistar rats were classified into homozygous HA and LA Wistar rats in terms of hepatic androsterone GT activity as described previously.[10] A female HA or LA Wistar rat (200—400 g) was decapitated and a 20% (w/v) liver homogenate was prepared in ice-cold 1.15% (w/v) KCl, with a Teflon/glass homogenizer. The microsomal fraction was obtained by differential centrifugation as described previously[10] and the microsomal pellets were suspended in 25 mM diethanolamine–HCl buffer, pH 9.5, containing 0.05% (w/v) Emulgen 911. The suspension was solubilized by further addition of 0.5 mg of Emulgen 911/mg of protein, stirred for 30 min, and then centrifuged at 105000 *g* for 30 min as described previously.[4] All procedures described above were done at 0—4 °C.

HPLC Chromatofocusing The liquid chromatograph consisted of a Shimadzu LC-6A liquid delivery pump equipped with a Shimadzu SPD-6A ultraviolet detector. A column of Mono P HR 5/20 (5 mm i.d. × 200 mm), a Superloop (50 ml) and a Valve V-7 were products of Pharmacia Fine Chemicals, Uppsala, Sweden. The solubilized microsomes (approximately 10 mg of solubilized protein, 5—15 ml) were applied through the Superloop to a Mono P column, which had previously been equilibrated with 25 mM diethanolamine–HCl buffer, pH 9.5, containing 0.05% Emulgen 911. The column was eluted with an elution buffer (pH 7.0) containing 0.05% Emulgen 911, which was made of Polybuffer 96 and Pharmalyte 8—10.5 according to the instructions of the supplier. The flow rate was 0.5 ml/min. The chromatography was carried out at room temperature and the fractions of 0.5 ml were collected in glass tubes, which were cooled in ice-water.

Assay Procedures GT activities toward androsterone and testosterone were determined by a modification of the method described previously.[10] The standard incubation medium contained 0.17 mM [^{3}H]androsterone (13 nCi) or 0.30 mM [^{3}H]testosterone (16 nCi), 2 mM UDP–glucuronic acid, 10 mM $MgCl_2$, 50 μM ethylenediaminetetraacetic acid (EDTA), 0.01% (w/v) phosphatidylcholine and 0.1 M Tris–HCl buffer, pH 7.4, in a total volume of 0.50 ml. The incubation was carried out for 30 min at 37 °C and the incubation mixture was treated as described previously.[10] GT activity toward 4-nitrophenol was assayed with 0.5 mM 4-nitro[^{14}C] phenol (8.4 nCi) for 30 min at 37 °C. The incubation was terminated by addition of 0.1 ml of 2 M phosphate buffer, pH 2.1. The resultant mixture was extracted with 6 ml of diethylether–chloroform (1 : 1, v/v) and centrifuged at 3000 rpm for 20 min. Then a portion (0.2 ml) of the aqueous phase was taken for the measurement of the radioactivity of the glucuronide. The blank value was obtained from the control incubation in which UDP–

glucuronic acid was omitted. To examine the effect of NEA, 10 mM NEA was added to the incubation mixture as described previously.[12]

Protein concentrations were determined by the method of Lowry *et al.*[13] or the method of Bradford[14] with bovine serum albumin as a standard.

Gel Electrophoresis Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was performed on a 10% polyacrylamide slab gel as described by Laemmli.[15] Proteins were identified by Coomassie Blue staining.

Results

Figure 1 shows a typical separation on a Mono P column of hepatic GT activities of HA and LA Wistar rats, which were solubilized from freshly prepared liver microsomes. A pH gradient was generated from approximately 9.5 to 7.0 and the elution was completed within 2 h. There were four fractions with different GT activities toward 4-nitrophenol, testosterone and androsterone in HA Wistar rats (Fig. 1A). The first GT activity appeared in the flow-through fraction of around pH 9.5 and had a high activity toward 4-nitrophenol (fraction 1 in Fig. 1A). This fraction was not further studied in this experiment. The second peak of GT activity was eluted at about pH 8.9 and had high activities toward 4-nitrophenol and testosterone (fraction 2 in Fig. 1A). The third peak of GT activity appeared at approximately pH 8.4 and exhibited high activities toward 4-nitrophenol, testosterone and androsterone. High activity toward androsterone and low activities toward 4-nitrophenol and testosterone were observed in the fourth fraction at about pH 8.0. Approximately 10% of the protein applied to the column was recovered in the pH gradient from 9.5 to 7.0. Recoveries of the GT activities toward 4-nitrophenol, testosterone and androsterone were around 30%, 70% and 60%, respectively on the basis of the GT activities of the solubilized microsomes. These active fractions were analyzed by SDS-PAGE as shown in Fig. 2A and GT isoenzymes were found to be highly purified by HPLC chromatofocusing on a Mono P column. Thus the fractions of pH 8.9 and 8.0 (fractions 2 and 4 in Fig. 1A) each exhibited a single protein band with an apparent subunit molecular weight (M_r) of 50000 and 52000, respectively. In the fraction of pH 8.4 (fraction 3 in Fig. 1A), two bands with apparent subunit M_r values of 50000 and 52000 were observed.

In the case of LA Wistar rats, which are deficient in androsterone GT isoenzyme,[10] the chromatographic profile of GT activities was distinct from that of HA Wistar rats. As shown in Fig. 1B, there were three active GT fractions. The first activity appeared in the flow-through fraction (pH 9.5) and showed high activity toward 4-nitrophenol (fraction I in Fig. 1B). The second fraction was eluted at approximately pH 8.7 and had high GT activities toward 4-nitrophenol and testosterone (fraction II in Fig. 1B). Low activities toward 4-nitrophenol and testosterone were observed in the fraction at around pH 8.3 (fraction III in Fig. 1B). Only negligible GT activity toward androsterone was present in fraction II (Fig. 1B). On SDS-PAGE, fraction II revealed three protein bands with apparent subunit M_r values of 48000, 50000 and 54000 (Fig. 2B). There was no protein band at around M_r 50000 in fraction III or in the fraction of pH 8.0, which corresponds to fraction 4 of HA Wistar rats.

When microsomes were stored at −80 °C in 0.25 M sucrose containing 0.1 M Tris-HCl pH 7.4 for 30 d, subsequently solubilized and applied to the Mono P column, no significant change was observed in the chromatographic profile. However, when microsomes were stored at −80 °C for 30 d in the solubilization buffer (25 mM diethanolamine-

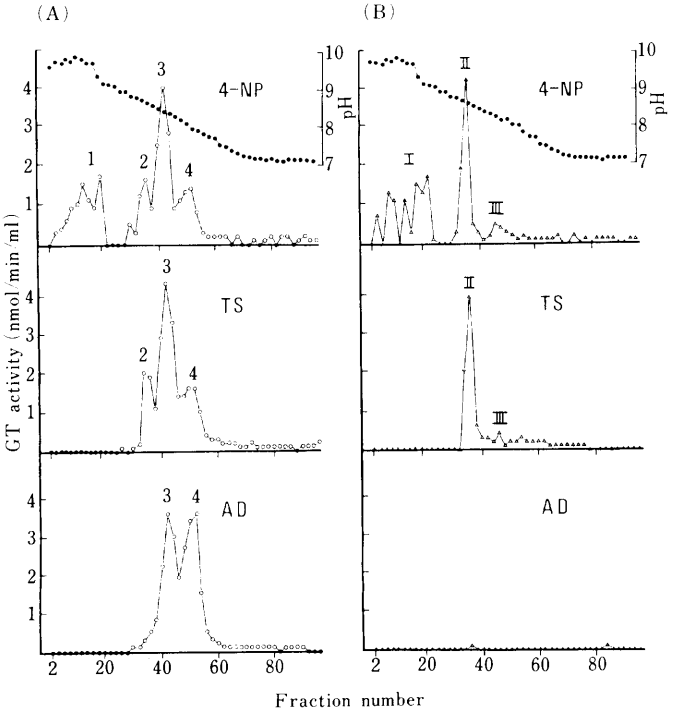

Fig. 1. HPLC Chromatofocusing of Solubilized Liver Microsomes from HA (A) and LA (B) Wistar Rats

The solubilized liver microsomes (10 mg) of HA and LA Wistar rats were applied to a Mono P column and the column was eluted as described in Materials and Methods, with a pH gradient from 9.5 to 7.0 (-●-). GT activities toward 4-nitrophenol (4-NP), testosterone (TS) and androsterone (AD) were determined (○- , △-).

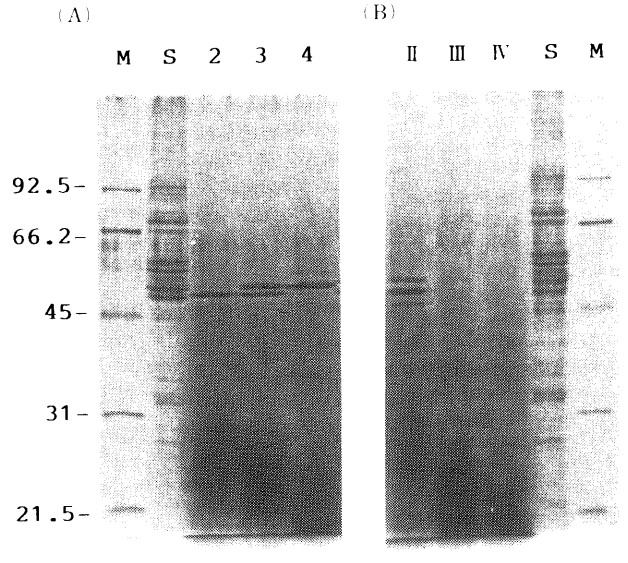

Fig. 2. SDS-PAGE of HA (A) and LA (B) Wistar Rat Liver GTs Purified by HPLC Chromatofocusing

Ten microliter portions of fractions 2, 3 and 4 in Fig. 1A and of fractions II and III in Fig. 1B were analyzed by SDS-PAGE. Column IV shows the fraction of pH 8.0 in Fig. 1B (fraction 52), which correponds to fraction 4 of HA Wistar rat in Fig. 1A. M, protein standards (kDa): phosphorylase B (92.5), bovine serum albumin (66.2), ovalbumin (45), carbonic anhydrase (31) and soybean trypsin inhibitor (21.5). S, solubilized microsomes.

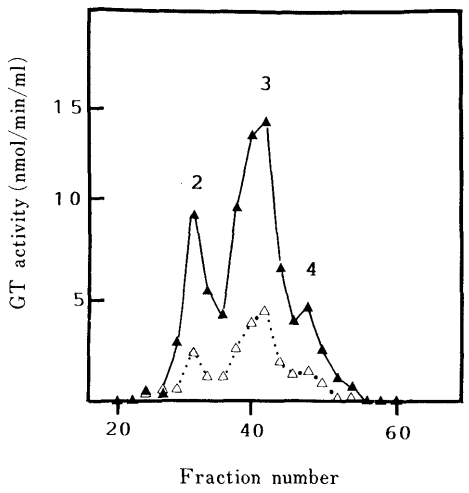

Fig. 3. Effect of NEA on GT Activities Separated by HPLC Chromatofocusing

Hepatic GT activities of HA Wistar rats were separated by HPLC chromatofocusing as described in Fig. 1. The GT activity toward 4-nitrophenol was determined in the fractions from 20 to 60 in the presence of 10 mM of NEA or in its absence. (+) 10 mM NEA, ▲—; (−) NEA, △· · · ·. Peaks 2, 3 and 4 correspond to the fractions 2, 3 and 4 in Fig. 1A, respectively.

HCl, pH 9.5 containing 0.05% Emulgen 911), chromatofocusing of the GT activities resulted in a broadening of each active peak and poorer resolution of GT activities (data not shown). Separation of GT activities was also impaired when microsomal proteins were stored at −80 °C for 30 d after solubilization with Emulgen 911.

In a previous paper,[12] we reported that NEA selectively activated the microsomal GT activities toward 4-nitrophenol and 2-aminophenol. Figure 3 shows the effect of NEA on 4-nitrophenol GT activities which were separated on the HPLC chromatofocusing. The GT activity toward 4-nitrophenol was determined in the presence or absence of 10 mM NEA. Addition of NEA enhanced 4-nitrophenol GT activities in fractions 2, 3 and 4.

Discussion

Separation and purification of rat hepatic GT activities by conventional chromatofocusing on PBE 94 have been previously reported.[4-8] The HPLC chromatofocusing on a MonoP column described in this report provided a similar chromatographic profile to that of the previous studies.[4,6] Although in the HPLC method, a relatively small amount (10 mg) of solubilized microsomal proteins was applied, elution was completed within 2 h. The conventional chromatofocusing requires about 40 h for the elution.[10] The active peaks also appeared sharply and reproducibly on HPLC chromatofocusing. The combination of conventional chromatofocusing on PBE 94 and UDP-hexanolamine affinity chromatography yielded highly purified forms of testosterone GT and androsterone GT isoenzymes.[4,6] These isoenzymes were eluted at pH 8.4—8.5 and pH 7.8 and displayed subunit M_r values of 50000 and 52000, respectively. The fraction with high GT activities toward androsterone and testosterone was eluted at about pH 8.0—8.1 and had apparent M_r values of 50000 and 52000. This fraction corresponds to fraction 3 in the HPLC method. Thus the fractions of pH 8.9 and 8.0 (fractions 2 and 4) obtained by the HPLC chromatofocusing correspond to the testosterone GT and androsterone GT isoforms, respectively. These isoforms were eluted on HPLC chromatofocusing at 0.2—0.5 higher pH values than those in conventional chromatofocusing.

HPLC chromatofocusing of hepatic GT activities of LA Wistar rats gave a similar chromatogram to that obtained by the conventional method[10,16] and confirmed the deficiency of androsterone GT isoenzyme in LA Wistar rats. Interestingly, three protein bands were observed in the fraction of pH 8.7 (fraction II) of LA Wistar rats, whereas in the corresponding fraction of HA Wistar rats, only a single protein band was demonstrated (Fig. 2, columns 2 and II). Previous reports [10,16] showed that a single protein with an apparent M_r of 50000 was obtained from the fraction, which corresponds to fraction II in this study, by further purification by UDP–hexanolamine affinity chromatography. This protein was presumed to be testosterone GT isoform.[10,16] Although we did not further purify fraction II by affinity chromatography, the proteins of M_r values of 48000 and 54000 observed in this fraction appear to be other than GT isoenzymes. Further study is required to clarify the reason for these aberrant chromatographic profiles in LA Wistar rats.

N-Nitrosodiethylamine (NEN) was reported to activate *in vitro* GT activities toward limited substrates such as 4-nitrophenol, 2-aminophenol and 4-methylumbelliferone.[1] This effect is presumably due to a direct interaction with the GT enzyme molecule, since the activation was also observed with purified GT isoforms.[17,18] In a previous report,[18] NEN was demonstrated to enhance 4-nitrophenol GT activity of specific GT isoenzymes. Details of the mechanism of the activation by NEN are still unknown. We previously reported that NEA, which is much less toxic than NEN, also activates GT activities toward 4-nitrophenol and 2-aminophenol in a similar way to that of NEN.[12] NEA enhanced 4-nitrophenol GT activities in all isoforms separated by HPLC chromatofocusing (Fig. 3). These results are somewhat contrary to the results of an activation study of GT isoenzymes utilizing NEN.[18] Further study seems necessary on the mechanism of the activation by NEN and NEA.

HPLC chromatofocusing described in this study is a rapid and reproducible method for the separation of GT isoenzymes, and could be useful for analysis and semi-preparative isolation of the isoenzymes. This method should aid further inquiry on the organization and multiplicity of the GT isoenzymes.

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