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Screening of inhibitors of uridine diphosphate glucuronosyltransferase with a miniaturized on-line drug-metabolism system

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Abstract

Inhibition of uridine diphosphate glucuronosyltransferase (UGT), a major drug-metabolyzing enzyme, has been studied using an on-line drug-metabolism system integrated into capillary electrophoresis. Microsomes isolated from rat liver were encapsulated in tetramethoxysilane (TMOS)-based silica matrices within a capillary in a single step under mild conditions. This microsome-immobilized capillary column allows both the metabolism of drugs and determination of the metabolites in a single capillary simultaneously, just by injecting the substrate–coenzyme mixture onto the column. Glucuronidation of acetaminophen, a widely used pharmaceutical analgesic and antipyretic agent, was investigated using this system. The glucuronidation was inhibited by 4-nitrophenol (4NP) or probenecid that was injected onto a column along with the substrate–coenzyme mixture. On the other hand, valproate did not inhibit the metabolizing reaction. The extents of inhibition using encapsulated UGT were almost the same as those obtained using free UGT. On the other hand, this electrophoretic enzyme–inhibitor assay in microfabricated devices consumes 10^4 less sample and 10^3 less microsome per experiment compared to the conventional reaction schemes. These results demonstrate that this on-line system can circumvent laborious procedures for the isolation and determination of drug metabolites from the reaction mixtures required in the conventional schemes and can provide an attractive alternative technique for the analysis of drug interactions in the metabolic pathways.

Keywords: Screening; Enzyme inhibitors; Uridine diphosphate glucuronosyltransferase

1. Introduction

Drug-metabolizing enzymes are separated essentially into two groups: phase-I enzymes including cytochrome P450s and phase-II enzymes including uridine diphosphate glucuronosyltransferases (UGTs) [1]. Although the effects of drugs on P450-mediated metabolism are routinely investigated in in vitro studies, their effects on UGT-mediated metabolism are reported only rarely [2]. Glucuronidation represents a major conjugation reaction, which is catalyzed by a family of UGTs [3]. UGTs catalyze the addition of the glycosyl group from a nucleotide sugar to a wide variety of endogenous and exogenous compounds. The products are polar, water-soluble β-D-glucuronides which are efficiently eliminated from the body in bile or urine. UGTs have been divided into two distinct subfamilies based on sequence identities, UGT1 and UGT2. The substrate specificities of various UGT1 isoforms have been widely examined and include bilirubin, amines, and planar and bulky phenol. The UGT2 subfamilies consist of numerous enzymes which catalyze the glucuronidation of diverse chemicals including steroids, bile acids, and opioids [3].

Acetaminophen (paracetamol, *N*-acetyl-*p*-aminophenol) is a widely used pharmaceutical analgesic and antipyretic agent. Although it is considered safe at normal doses, at higher doses it is associated with a predictable, dose-dependent centrilobular hepatotoxicity by a mechanism involving its metabolism to a toxic quinone imine that is formed via cytochrome P-450-based oxidation [4,5]. Acetaminophen

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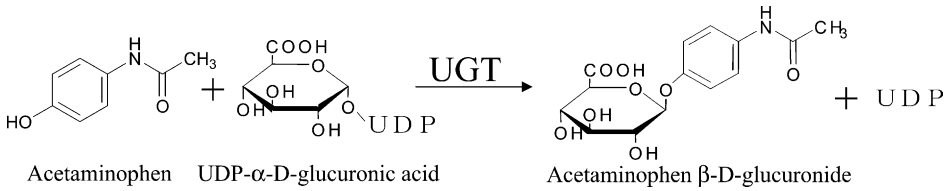

Fig. 1. Scheme of the glucuronidation of acetaminophen.

is mainly metabolized by direct conjugation with glucuronic acid to nontoxic agents in human and rat liver, which then are eliminated in bile or urine (Fig. 1) [6]. Therefore, compounds that compete with acetaminophen for glucuronidation may have therapeutic as well as toxicological implications, especially at higher acetaminophen doses.

New technologies such as combinatorial chemistry and genomics have increased the number of compounds and the pharmacological targets in the drug discovery process. This has led to the re-evaluation of traditional in vitro techniques, which are laborious and require sample preparation to handle the increased flow of drugs through the discovery pipelines [7].

Recently, a capillary electrophoresis (CE) technique has attracted considerable attention because that can speed up the in vitro screening process [8]. Especially, CE-based assays are ideal for enzyme kinetics studies [8–11]. First, CE offers fast analysis time and requires extremely small amounts of sample. Second, CE provides the capacity for highly efficient separation of the reaction products from the substrates in short times. This is advantageous in situations where spectroscopic methods fail to detect differences between the substrate and products simultaneously. This separation also offers the advantage that the presence of inhibitors, which may interfere with detection, will be separated from the reactant or products being measured. From these reasons, CE technique is an excellent tool for studying enzyme reactions, kinetics, and the influence of potential enzyme inhibitors. However, in most cases of enzyme-based assays using CE, the reaction mixture containing enzyme and substrates are directly injected onto a column, which is not desirable when the enzyme is expensive. Also, there is a possibility that the enzyme may interfere with the product detection.

To overcome these problems, we recently developed a novel protein-encapsulation technique using the sol–gel method for the immobilization of proteins into a capillary [12–14]. A variety of proteins including enzymes were encapsulated into the gel matrix without loss of protein activities. Using this technique, a microsome-encapsulated column was fabricated, and an on-line drug-metabolism analytical system was developed by integration into CE [15]. This analytical system allows both the metabolism of drugs and determination of the metabolites in a single capillary simultaneously, just by the injection of the substrate–coenzyme mixtures, and the immobilized microsome can be used repeatedly. Microsomes isolated from rat liver were encapsulated in tetramethoxysilane (TMOS)-based silica matrices within a capillary in a single step under mild conditions. The availability of this system was evaluated using UGT, and 4-nitrophenol (4NP) and testosterone, which were metabolized by the different isoforms of UGT, as substrates. The encapsulated UGT showed enzymatic activity at the same level as that of the soluble form. The following separation of the unreacted substrates and metabolites in the same capillary also showed high selectivity.

The aim of the present study was to develop an on-line system for screening the inhibitors of UGT using the microsome-encapsulated column and CE system. This system was expected to enable a high-throughput screening system for enzyme–inhibitor assay using nano-scale samples. Acetaminophen was used as substrate and the extent of inhibition by some xenobiotics and the experimental procedures were compared with those by the conventional schemes.

2. Experimental

2.1. Materials and chemicals

The fused-silica capillary (75 μm I.D.) was obtained from Polymicro Technologies (Phoenix, AZ, USA). TMOS and methacryloxypropyltrimethoxysilane (MPTMS) were purchased from Tokyo Kasei (Tokyo, Japan). Uridine 5′-diphosphate glucuronic acid (UDPGA), 4NP, and acetaminophen β-D-glucuronide were purchased from Sigma–Aldrich (Milwaukee, WI, USA). Acetaminophen, sodium valproate, and probenecid were purchased from Wako Pure Chemicals (Osaka, Japan).

2.2. Preparation of microsomes

The microsomes were isolated from rat liver by a modification of the described procedures [16,17]. Briefly, male Wister rats, (5 weeks old, Clea Japan, Tokyo, Japan) were killed after light anesthesia with ether. The livers were excised, rinsed in 0.15 M KCl until free of blood and cut into pieces. The pieces were homogenized with 7-volume of ice-cold 0.1 M potassium phosphate buffer, pH 7.4, containing EDTA and dithiothreitol at a concentration of 0.1 mM and glycerol (20%) (isolation buffer). The homogenate was centrifuged at 12 000 × *g* for 30 min and the precipitate was discarded. The supernatant fraction was centrifuged at 105 000 × *g* for 60 min in a refrigerated ultracentrifuge (Hitachi Himac Centrifuge SCP85H2). The microsomal pellet obtained was rinsed with isolation buffer, resuspended in the same medium and recentrifuged at 105 000 × *g* for 60 min. The washed

microsomal pellet was suspended in the isolation buffer and stored at −80 °C until use. All operations were carried out at 0–4 °C. The protein was determined by the method of Lowry et al. [18] using bovine serum albumin (BSA) as standard.

2.3. Capillary pretreatment

A capillary column (40 cm) was pretreated with MPTMS, which covalently anchors the capillary wall to the silanol groups of the silicate matrix [12]. In the preliminary experiment, this pretreatment proved to be very effective in preventing the gel from leaking out of the capillary [12].

The polyimide coating of the treated capillary is burned with fuming sulfuric acid to create a detection window.

2.4. Monolithic capillary column preparation

The sol–gel reaction basically followed the procedures developed by Ellerby et al. [19] and described in our previous report [15].

The monomer solution was obtained by mixing the following reagents just prior to use: (1) 761 μl of TMOS, (2) 169 μl of water and (3) 11 μl of 0.04 M HCl. This monomer solution was stirred for 20 min so that hydrolysis proceeds to form a fully or partially hydrolyzed silane, $SiOH_{4-n}(OMe)_n$.

The microsomal suspension was diluted with the same volume of 20 mM phosphate buffer (pH 7.5), and a 120-μl aliquot was added to 20 μl of the hydrolyzed solution. After mixing and ultrasonication for 5 s, the mixture solution was aspirated with a 1.0-ml disposable syringe from the inlet of the capillary, which was filled in advance with 20 mM phosphate buffer (pH 7.5), until the sol plug was 2.0 cm long under microscopic observation. Introducing the running buffer in advance is very useful in order to shorten the conditioning time. Both ends of the capillary were sealed and held at 4 °C for more than 4 days.

Once fabricated, the capillary was carefully installed in a CE cartridge and conditioned electrokinetically (−2 kV) with 20 mM phosphate buffer (pH 7.5) for 1 h to eliminate the resultant methanol or proteins which were not encapsulated in the sol–gel matrix. The average amount of microsomal protein in the capillary was c.a. 0.18 μg/cm gel, which was measured by the method of Lowry et al. [18] using BSA as standard [15].

2.5. UGT activity using free microsome

The free UGT activity was assayed according to a previously published method [20]. The reaction mixture contained a microsomal suspension (equal to 0.37 mg microsomal protein), 20 mM UDPGA, 5 mM acetaminophen, 10 mM $MgCl_2$, and 20 mM phosphate buffer (pH 7.5) in a total volume of 0.10 ml. The incubation was carried out at 25 °C. The reaction was stopped by dipping the mixture in boiling water for 2 min and the reactant was centrifuged at 10 000 rpm for 5 min. The supernatant solution was filtered through a 0.22-μm membrane. The filtrate was injected onto a fused-silica capillary without gel for the determination of glucuronide. The background electrolyte used was 20 mM phosphate buffer (pH 7.5) and the applied voltage was 4 kV. The glucuronide formation rate was expressed as the total amount of glucuronide formed/mg protein during incubation period (min). The extents of inhibition of acetaminophen glucuronidation were assessed by adding the same concentration of acetaminophen and test compounds to the reaction mixture. The following procedures were the same as those mentioned above.

2.6. UGT activity using encapsulated microsome

The enzymatic activity of the encapsulated UGT was determined using 2 mM acetaminophen along with 20 mM UDPGA and 10 mM $MgCl_2$ as substrate solution. In the preliminary experiment, the concentrations of acetaminophen and UDPGA used for assay were optimized to provide sufficient activity. The substrate solution was introduced electrokinetically (4 kV, 20 s) from the inlet side of the capillary and electroosmotic flow (EOF) was stopped for 10–90 min, enabling the substrates and UDPGA to be in contact with the encapsulated microsomes. The enzymatic reactions were carried out at the microsome-encapsulated matrix, and the substrates were converted to metabolites. After an appropriate duration, voltage (4 kV) was again applied, and the metabolites and resultant substrates were separated and determined at the part of the capillary downstream of the gel.

In the inhibition study, test compounds at a concentration of 2 mM were added to the 2 mM acetaminophen-containing solution, and the mixture were injected onto the column. The following procedures were the same as described above.

Each peak in CE was identified using the peak spectrum obtained by a diode array detector and the migration time of authentic sample.

3. Equipment

CE experiments were carried out on a Hewlett Packard ^{3D}CE system (Palo Alto, CA, USA) equipped with a diode array detector. Substrates were introduced electrokinetically at the anodic side (4 kV, 20 s). A voltage of 4 kV was applied. The temperature was kept at 25 °C in all experiments. The background electrolyte was 20 mM phosphate buffer (pH 7.5). All solutions were filtered through a 0.22-μm membrane (Millipore, Bedford, MA, USA) and degassed by ultrasonication. Water was purified by Milli-Q apparatus (Millipore).

4. Results and discussion

4.1. Scheme of the on-line analytical system of drug metabolites

Fig. 2 is a schematic illustration of the capillary enzyme reactor. At the inlet of the capillary, microsomes are encapsu-

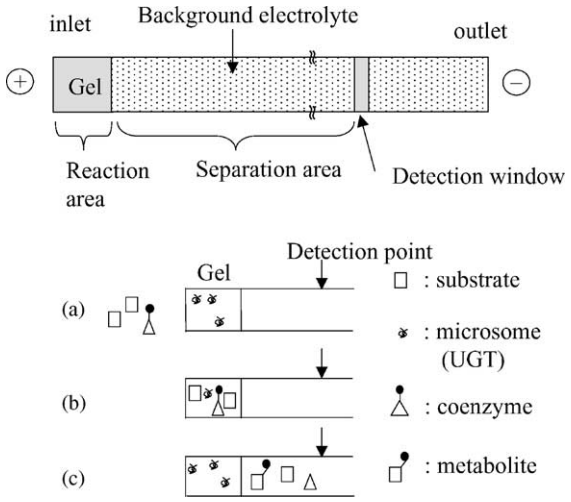

Fig. 2. Scheme of an on-line analytical system of drug metabolites. (a) Substrates are introduced electrokinetically into the microsome-encapsulated reactor; (b) substrates are metabolized by UGT while they flow through the microsome-encapsulated gel by electrophoresis and EOF; (c) the metabolites and unreacted substrates were separated by electrophoresis and determined at the separation section of the capillary.

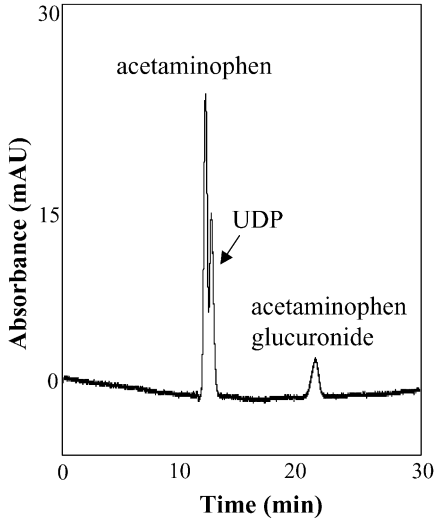

Fig. 3. Electropherogram of an on-line acetaminophen metabolism. Conditions: sample: 2 mM acetaminophen, 20 mM UDPGA, and 10 mM $MgCl_2$; injection: 4 kV, 20 s; microsome-encapsulated gel length: 2.0 cm, total length: 34 cm; background electrolyte: 20 mM phosphate buffer (pH 7.5), applied voltage: 4 kV; detection: 254 nm; UDP: uridine 5′-diphosphate.

lated in the gel matrix. The substrate solution containing substrates and coenzyme is introduced electrokinetically from the inlet of the capillary. The substrates are converted into metabolites by encapsulated UGT at the reaction area. Finally, the unreacted substrates and metabolites are separated by electrophoresis and detected at the part of the capillary downstream of the gel.

4.2. Enzymatic activity of encapsulated UGT

The activity of acetaminophen glucuronidation was evaluated using the encapsulated microsomes. The substrate solution containing acetaminophen, UDPGA and $MgCl_2$ was introduced electrokinetically from the inlet of the capillary, which is filled with microsome-encapsulated sol–gel matrix. This enzyme reactor system was evaluated by the production of acetaminophen glucuronide and its separation from the unreacted acetaminophen. The substrate solution should contain UDPGA as coenzyme and $MgCl_2$ as cofactor along with substrate, acetaminophen. The UDPGA was used in excess so that the UDPGA and acetaminophen could contact each other in the column after injection.

Fig. 3 illustrates a typical electropherogram of acetaminophen and its glucuronide, when 2 mM acetaminophen was introduced into the capillary as substrate. Encapsulated UGT successfully transferred glucuronic acid to acetaminophen. Because the reaction rate of UGT is intrinsically slow, the flow had to be stopped after injection to obtain the appropriate reaction time. Table 1 shows the production of the glucuronide as a function of the resident time of substrates in the column. The glucuronide was produced in correlation with the resident time, followed by the slope saturation after 40 min. Run-to-run repeatability of the activity was acceptable, and the RSD of the values was 4% (n = 3). Based on the results of Table 1, the initial velocity was calculated using the data obtained after a 10-min stop time for rapid screening. Table 2 shows the reaction velocity of the encapsulated microsome and the free microsome. The UGT activity of encapsulated microsome was obtained using three different columns, and the value was 1.34 nmol/min/mg protein, which was slightly smaller than that of free UGT, 1.76 nmol/min/mg protein. The column-to-column repeatability was good and the RSD of the activity was 3% (n = 3). It is notable that the sample and microsome amount required for one analysis decreased by about four and three orders of magnitude, respectively, from a conventional reaction scheme in free solution due to the employment of CE. Furthermore, be-

Table 1
Acetaminophen productions as a function of the resident time of substrates in the microsome-encapsulated column

Time (min)	Glucuronide (mM)
0	0
10	0.15
20	0.28
45	0.44
90	0.42

Conditions are the same as in Fig. 3.

Table 2
UGT activities of encapsulated and free microsome

	Encapsulated	Free
Activity (nmol/min/mg protein)[a]	1.34 ± 0.02	1.76 ± 0.11
Sample size (mol)[b]	4.69×10^{-11}	5.00×10^{-7}
Microsome (μg/analysis)	0.35/column	368

[a] The enzymatic activities were determined using acetaminophen as a substrate. Values are expressed as mean ± S.E.M.; n = 3.
[b] Substrate amount required for one analysis.

cause this microsome-encapsulated column can be used repeatedly without loss of its activity at least for two days, this on-line system is applicable to a high-throughput screening system.

4.3. Inhibition study using the microsome-encapsulated column

In drug development research, it is very important to examine the drug interactions, especially in the metabolic pathway, because it gives the toxicological or therapeutic effects on the patients. The inhibition of drug-metabolizing enzymes as a side effect of drug therapy is of great clinical interest because the resulting increase in blood levels in patients very often causes unexpected severe toxic side effects. On the other hand, the induction of drug-metabolizing enzymes by various compounds can accelerate the metabolism of the drugs biotransformed by the enzymes and can cause a decrease in pharmacological actions or cause inefficacy.

It was expected that the ability to examine UGT activity on this on-line system allows for the examination of possible inhibitors of the metabolism, just by injecting the tested compounds along with substrate–coenzyme mixtures onto a column. Two injection methods of the inhibitors onto the column were examined. Firstly, the injection study was carried out using injections of a series of substrate–coenzyme–inhibitor mixtures. Secondly, the solution that contains only inhibitor was injected, followed by the injection of the substrate–coenzyme–inhibitor solution for the same injection periods of time. In both ways, the extents of inhibition were the same. Therefore, only the mixture solution was injected onto the column. The test compounds studied in this report were probenecid, valproate, and 4NP, and their effects on acetaminophen glucuronidation are summarized in Table 3. Because the inhibitors migrated after acetaminophen glucuronide and did not interfere with the product peak, the change in the peak area of acetaminophen glucuronide with or without test compounds was assessed as the effect of inhibition.

Probenecid is a classic inhibitor of organic anion transport in the kidney [21] and has been used clinically to inhibit renal secretion of penicillin and cephalosporin antibiotics [22]. Clinical studies have suggested that probenecid may impair the glucuronidation of acetaminophen [23]. The inhibitory function was also reported in an in vitro experiment using rat liver microsomes [24]. Using our on-line system, it was found that the acetaminophen glucuronidation was decreased to 42% at the same concentration of probenecid, compared with the control (Table 3). Using the free microsome, the reaction was inhibited to 44% (Table 3). The effect of the concentration of probenecid on the enzymatic activity of immobilized UGT is presented in Table 4. As the inhibitor concentration increased, the amount of metabolite decreased, indicating an increase in enzyme inhibition.

Table 3
Effect of various xenobiotics on the acetaminophen glucuronide

Inhibitor	Remaining activity (%)	
	Encapsulated UGT	Free UGT
Probenecid	41.6	43.5
Valproate	93.7	98.0
4NP	50.8	48.7

Conditions: sample: 2 mM acetaminophen, 2 mM inhibitors, 20 mM UDPGA, and 10 m $MgCl_2$. Injection: 4 kV, 20 s. Other conditions are the same as in Fig. 3.

Table 4
Effect of probenecid concentration on the UGT activity

Probenecid (mM)	Relative activity (%)
0	100
1	69.6
2	43.5
3	29.5

Conditions: sample: 2 mM acetaminophen, 20 mM UDPGA, and 10 m $MgCl_2$. Injection: 4 kV, 20 s. Other conditions are the same as in Fig. 3.

Valproate has been widely used as anticonvulsant and undergoes metabolism by a variety of conjugation and oxidative process [25]. It is also known to inhibit the glucuronide conjugation of some drugs, such as lamotrigine, lorazepam, and zidovudine [26]. Therefore, its effect on the acetaminophen glucuronidation was examined. As shown in Table 3, valproate essentially did not impair the glucuronidation of acetaminophen, and the activity was 94% compared with control, whereas in the free microsome, the activity was 98%. This result coincides with the fact that the UGT isoforms for acetaminophen and valproate are different [3].

4NP is a classic substrate used to analyze UGT activity. Acetaminophen is thought to be a specific substrate for UGT1A6, which is one of the UGT isoforms and catalyzes planar phenols. Because 4NP is also catalyzed by UGT1A6, it is expected that 4NP competes with acetaminophen in the reaction. As shown in Table 3, the glucuronidation of acetaminophen was 49% inhibited by concomitant injection of 4NP. In the free microsome, the glucuronidation was 51%.

Concerning the three xenobiotics studied, this on-line system showed a similar extent of inhibition as those using the free microsome. This indicates that this on-line system can be an alternative to the conventional techniques for the screening and characterization of potent inhibitors to elucidate the drug interactions. It is also noted that this system does not require the continuous flow of the inhibitor nor enzyme in the running buffer. To our knowledge, there are no other capillary electrophoresis enzyme–inhibitor assays, where the enzyme is immobilized in a capillary and the inhibition study is performed only by injection of a substrate–inhibitor mixture. Most of the enzyme-related assays using CE or microchip electrophoresis require the injection of a substrate–enzyme mixture, which is not desirable when the enzyme is expensive or disturbs the separation and detection [8–11,27,28].

5. Conclusions

Inhibition of UGT has been studied by an on-line drug-metabolism system. Using this approach, the extent of inhibition can be analyzed using quantitative information that can be obtained from an electropherogram. Because the values were almost the same as those obtained by the conventional method, it can be said that the inhibition of glucuronidation was correctly estimated by this technique. This electrophoretic enzyme–inhibitor assay in microfabricated devices consumes 10^4 less sample and 10^3 less microsome per experiment compared to the conventional reaction schemes. The immobilization of microsomes in the capillary not only greatly simplified the procedures for the isolation and determination of drug metabolites from the reaction mixtures required in the free solutions but also enabled the repeated uses of microsomes. Because the sol–gel encapsulation technique is applicable to a variety of proteins by optimizing the encapsulation conditions, such as buffer pH, ionic strength, or alkoxysilane monomers [12–15], this enzyme–inhibitor system can be applied to various enzymes for drug discovery by encapsulating the enzymes into capillaries. Further studies will be continued for the transformation of this system into a microchip-based platform, which is required for higher performance and the development of a high-throughput analytical system.

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