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The covalent immobilization of microsomal uridine diphospho-glucuronosyltransferase (UDPGT): Initial synthesis and characterization of an UDPGT immobilized enzyme reactor for the on-line study of glucuronidation

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Abstract

The microsomal fraction of rat liver containing uridine diphospho-glucuronosyltransferase (UDPGT; EC 2.4.1.17) has been covalently immobilized on a high performance chromatographic support. In this study Nucleosil Si-500 silica was converted into diol-bonded silica and subsequently converted into an aldehyde form through oxidation with sodium periodate. The microsomal fraction was immobilized via Schiff base formation followed by reduction with sodium cyanoborohydride. The resulting immobilized enzyme reactor (IMER) was placed in a multi-dimensional chromatographic system which utilized a mixed mode (C_{18} and anion exchange) column to trap the parent compound and glucuronide and a C_{18} column to separate the substrate and product. The IMER system was used for the online glucuronidation of 4-methylumbelliferone (4Me7OHC) and acetaminophen (APAP). The Michaelis-Menten kinetic parameters (K_m and V_{max}) associated with the formation of 4Me7OHC and APAP glucuronides demonstrated that the immobilization had not significantly affected the enzymatic activity of the UDPGT relative to the non-immobilized enzyme. The IMER retained enzymatic activity for more than 6 weeks. The results of this study demonstrate an easy and convenient way to identify compounds which may be glucuronidated and to synthesize and characterize the resulting products.
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Keywords: Glucuronides; Immobilized enzyme reactors; Rat liver microsomes

1. Introduction

The glucuronidation of compounds by uridine diphospho-glucuronosyltransferase (UDPGT; EC 2.4.1.17) is a major detoxification pathway found in all vertebrates [1–4]. The UDPGTs are a superfamily of membrane bound enzymes that are responsible for transfer of the glucuronyl group from uridine 5′-diphosphoglucuronic acid (UDPGA) to available substrates forming β-D-glucuronides, Fig. 1. UDPGTs are localized primarily in the endoplasmic reticulum of liver and, to a lesser extent, in all other mammalian tissues such as kidney and intestine. More than 50 mammalian UDPGT isozymes (16 in human) have been identified and have been grouped into two families [1,2,4–6]. Several studies have shown that UDPGTs are differentially regulated and have distinct but often overlapping substrate specificities [6].

The addition of a glucuronyl group to a compound increases its polarity resulting in an enhanced partitioning into the aqueous intra- and extracellular compartments of the body. Glucuronidation facilitates the transport of lipophilic compounds to excretory organs and their subsequent elimination through the bile and urine [6,7]. Since β-D-glucuronides can be formed using a variety of functional groups, e.g. oxygen, nitrogen, sulphur and carbon, UDPGTs can play an

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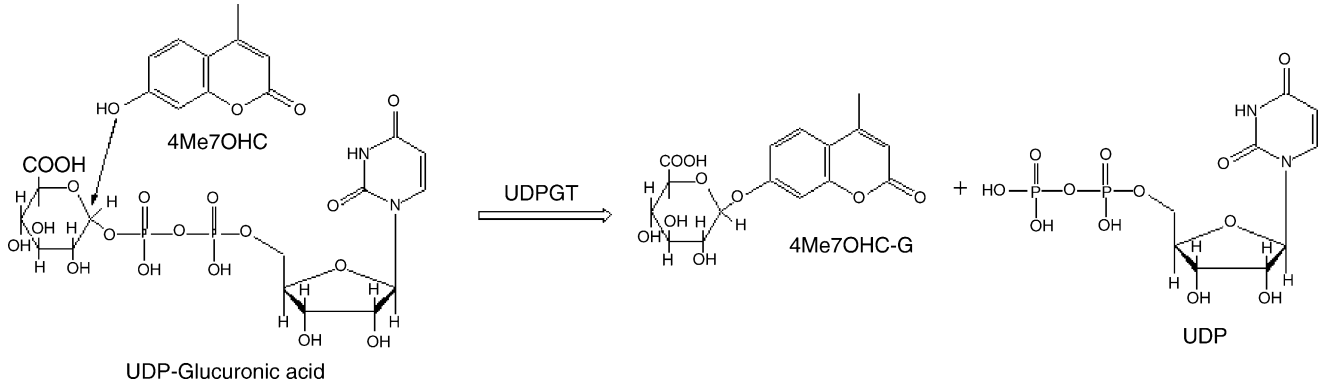

Fig. 1. General reaction scheme for glucuronidation of 7-hydroxy-4-methylcoumarin (4Me7OHC) by UDPGT.

important role in the metabolism and disposition of a wide variety of compounds [2,3,6–8]. Thus, the determination of whether these enzymes interact with parent molecules and Phase I metabolites is a key part of drug discovery and development programs.

The role that UDPGTs play in the metabolism and elimination of drugs has been the subject of numerous studies [1,3,7,9]. The majority of these studies involve the in vitro production of glucuronides in which substrates are incubated with UDPGA and microsomal fractions containing UDPGTs and the products are then analyzed using liquid chromatography [10–13]. Solubilized microsomal UDPGT has also been immobilized through covalent immobilization on Sepharose beads [14,15] and by entrapment into algenate beads in the presence of polyethyleneimine [16]. The resulting immobilized enzyme reactors (UDPGT-IMERs) retained enzymatic activity, but could not be used in-line in HPLC systems.

The production of an in-line UDPGT-IMER was accomplished through the immobilization of nonsolubilized rat liver microsomes on an immobilized artificial membrane (IAM) liquid chromatographic stationary phase [17]. The resulting UDPGT-IMER was active and could be used in-line for the production and identification of glucuronides. However, the IAM support is extremely hydrophobic and inefficient resulting in substantial nonspecific binding, which limits the utility of UDPGT-IMERs based upon this support.

In this study we report covalent immobilization of nonsolubilized rat liver microsomal UDPGT onto the surface of an activated diol-bonded silica liquid chromatographic support. The resulting UDPGT-IMER was coupled via a switching valve to a C_{18} column, which was used to separate and quantify the parent compound and the β-D-glucuronides. The results of the study demonstrate that the UDPGT-IMER was active and could be used for the on-line β-D-glucuronidation of 4-methylumbelliferone (4Me7OHC) and acetaminophen. In addition, the resulting multi-dimensional liquid chromatographic system can be coupled to a mass spectrometer and used for the rapid on-line screening of lead drug candidates.

2. Materials and methods

2.1. Materials

4-Methylumbelliferone (7-hydroxy-4-methylcoumarin, 4Me7OHC), 4-methyl-umbelliferone β-D-glucuronide, 4Me7OHCG), acetaminophen (*N*-acetyl-*P*-aminophenol, APAP), acetaminophen β-D-glucuronide (APAPG), trizma hydrochloride (Tris–HCl), magnesium chloride hexahydrate ($MgCl_2{\cdot}6H_2O$), uridine 5′-diphosphoglucuronic acid (UDPGA) and CHAPS (3-[(3-cholamidopropyl)-dimethylammonia]-1-propane sulfonate) were purchased from Sigma (St. Louis, MO, USA). Sodium periodate, sodium cyanoborohydride and sodium borohydride were purchased from Aldrich (Milwaukee, WI, USA). Acetonitrile (HPLC grade) was from Fisher (Pittsburgh, PA, USA). Protease inhibitor cocktail set III was obtained from Calbiochem (San Diego, CA, USA). The Nucleosil Si-500 (7 μm particle diameter, 500 Å pore size) was obtained from Machery Nagel (Düren, Germany). Reagents for the bicinchoninic acid (BCA) protein assay were obtained from Pierce (Rockford, IL, USA). All other chemicals were of the highest purity available. All aqueous solutions were prepared using water from a Milli-Q water system (Millipore, Billerica, MA, USA) and filtered using Osmonics 0.22 μm nylon filters purchased from Fisher.

2.2. Apparatus

A schematic diagram of the chromatographic system used in this study is depicted in Fig. 2. System 1 consisted of a Shimadzu (Columbia, MD, USA) LC-10AD isocratic pump, a Shimadzu SIL-10 AD auto-injector with a 20 μL sample loop, a Shimadzu FCV-12AH six-port 2 way position switching valve, a Shimadzu CTO-10AS column oven, an IMER column (30 mm × 4.6 mm I.D.) and a Primesep D mixed mode C_{18}/anion exchange column (10 mm × 4.6 mm I.D.) obtained from SIELC (Prospect Heights, IL USA). System 2 consisted of two Shimadzu LC-10AD isocratic pumps with a low-pressure mixer, an Alltech Prevail C_{18} Rocket

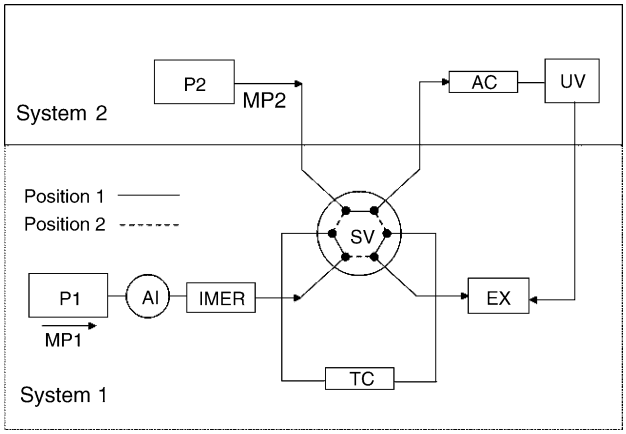

Fig. 2. Schematic diagram of chromatographic set-up for performing on-line glucuronidation and separation; where: P1—isocratic pump, P2—two isocratic pumps with low pressure mixer, MP1—mobile phase 1, MP2—mobile phase 2, AI—auto injector, SV—switching valve, IMER—immobilized enzyme reactor, TC—trapping column (in this experiment containing a mixed mode C_{18} and anion exchange phase), AC—analytical column (in this experiment a C_{18} column), UV–vis detector, EX—excess.

or Platinum C_{18} Rocket column (50 mm × 7 mm I.D.) and a Shimadzu SPD-10AV UV absorbance detector. Chromatographic data were collected and processed using Class-VP software Version 5.032 from Shimadzu. Empty columns used to prepare the IMER columns were purchased from Alltech and columns were downward slurry packed using an Alltech Slurry Packer (Deerfield, IL, USA). BCA protein assay was performed using a Model 680 microplate reader from BioRad (Hercules, CA, USA).

2.3. Isolation of rat liver microsome

The microsomal fraction from rat liver was isolated using a previously reported method [18], which was modified to meet the experimental requirements of this study. All steps in the isolation procedures were done at 4 °C unless stated otherwise.

Fresh rat liver was minced and homogenized in 30 ml of ice-cold potassium phosphate buffer (250 mM, pH 7.4) containing 150 mM KCl and 1ml protease inhibitor cocktail set III (Calbiochem). The mixture was homogenized for 30 s using a Polytron PT 2100 homogenizer at setting 15. The homogenate was centrifuged at 14,500 × *g* for 20 min using a Beckman XL 90 Ultracentrifuge, the supernatant was recovered, centrifuged again at 14,500 × *g* for 20 min, the resulting supernatant was further centrifuged at 100,000 × *g* for 60 min, and the subsequent supernatant was discarded. The remaining microsomal pellet was washed twice in 10 ml ice-cold potassium phosphate buffer (250 mM, pH 7.4) containing 20% glycerol. The pellet was resuspended in the same buffer (5 ml) and further homogenized with a Fisher glass micro tissue grinder. The rat liver microsome solution was aliquoted into 1 ml aliquots and stored at −80 °C until further use.

The disruption of UDPGT-containing membranes from the rat liver microsomal solution was accomplished using a modified version of the procedures described by Parikh et al. [19]. Briefly, the rat liver microsomal solution (1 ml) was combined with 9 ml of potassium phosphate buffer (67 mM, pH 7.4) in an ice bath and the resulting solution was sonicated for five bursts of 10 s each with 30 s intervals between each burst using a Fisher Sonic Dismembrator Model 100 at setting 3. The sonicated solution was centrifuged at 100,000 × *g* for 60 min and the supernatant containing UDPGT was removed and stored at −80 °C until further used.

2.4. Immobilization of rat liver microsome onto silica support

Nucleosil Si-500 silica was converted into a diol-bonded form according to a previous procedure [20]. This diol-bonded silica was then used in a Schiff base method for the immobilization of rat liver microsome or UDPGT. This was accomplished by first converting the diol-bonded silica into an aldehyde form through oxidation with sodium periodate [20], then 0.7 g of the aldehyde activated silica was transferred to 10 ml Falcon tube, suspended in 4 ml of potassium phosphate buffer (67 mM, pH 7.4) followed by the addition of rat liver microsomal solution (1 ml) and sodium cyanoborohydride (50 mg). This step was carried out in three different approaches:

(1) Sonication was used while mixing the silica and microsomal solution. In this approach, mixture was sonicated while in an ice bath for five bursts of 10 s each with 30 s intervals between each burst using Fisher Sonic Dismembrator Model 100 at setting 3 (Approach 1).
(2) CHAPS (10 mM in final concentration) was added to while mixing the silica and the microsomal solution (Approach 2).
(3) The silica and the microsomal solution were just gently agitated (Approach 3).

In addition, the solution obtained after the disruption of the UDPGT-containing membranes (see Section 2.3) was separately mixed with aldehyde silica in the same manner as the Approach 3 (Approach 4).

The immobilization reaction was allowed to proceed for 3 days at 4 °C. The silica was then washed with potassium phosphate buffer (67 mM, pH 7.4) and treated with three portions of 10 mg sodium borohydride to convert the excess aldehyde groups on the support into alcohols. The support was then washed several times with potassium phosphate buffer (67 mM, pH 7.4) and stored in this buffer at 4 °C until use.

A control support was prepared by performing the Schiff base method on a separation portion of the diol-bonded silica, but with no rat liver microsomal solution added during the immobilization step. This control material was washed and stored in the same manner as the immobilized rat liver microsome support.

A 0.25 ml portion of either the silica containing the immobilized microsomal solution or the control support was washed several times with deionized water (1.5 ml each) using Eppendorf centrifuge (Model 5415) and dried under vacuum at room temperature. The dried samples were weighed and analyzed in triplicate using a BCA protein assay kit in which BSA was the standard and the control silica acted as the blank. Briefly, test samples were prepared by adding 10 μl of the microsomal solution to 490 μl of potassium phosphate buffer (67 mM, pH 7.4) or by adding dried silica samples (∼1.2 mg) to 500 μl of the same buffer. In addition, 500 μl BSA standard solutions were prepared in concentrations ranging from 0 to 40 μg/ml. The solutions were combined with 500 μl of BCA working reagent and then incubated for 60 min at 60 °C. The incubated samples were then cooled in ice-cold water for 10 min and the absorbance of each solution was determined at 560 nm by using the microreader plate.

The IMER silica and control support were downward slurry packed at 2000 psi (122 bar) into 30 mm × 4.6 mm I.D. stainless steel columns using Tris–HCl buffer (50 mM, pH 7.4) containing 10 mM $MgCl_2$ as the packing solvent.

2.5. On-line production of glucuronides with IMER

Stock solutions (100 mM) of 4Me7OHC and APAP were prepared in 100% methanol and diluted with Tris–HCl buffer solution (50 mM, pH 7.4) to produce concentrations of 10, 20, 40, 60, 80 and 100 μM while APAP was diluted with water to produce concentrations 1, 2.5, 7.5, 10 and 25 mM. A 10 μl aliquot of each of the 4Me7OHC samples was added to 990 μl of 5 mM UDPGA in M Tris–HCl buffer (50 mM, pH 7.4) supplemented with 10 mM of $MgCl_2$. A 20 μl sample of the resulting solution was injected into the chromatographic system with the switching valve set a position 1 (System 1, Fig. 2). In this configuration, the eluent from the IMER was directed to a trapping column (TC) where the glucuronides and parent compounds were retained. The TC was a Primesep D mixed mode C_{18}/anion exchange column.

The effect of flow rate on the activity of the IMER was investigated at flow rates at 0.1, 0.167, 0.25, 0.5, 1 and 2 ml/min (4Me7OHC) and 0.05, 0.1, 0.2, 0.5 and 1 ml/min (APAP). The temperature of IMER was set to 37 °C using Shimadzu CTO-10AS column oven.

The same procedure was followed when the glucuronidation of APAP was studied except for the composition of the buffer, which was Tris–HCl (20 mM, pH 7.4) supplemented with 5 mM $MgCl_2$.

2.6. Separation of glucuronides and parent compounds

After a set time, the switching valve was rotated to position 2 (Fig. 2, System 2) and the glucuronides and parent compounds retained in the TC were eluted onto a C_{18} column at 1.0 ml/min with initial HPLC mobile phase composition (see below). The glucuronides and parent compounds were then separated and quantified using a gradient elution condition. This was demonstrated in Fig. 2 (System 2, switching valve position 2). The analytical separations were carried out at room temperature using a flow rate of 1 ml/min and the analytes were detected using a UV detector set at λ = 320 nm for 4Me7OHC/4Me7OHCG and λ = 260 nm for APAP/APAPG.

All mobile phases for the chromatographic studies were degassed at least 15 min prior to use. The initial HPLC elution conditions for separation of 4Me7OHC and 4Me7OHCG were 95% of a 0.1% ammonium acetate in water (mobile phase A) and 5% of 100% acetonitrile (mobile phase B). The concentration of mobile phase B was increased to 30% over 9 min and continued for additional 8 min. Then the mobile phase composition was recycled back to the initial HPLC condition within 3 min.

For the separation of APAP and APAPG, the initial HPLC conditions were 100% of a 0.50% formic acid in water (mobile phase A). The concentration of mobile phase B (100% acetonitrile) was increased to 30% over 7 min and continued for additional 8 min. Then the mobile phase composition was recycled back to the initial HPLC condition within 5 min.

The quantifications of 4Me7OHCG and APAPG were achieved by comparing to the absorbance of a standard curve for 4Me7OHCG and APAPG. The standard solutions of 4Me7OHCG and APAPG were prepared in water in triplicate at concentrations of 2.5, 5.0, 10, 25, 50, 100 μM (4Me7OHCG) and 5, 10, 20, 50, 100, 200 μM (APAPG), respectively. The data was analyzed using GraphPad Prism 4 software and linear regression lines were obtained for 4Me7OHCG with an $r^2 = 0.994$ ($p = 0.0001$) and for APAPG with an $r^2 = 0.9998$ ($p = 0.0001$). Sample solutions containing 4Me7OHCG and APAPG were prepared daily.

2.7. Production of glucuronides with non-immobilized microsomes

The glucuronidation of 4Me7OHC and APAP using non-immobilized rat liver microsomes was performed in similar manner as glucuronidation within IMER. In this method, a 1.0 ml reaction mixture containing 10 μl of 4Me7OHC or APAP (0.5 mM to 10 mM), 10 μl of rat liver microsome solution (39.12 mg/ml protein) and 0.980 ml of 5 mM UDPGA dissolved in Tris–HCl buffer (50 mM, pH 7.4) supplemented with 10 mM of $MgCl_2$ was incubated at 37 °C for 0.5 min (4Me7OHC) and 20 min (APAP). After incubation, the reaction was stopped by the addition of 0.5 ml of a solution acetonitrile:glacial acetic acid (94:6, v/v) and centrifuged at 15,000 × *g* for 5 min. The supernatant solution was carefully removed and 20 μl of this solution was analyzed directly on the C_{18} analytical column, System 2 (Fig. 2).

2.8. Kinetic analysis of glucuronide formation from immobilized and non-immobilized enzymes

Enzymatic parameters associated with the production of the glucuronides were evaluated by injecting 20 μl of

4Me7OHC and APAP (5 to 100 μM in final concentrations) in 1 ml UDPGA (5 mM) solution containing $MgCl_2$ (10 mM) in pH 7.4, 50 mM Tris–HCl buffer at different flow rates (0.05–2.0 ml/min) which correspond to incubation times of 0.21–8.4 min. The amount of glucuronides produced was quantified by comparing to standard curves of corresponding glucuronides. The Michaelis-Menten constants K_m and V_{max} were calculated from the data using Lineweaver-Burk reciprocal plots.

3. Results and discussion

3.1. General characteristics of IMER

The amount of protein immobilized on the activated silica support varied for the four different immobilization approaches and ranged from 14.5 (Approach 3) to 40.8 (Approach 4) mg/g silica, Table 1. In Approach 3, the integrity of the initially obtained microsomal membranes remained virtually intact. The low amount of total protein immobilized on the column most likely reflects the ratio between membrane proteins and membrane lipids and the effect these lipids have on the accessibility of protein coupling sites on the activated silica. Approach 4 used a solution in which the initially obtained microsomal membranes had been extensively disrupted. The three-fold increase in total immobilized protein indicates that this procedure had substantially reduced the amount of lipids associated with the immobilized protein and, therefore, the steric effect of these lipids.

The amount of protein immobilized onto the activated silica was similar when sonication (Approach 1) or addition of CHAPS detergent (Approach 2) was performed while mixing the microsomal solution with the activated silica. In both cases the amount of immobilized protein was significantly greater than the amount obtained using Approach 3 and slightly less than the amount achieved with Approach 4, Table 1. This is probably caused by the gentile disturbance of the membrane structure (i.e., decreasing overall size of membrane) in both methods, which resulted in a higher number of protein and membrane complexes accessible to possible coupling sites on the silica.

The silica supports produced using Approaches 1–4 were used to created IMER-1, IMER-2, IMER-3 and IMER-4, respectively. The activities of the immobilized UDPGTs contained within the IMERs were compared by determining the specific activities of the enzymes, which was defined as the nmoles of 7OHMeCG produced per mg of immobilized protein, Table 1. Although the amount of protein immobilized was the highest when the microsomal membranes were extensively disrupted (Approach 4), the specific activity of the resulting IMER-4 was the lowest. In contrast, there was no significant difference in the specific activity of IMER-1, IMER-2 and IMER-3, even though there was ∼50% less protein immobilized on IMER-3. The data indicate that membrane lipids play a significant role in maintaining UDPGT activity and their integrity should be maintained during the immobilization process.

The importance of maintaining membrane integrity was also demonstrated by the stability of the IMERs, which was evaluated over a 45 day period using 4Me7OHCG formation as the probe. All four IMERs were used equally over the evaluation periods and all four IMERs showed decreasing specific activity during this period, Fig. 3. The greatest loss of activity was observed with IMER-4, which was essentially inactive by Day 20. IMER-1 retained ∼75% of its initial activity over the 45-day period, while IMER-2 and IMER-3 retained 47% and 53% of their initial activity, respectively. While the reason for the loss of UDPGT activity is currently not known, the results suggest that the integrity of the membrane is lost over time which could be due to mechanical disruption caused by the friction associated with the flowing mobile. No attempt was made to improve overall stability of enzyme activity in IMER.

Immobilization Approach 1 was chosen to be the optimum approach for preparing rat liver microsomal IMER. This approach showed the highest specific activity and stability for 4Me7OHCG formation. IMERs prepared using Approach 1 were used for all of the other experiments performed in this study. Several IMERs were prepared using Approach 1. These IMERs retained similar UDPGT enzyme activities for formation of 4Me7OHCG even if they were used at different times during a 3 month period. Given the fact that the activity of the immobilized UDPGT decreases over the time, it is advisable to calibrate the enzymatic on a weekly basis. In this study, the glucuronidation of 4Me7OHC was used as the probe as it is a probe for multiple UDPGT isoforms [6]. If a specific isoform was of interest, then a specific probe could

Table 1
General characteristics of the IMERs studied in this project where: IMER-1 was synthesized using Approach 1, IMER-2 was synthesized using Approach 2, IMER-3 was synthesized using Approach 3, IMER-4 was synthesized using Approach 4

IMER	Amount of protein (mg protein/g silica)	Initial activity UDPGT (nmol, 4Me7OHC-G/g silica)[1]	Specific activity UDPGT (nmol, 4Me7OHC-G/mg protein)
IMER-1	30.4 (±2.1)	2.36 (±0.02)	0.078 (±0.006)
IMER-2	36.2 (±2.8)	2.26 (±0.02)	0.062 (±0.005)
IMER-3	14.5 (±2.3)	0.92 (±0.04)	0.063 (±0.013)
IMER-4	40.8 (±3.4)	1.16 (±0.03)	0.028 (±0.003)

The initial activity of IMER column was obtained by on-line production of 7-hydroxy-4-methylcoumarin glucuronide (4Me7OHC-G) using an incubation flow rate of 0.25 ml/min at 37 °C. The values in parentheses represent a range of ±1 S.D. with an $n = 3$.

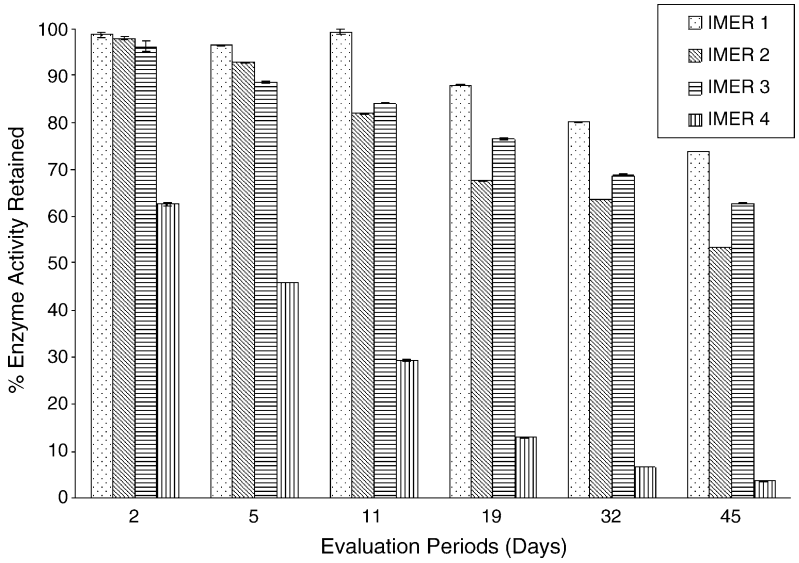

Fig. 3. The stabilities of UDPGT IMERs prepared by the immobilization approaches utilized in this study; where: the stability is expressed as percent of the initial specific activity (set at 100%) of the immobilized UDPGT determined by the conversion of 7-hydroxy-4-methylcoumarin (4Me7OHC) to 7-hydroxy-4-methylcoumarin glucuronide (4Me7OHCG), and where IMER-1 was produced using immobilization approach 1, IMER-2 was produced using immobilization approach 2, IMER-3 was produced using immobilization approach 3, IMER-4 was produced using immobilization Approach 4.

be used. For example, bilirubin is specifically glucuronidated by UDPGT 1A1 and this substrate could be utilized to specifically probe the activity of this isoform. The determination of specific isoform activity is currently under investigation in this laboratory and the results will be reported elsewhere.

3.2. On-line extraction of glucuronides

A key component of the on-line analysis of UDPGT activity was the trapping of the substrates and glucuronides after their elution from the IMER. This is complicated by the difference in the polarities between the substrate and product produced by the addition of a glucuronyl group by the UDPGT. In the chromatographic system used in this study, the substrates and products were trapped using a mixed mode C_{18}/anion exchange column and the results are presented in Fig. 4.

The benefit of using a mixed mode column is illustrated by the observation that 4Me7OHC, 4Me7OHCG and APAP could be retained using a C_{18} trapping column. However, APAPG was not retained on this column, most probably due to its low hydrophobicity. An alternative approach to the retention of glucuronides is to utilize the carboxylic acid moiety present in the glucuronyl group by employing an anion exchange column. When an anion exchange extraction column was used as the trapping column, APAPG and 4Me7OHCG were efficiently extracted, but APAP and 4Me7OHC were not.

3.3. Separation of glucuronides and parents compounds

The eluents from the trapping column were directed onto the C_{18} column where the glucuronides and parent compounds were separated using the chromatographic system labeled as System 2 in Fig. 2. Under the chromatographic conditions used in this study, the retention times of 4Me7OHCG and 4Me7OHC were 3.8 and 12.0 min, respectively, Fig. 5A, and the retention times of APAPG and APAP were 3.8 and 11.6 min, respectively, Fig. 6A. The total analysis time including glucuronide formation in the IMER was less than 30 min depending on incubation flow rates and the retention times varied less than 0.5% over the course of study.

The retention times of APAPG and 4Me7OHCG were confirmed by injecting standard solutions of both compounds. In addition control experiments were conducted by injecting the parent compound without the UDPGA cofactor and the resulting chromatograms contained only the peaks corresponding to the parent compounds (Figs. 5B and 6B) and

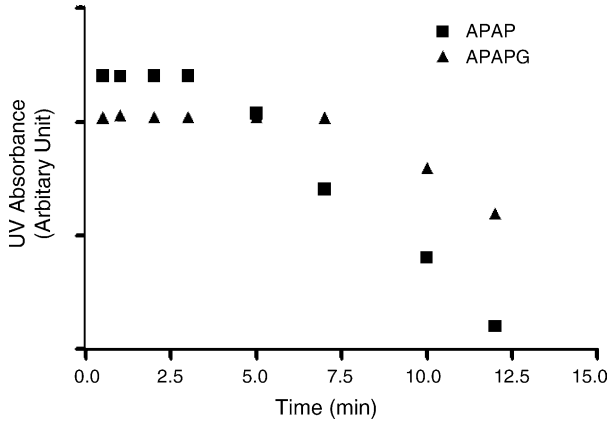

Fig. 4. The extraction profiles of *N*-acetyl-*P*-aminophenol (APAP) and *N*-acetyl-*P*-aminophenol glucuronide (APAPG) on a mixed mode (C_{18} and anion exchange) extraction column.

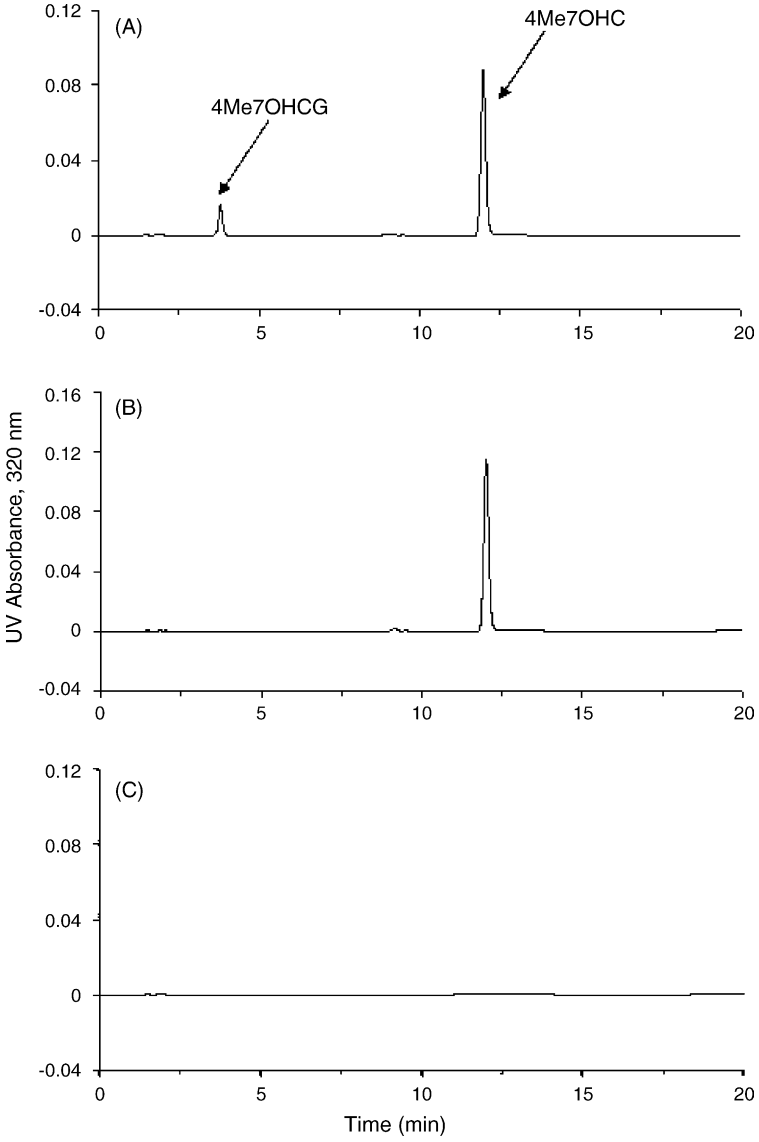

Fig. 5. Representative chromatograms on of 7-hydroxy-4-methylcoumarin (4Me7OHC) and 7-hydroxy-4-methylcoumarin glucuronide (4Me7OHCG) eluted from UDPGT-IMER. (A) IMER incubation containing 4Me7OHC and UDPGA. (B) IMER incubation containing only 4Me7OHC. (C) IMER incubation containing only UDPGT.

by injecting only the buffer, which produced chromatograms containing no observable peaks (Figs. 5C and 6C).

3.4. Comparison of the enzymatic activities of the immobilized and non-immobilized UDPGT enzymes

The enzymatic activities of the immobilized and non-immobilized UDPGT enzymes were compared through the determination of the Michaelis-Menten kinetic parameters, K_m (affinity if the substrate) and V_{max} (velocity of the reaction) for the formation of APAPG and 4Me7OHCG. The initial experiments investigated the effect of flow rate on the production of APAPG and 4Me7OHCG in order to determine the best parameters for the calculation of the initial velocities, i.e. the linear portions of the kinetic curves. Flow rates through the IMER from 0.05 to 2.00 ml/min were used and corresponded to substrate-enzyme contact times ranging from 8.40 to 0.21 min. With both APAP and 4Me7OHC, the production of glucuronides leveled off as the contact time increased, Fig. 7. This behavior was observed for all substrate concentrations used in this study.

The initial velocities used in the Lineweaver-Burke plots used to calculate K_m and V_{max} were determined using the slope of curve produced by contact times of 0.21–2.5 min for 4Me7OHGC and 0.21–4.70 min for APAPG. Accordingly, Lineweaver-Burke reciprocal plots were constructed for the production of 4Me7OHCG and APAPG and were linear over the study time with $r^2 = 0.9982$ ($n = 6$) and $r^2 = 0.9996$ ($n = 6$), respectively. The kinetic parameters obtained from the Lineweaver-Burke plots for the immo-

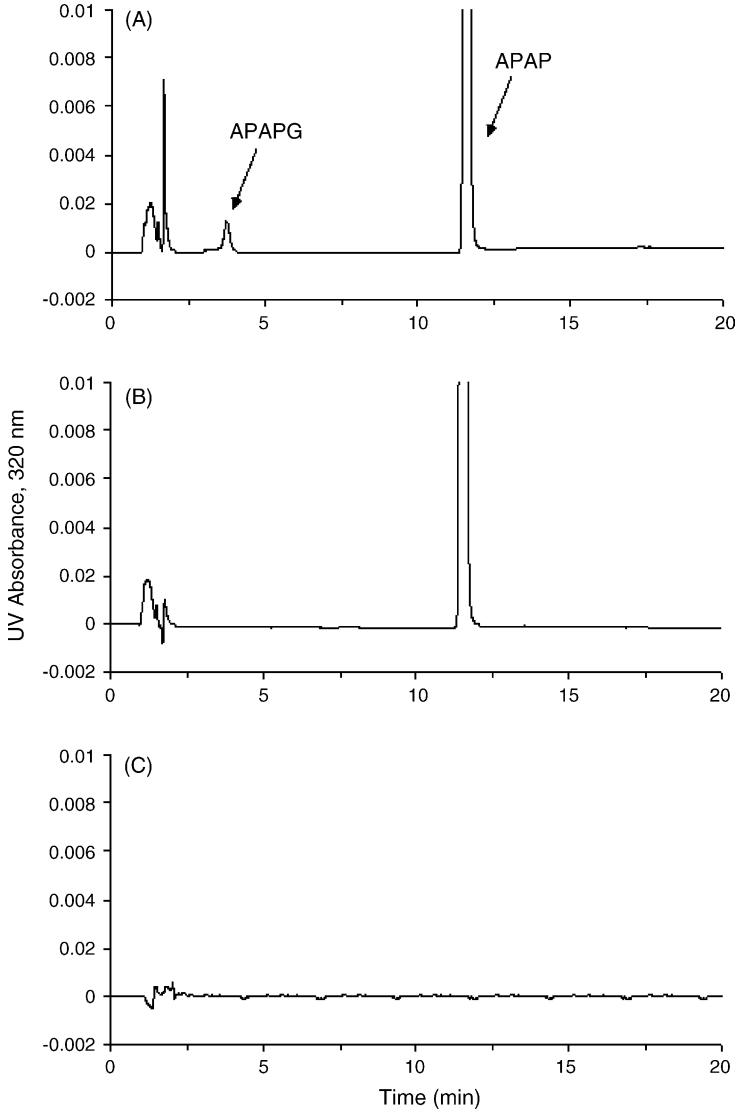

Fig. 6. Representative chromatograms on of *N*-acetyl-*P*-aminophenol (APAP) and *N*-acetyl-*P*-aminophenol glucuronide (APAPG) eluted from UDPGT-IMER. (A) IMER incubation containing APAP and UDPGA. (B) IMER incubation containing only APAP. (C) IMER incubation containing only UDPGT.

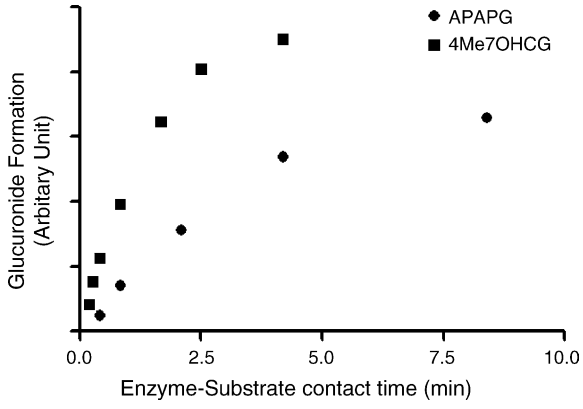

Fig. 7. The effect of enzyme-substrate contact times (flow rates) on glucuronidation formation profiles of 4Me7OHCG and APAPG.

bilized and non-immobilized UDPGT are presented in Table 2.

The immobilization of the UDPGT appeared to increase the K_m values for 4Me7OHCG and APAPG relative to soluble enzymes. In the case of 4Me7OHC, there was a 1.18-fold

Table 2
The Michaelis-Menten kinetic parameters, K_m and V_{max}, determined for the glucuronidation of 7-hydroxy-4-methylcoumarin (4Me7OHC) and *N*-acetyl-*P*-aminophenol (APAP) by UDPGT immobilized in IMER-1 (created using immobilization approach 1) and by non-immobilized UDPGT

Substrates	K_m	V_{max} (nmol/min/mg protein)
4Me7OHC (IMER-1)	217 (±38) μM	2.8 (±0.5)
4Me7OHC (non-immobilized)	184 (±21) μM	1.3 (±0.4)
APAP (IMER-1)	23 (±3) mM	0.82 (±0.07)
APAP (non-immobilized)	17 (±3) mM	0.29 (±0.04)

The values in parentheses represent a range of ±1 S.D., where $n = 3$.

difference and for APAPG the difference was about 1.35-fold. These differences are relatively small and do not reach statistical significance. It appears that the immobilization of UDPGT on IMER format does not significantly change the ability of the substrate and cofactor to access the enzyme or their ability to bind to the enzyme. Similar trends have been seen in other immobilized enzyme systems; for example, the K_m values for both L-glutamine and D-glutamine on the glutamine synthetase IMER were increased by a factor of 2.5 compared to soluble glutamine synthetase [21].

The V_{max} values obtained with 4Me7OHC and APAP on IMER were increased relative to the results from the non-immobilized enzyme, Table 2. As observed with the K_m values, the calculated differences were less than a three-fold difference and suggest that although there was a difference in the V_{max} values, the immobilization did not significantly affect the activity of the UDPGT. This trend has been previously observed with other IMER systems [22–24]. The differences in the calculated V_{max} values may be due to the experimental format. The UDPGT catalyzed enzymatic conversion is a tri-molecular process requiring substrate, cofactor and enzyme. In the on-line format, the substrate and cofactor are injected together as a bolus and travel down the column in a concentration gradient, while reactions with the non-immobilized enzyme are carried out in a solution. The chromatographic conditions should increase the probability that the substrate and cofactor will encounter the UDPGT, and therefore increase the calculated V_{max}, relative to the solution producing the observed variation in the enzymatic activity.

4. Conclusions

This study describes a method for the on-line production and analysis of glucuronides using an IMER based upon immobilized rat liver microsomes, which can be used for the online screening of lead drug candidates and their metabolites. The compounds used to establish the enzymatic activity of the immobilized UDPGTs, 4Me7OHC and APAP, are well-characterized substrates of this class of enzymes. However, they are also substrates of multiple UDPGT isoforms; 4Me7OHC is a substrate for UGT 1A3, 1A6, 1A2, 1A9 and 2B15 while APAP is a substrate for UGT 1A6 and 1A9 [6]. Thus, while this study has established that the IMER contains active UDPGTs, the data does not allow for the determination of which UGT isoforms are active. This will be approached using multiple isoform-specific substrates and will be the subject of a later report.

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