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Accelerated Articles

On-Line Drug Metabolism in Capillary Electrophoresis. 1. Glucuronidation Using Rat Liver Microsomes

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A rat liver microsome pseudostationary phase has been used for the on-line capillary electrophoresis monitoring of glucuronidation. Uridine diphosphate glucuronosyltransferase (EC 2.4.1.17) containing microsomes was isolated from rat liver and directly injected onto neutrally coated capillary containing polymeric replaceable gels followed by injection of the substrate mixture. On-line glucuronidation was observed within 15 min without any sample preparation. The factors affecting the separation of glucuronides and parent compounds were investigated by varying the applied electric fields and the size (length and internal diameter) of capillary. The Michaelis−Menten parameters (K_m and V_{max}) for the glucuronidation of 4-methyl-7-hydroxy coumarin and 4-nitrophenol were determined using the CE method and by off-line microsomal incubation. No significant differences were observed for K_m and V_{max} values for 4-methyl-7-hydroxycoumarin and 4-nitrophenol between on-line and off-line glucuronidation of these two compounds. This method was also used to determine the inhibition constant (IC_{50} value) for the competitive inhibition of morphine glucuronidation by codeine, IC_{50} (on-line) = 170 vs 580 μM (off-line). The results demonstrate that this method can be used to screen for the glucuronidation of test compounds and should reduce the time required for this screening process.

Glucuronidation is a key phase II metabolic process that plays a major role in the clearance of many endogenous and xenobiotic compounds.[1−4] The process is mediated by uridine diphosphate glucuronosyltransferases (UDPGTs; EC 2.4.1.17), an enzyme superfamily that catalyzes the conjugation of uridine diphosphate glucuronic acid (UDPGA) to nucleophilic functional groups on substrate molecules. This process transforms lipid-soluble molecules into water-soluble molecules, which are excreted from the body.

UDPGTs are found in the membranes of the endoplasmic reticulum and show a broad tissue distribution with the liver being a major site. Interindividual variation in UDPGT expression plays a role in drug efficacy and xenobiotic toxicity, as well as in hormonal regulation and certain diseases such as Crigler−Najjar syndrome.[5,6] Since glucuronidation affects systemic exposure, efficacy, and toxicity, it is important to assess the role that this metabolic route will have on new drug candidates early in the preclinical drug development stage.

The traditional approach to the assessment of the role glucuronidation plays in the metabolism of a target compound involves off-line incubations with microsomal preparations or hepatocytes followed by chromatographic separation and identification of the products.[7−9] Several on-line liquid chromatography methods have

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been reported in which microsomal UDPGTs were immobilized on chromatographic supports to create immobilized enzyme reactors (IMERs).[10−13] The on-line use of UPDGT-IMERs typically reduced analysis time and labor and allowed for the reuse of the UDPGTs, but required a number of steps to prepare. In addition, in order to separate substrates and products, it was necessary to couple the UDPGT-IMERs to analytical columns.

The use of capillary electrophoresis (CE) is an alternative approach to the on-line formation and monitoring of glucuronidation. CE generally requires small amounts (nL range) of reagents and provides relatively fast analysis times with highly efficient separations. An on-column immobilized enzyme microreactor has been used with CE in several studies.[14−16] Sakai-Kato et al. have reported a miniaturized on-line glucuronidation system based on encapsulation and immobilization of UDPGTs on monolithic capillary columns.[17] The columns were placed in a CE system and used to carry out enzymatic glucuronidations. However, this method required an on-line incubation of 10−90 min, which was accomplished by stopping the electroosmotic flow. Similar approaches were also reported by Righetti and co-workers using enzymes that were isoelectrically trapped in membranes and enzymatic products were later migrated to the detection window by differential charges.[18,19] In these reports, the reactions were not carried out under the conditions of an applied electric field or continuous flow.

We now report the development of an on-line capillary electrophoresis method for the rapid production and separation of glucuronides that does not require an incubation period. In this approach, rat liver microsomes were used with a replaceable polymeric run buffer to create a pseudostationary phase in linear polyacrylamide-coated capillary column. The substrates and necessary cofactors were then injected onto the system and the glucuronides were formed and separated. The results of this study demonstrate that this technique can be applied to the screening of new drug candidates and should reduce overall analysis time and increase throughput. This approach can be expanded to other microsomal and membrane-bound enzymes.

EXPERIMENTAL SECTION

Reagents. Morphine, morphine glucuronides (3 and 6), codeine, codeine 6-glucuronides, 4-nitrophenol (4NP), 4-nitrophenol β-D-glucuronide (4-NPG), 4-methylumbelliferone (4-methyl-7-hydroxycoumarin, 4Me7OHC), 4-methylumbelliferone β-D-glucuronide (4Me7OHCG), boric acid, Trizma hydrochloride (Tris-HCl), tris(hydroxymethyl)aminomethane, magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), and uridine 5′-diphosphoglucuronic acid (UDPGA) were purchased from Sigma-Aldrich (St. Louis, MO). Ammonium persulfate, (γ-methacryloxypropyl)trimethoxysilane, polyacrylamide (average M_w ~10 000), acrylamide, and TEMED were purchased from Aldrich (Milwaukee, WI). Methanol (HPLC grade) was from Fisher Scientific (Pittsburgh, PA). Protease inhibitor cocktail set III was obtained from Calbiochem (San Diego, CA). Reagents for the bicinchoninic acid (BCA) protein assay were obtained from Pierce Chemical Co. (Rockford, IL). All other chemicals were of the highest purity available. All aqueous solutions were prepared using water from a Millipore Milli-Q water system (Billerica, MA) and filtered using Osmonics 0.22-μm nylon filters purchased from Fisher Chemical Co.

Capillary Electrophoresis. CE was performed on a Beckman P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Inc., Fullerton, CA) controlled by 32 Karat software (Version 5.0, Beckman Coulter, Inc.). The CE instrument was equipped with PDA detector. The electrophoretic separations were performed in a 100-μm-i.d., 40-cm-total length (30 cm to the detector window) neutrally coated columns. The preparation of neutrally coated capillary (i.e., linear polyacrylamide coating) was followed.[20] The bare silica capillary was obtained from Polymicro Technologies (Phoenix, AZ). The capillary was cut with capillary cutting tool, and a capillary window (0.3 cm) was prepared by burning with flame and removing burned residues. The capillary was filled with 0.1 M NaOH for 3 h and rinsed with water to maximize free silanols on capillary surface. The capillary was again filled with 0.1 M HCl for 5 min and rinsed with water. Finally the capillary was filled with 3% (v/v) (γ-methacryloxypropyl)trimethoxysilane in 60% (v/v) acetone/water mixture for overnight to neutralize the capillary surface. The silane reagent attached to the capillary was further reacted with acrylamide (3%, w/v) in the presence of TEMED (0.8 μL/mL) and ammonium persulfate (2 mg/mL) in water. The capillary was sealed for 8 h after which the unreacted reagents were removed with water. CE Separation polymeric run buffer was prepared by mixing 80% (v/v) polyacrylamide, 0.1% (w/v) acrylamide, 10% (w/v) tris(hydroxymethyl)aminomethane, and 10% (w/v) boric acid in water.

Microsomal Fraction Preparation. The UDPGT-containing microsomal fraction of rat liver was isolated using a previously reported method,[21] which was modified to meet the experimental requirements of this study. All steps in the microsomal fraction preparation were done at 4 °C unless stated otherwise. Briefly, fresh liver from an 8-week-old Sprague−Dawley rat was minced and rinsed several times with 50 mL of ice-cold potassium phosphate buffer (homogenized buffer, 250 mM, pH 7.4) containing 150 mM KCl to remove excess blood. The minced liver was placed in 30 mL of ice-cold homogenized buffer with 1 mL of protease inhibitor cocktail set III. The minced liver mixture was homogenized for 30 s using a Polytron PT 2100 homogenizer (Kinematica AG, Luzern, Switzerland) at setting 11. The homogenate was centrifuged at 14500*g* for 20 min using a Beckman XL 90 Ultracentrifuge (Beckman Coulter, Inc.), the supernatant was recovered, centrifuged again at 14500*g* for 20 min, the resulting supernatant was further centrifuged at 100000*g* for 60

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min, and the subsequent supernatant was discarded. The remaining microsomal pellet was washed twice in 10 mL of 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 microtissue grinder (Fisher Scientific). The rat liver microsome solution was aliquoted into 1-mL aliquots and stored at −80 °C until further use.

Off-Line Glucuronidation. The glucuronidation of 4Me7OHC and 4-NP using off-line incubation was performed in a typical enzymatic incubation system.[22−24] In this method, a 120-μL reaction mixture containing 10 μL of 4Me7OHC or 4-NP (0−0.1 mM), 10 μL of rat liver microsome solution (28.12 mg/mL protein), and 100 μL of 10 mM UDPGA dissolved in Tris-HCl buffer (50 mM, pH 7.4) supplemented with 10 mM $MgCl_2$ was incubated in a 1.5-mL Eppendorf tube at 37 °C for 10 (4Me7OHC) or 15 min (4-NP). After incubation, the reaction was stopped by the addition of 80 μL of a solution of acetonitrile/glacial acetic acid (94:6, v/v) and centrifuged at 15000*g* for 5 min. The supernatant solution was carefully removed and analyzed in the CE system with the 50 mM borate buffer (pH 9.0) as the CE separation buffer. The quantifications of 4Me7OHCG and 4-NPG were performed by comparing to the absorbance of a standard curve for 4Me7OHCG and 4-NPG. The standard solutions of 4Me7OHCG and 4-NP were prepared in water in triplicate at concentrations of 0, 5.0, 10, 25, 50, 100, 500, and 1000 μM. The glucuronide peak area was calculated using 32 Karat (version 5.0) software from Beckman Instruments and the data were analyzed using GraphPad Prism 4 software (GraphPad Software, Inc., San Diego, CA). Sample solutions containing 4Me7OHCG and 4-NPG standards were prepared daily.

On-Line Glucuronidation. Prior to separation, the capillary was rinsed with CE separation polymeric run buffer at pressure of 20 psi for 5 min. A 24-nL aliquot of the UDPGT-containing microsomal mixture, representing 674.9 ng of total protein obtained by BCA protein assay, was applied into the capillary at a pressure of 1 psi for 15 s. Stock solutions (100 mM) of 4Me7OHC and 4-NP were prepared in 100% methanol and diluted with 100% methanol to produce concentrations of 0.75, 1, 2.5, 5, 10, 25, and 50 mM. A 10-μL aliquot of each of the 4Me7OHC or 4-NP samples was added to 90 μL of 10 mM UDPGA in Tris-HCl buffer (50 mM, pH 7.4) supplemented with 10 mM $MgCl_2$. A 10-nL sample of the mixture solution was injected into the capillary with a pressure injection at 0.5 psi for 10 s. The separation of 4Me7OHC or 4-NP, the respective glucuronide, UDPGT, and any interfering compounds was accomplished using an applied voltage of 9 kV unless stated otherwise, and this voltage produced roughly 26 μA of current. The capillary cartridge temperature was kept at 37 °C unless stated otherwise. Assuming the electrophoretic velocity of microsome was negligible compared to that of a small substrate under the above experimental conditions, the on-line enzyme reaction time was approximated to be 12.7 s for 4Me7OHC and 17.1 s for 4-NP from the microsome plug length (6 mm in a 40-cm capillary column) and velocities of substrates in a 40-cm capillary column using an applied voltage of 9 kV. The online glucuronidation of 4Me7OHC and 4-NP was monitored at 310 nm.

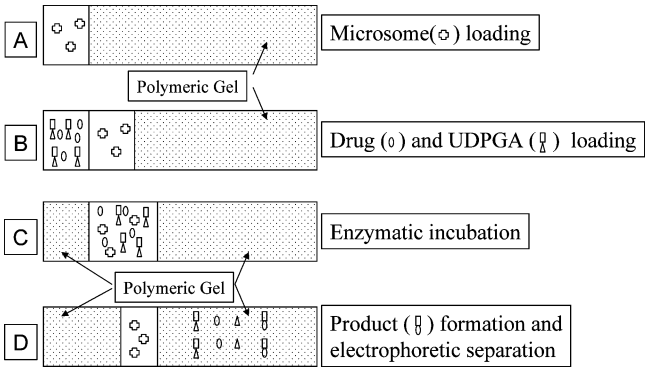

Figure 1. Principle of capillary gel electrophoresis enzymatic glucuronidation and separation based on rat liver microsome as pseudostationary phase.

Competitive Inhibition Study. On-line and off-line competitive inhibition studies of morphine and codeine were performed in a manner similar to that of the glucuronidation of 4Me7OHC and 4-NP except one of two compounds (either morphine or codeine) was added to the UDPGA solution. For off-line inhibition studies, a 100-μL reaction mixture containing 10 μL of morphine or codeine (0−100 mM), 10 μL of rat liver microsome solution (39.12 mg/mL protein), and 100 μL of 10 mM UDPGA dissolved in incubation buffer (See above) with varying amounts of morphine or codeine incubated in a 1.5-mL Eppendorf tube at 37 °C for 30 min. After incubation, the reaction was stopped by the addition of 80 μL of a solution of acetonitrile/glacial acetic acid (94:6, v/v) and centrifuged at 15000*g* for 5 min. The supernatant solution was carefully removed and analyzed in the CE system in the same manner as the off-line glucuronidation study discussed above.

For on-line inhibition studies, varying amounts of morphine or codeine were added to the UDPGA-containing incubation buffer and the resulting solution was applied with pressure injection at 1.0 psi for 15 s after the initial application of the CE separation gel buffer. Unless stated otherwise, all other experimental procedures were the same as described above.

RESULTS AND DISCUSSION

Development of the On-Line CE System. The general approach used in this study is presented in Figure 1. In this method, the microsomal preparation was injected at the inlet (cathodic side) of the capillary followed by a mixture of substrate and UDPGA. The viscous CE separation buffer acts as a sieving medium, and the relative mobility of the microsomes is negligible relative to the substrate and UDPGA. Thus, the substrate and UDPGA migrate through the microsomal mixture during which the substrate is glucuronidated. The substrate, UDPGA, and glucuronide are then resolved as they migrate to the outlet (anodic side) of the capillary.

The initial studies utilized an unmodified fused-silica capillary column and either an aqueous run buffer (sodium phosphate buffer 50 mM, pH 7.0) or a polymeric run buffer (See Experimental Section). With both buffers, the run-to-run variation of migration times for the glucuronides and parent compounds was too large to perform peak identification. This was expected since proteins tend to be adsorbed onto an unmodified capillary surface (negatively charged surface) at neutral pH, producing variations in migration behavior.

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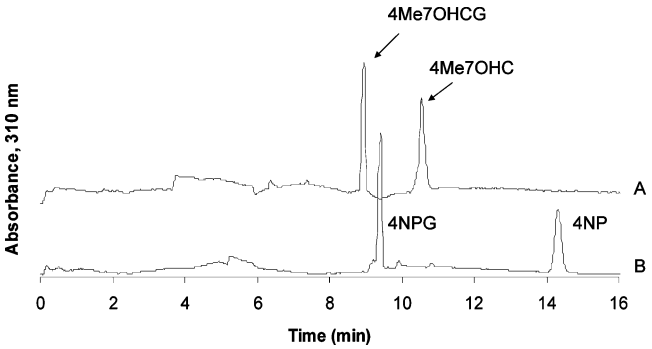

Figure 2. Representative electropherogram on glucuronidation of 4-methyl-7-hydroxycoumarin (4Me7OHC) and 4-nitrophenol (4NP). The concentration of 4Me7OHC and 4NP in the reaction mixture was 5 mM, and detection was made at 310 nm.

In order to overcome this problem, the uncoated capillary was replaced with a linear polyarcylamide (LP)-coated capillary (neutral capillary surface). When the aqueous run buffer was used, the amount of protein adsorbed onto LP capillary surface was minimal due to the presence of a neutral LP coating. This improved the reproducibility of the electropherograms, but the microsomal proteins, substrates, and glucuronides were not resolved.

When the LP-coated capillary was used with a polymeric run buffer, the microsomal proteins were separated from the small molecules and the inter-run variability was reduced. The electropherograms obtained from the on-line CE system for glucuronidation of 4Me7OHC and 4-NP are presented in Figure 2. The migration times of glucuronides and parent compounds were confirmed by injecting the individual standard compound without the UDPGA cofactor. Under these conditions, it appears that the viscous CE separation buffer acted as a sieving medium and the relative mobility of the microsomal proteins was small compared to that of small substrates due to complete removal of electroosmotic flow by the presence of a neutral polymeric coating in the capillary surface.

Optimization of the On-Line Glucuronidation. Substrate−enzyme contact times (i.e., incubation times) are dependent upon the relative mobilities of the microsomal proteins and substrates. Since the mobility of a compound is affected by the applied voltage and column length, these variables were examined as part of the optimization of the on-line glucuronidation of 4Me7OHC. The applied voltage was varied between 2.5 and 17.5 kV, and maximum glucuronide formation, as determined by UV absorption, was observed at 2.5 kV, with an ~2-fold difference between the two voltage extremes, Figure 3A. The total analysis time was also examined over this voltage range, with an inverse effect; i.e., the higher the applied voltage the shorter the retention time, Figure 3B. The optimum CE separation voltage was found to be 9 kV at which the glucuronide production and analysis time were reduced by 25 and 50%, respectively, relative to the results at 2.5 kV, Figure 3A and B. Although applied voltages of greater than 9 kV still resulted in the production of significant amounts of glucuronide and further reduced the analysis time, voltages higher than 9 kV were avoided since there was an increase in the possibility of current drops due to the formation of bubbles.

The diameter of the capillary column directly affected glucuronide formation, as determined by UV absorption, and the larger the diameter the greater the amount of the observed glucuronide, Figure 3C. Although the 150-μm-diameter column produced the greatest amount of glucuronide, a 100-μm-diameter column was chosen for the studies as elevated capillary currents and resulting current drops were observed with the150-μm-diameter column. In addition, columns with a diameter of less than 100 μm were prone to clogging.

The length of the capillary was also directly proportional to the amount of detected glucuronide, and the longer the column, the higher the measured UV response (data not shown). However, the analysis time also increased with column length, Figure 3D. While it was possible to reduce the analysis time by increasing the applied voltage, this produced sporadic current drops. A

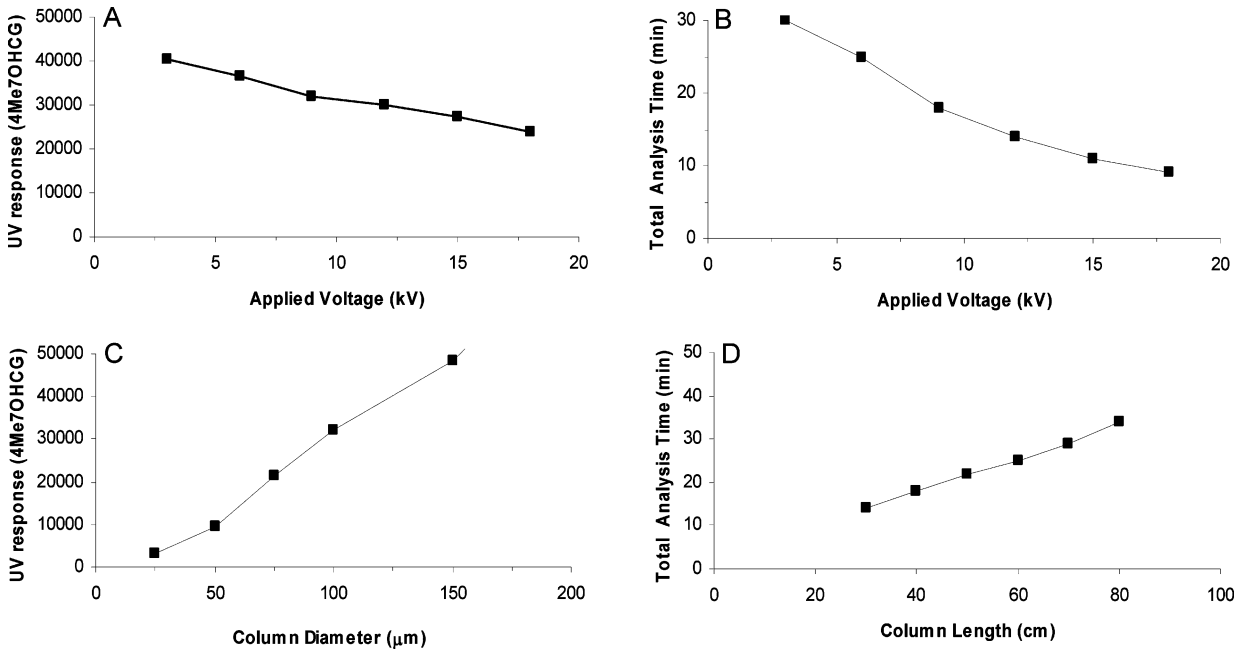

Figure 3. Optimization of the on-line glucuronidation on different experimental conditions. (A) 4Me7OHG formation on various applied voltages, (B) total analysis times on various applied voltages, (C) 4Me7OHG formation on various column diameter, and (D) total analysis times on various column lengths.

column length of 40 cm (length to the detection window, 30 cm) proved to be optimal and was used for the rest of the experiments.

Using 9.5-kV applied voltage and a 40 cm × 100 μm (i.d.) column, the experiment was complete in ~20 min. This included a 15-min analytical run and a 5-min capillary column washing. The relative standard deviations (RSDs) for migration time and peak area were determined for a set of three replicates. The RSD values were lower than 3.8% for migration times and lower than 4.4% for peak areas.

The volume and concentration of microsome injected onto the CE system were used as maximum values since these two parameters were associated with the separation and detection of the CE system. The maximum volume of the microsome plug injected at optimized capillary column length (i.e., 40 cm) was 24 nL, and volumes above this amount resulted in the loss of resolution between substrates and products. The concentration of microsome injected was set as the maximum value because lower concentrations resulted in the loss of detectability at low substrate concentrations.

Comparison of On-Line and Off-Line Glucuronidation. The effect of the CE conditions on the activity of the microsomal enzymes was evaluated by determining the Michaelis−Menten parameters (K_m and V_{max}) associated with the glucuronidation of 4Me7OHC to form 4Me7OHCG and 4-NP to form 4-NPG. These compounds were chosen because they have been extensively used to assess UDPGT activity. In these studies, the reaction incubation times were set to give linear response in the formation of glucuronides and the enzyme kinetics studies were conducted by measuring the changes in absorbance signals of glucuronide produced by different substrate concentrations, and the conditions were within the linear portion of the product formation−substrate concentration curves. The data wered analyzed using a nonlinear curve-fitting program (Prism) to produce Michaelis−Menten plots of 4Me7OHCG and 4NPG formation versus substrate concentrations, Figure 4. The correlation of variance (r^2) values of the two plots were 0.989 (4Me7OHCG) and 0.991 (4-NPG). The Michaelis−Menten parameters (K_m and V_{max}) for the formation of 4Me7OHCG and 4-NPG in an on-line CE system are presented in Table 1.

The enzyme kinetic data obtained from the on-line CE glucuronidation study were compared to the results obtained using the same microsomal preparations and a typical off-line enzyme incubation system. In the off-line CE study, the substrate and products were separated and quantified using an unmodified fused-silica capillary and a borate (50 mM, pH 9.0) running buffer. This was possible because the microsomal proteins were precipitated prior to CE analysis. The off-line glucuronide peak areas were obtained using 32 Karat (version 5.0) software from Beckman Instruments, and the data were analyzed using GraphPad Prism 4 software. The Michaelis−Menten parameters (K_m and V_{max}) for the formation of 4Me7OHCG and 4-NPG in the off-line incubations were obtained from nonlinear regression lines with correlations of variance (r^2) of 0.994 (4Me7OHCG) and 0.9998 (4-NPG). The data are presented in Table 1.

The calculated binding affinities, expressed as K_m values, obtained on the on-line system for 4Me7OHC and 4-NP were stronger, i.e., lower, than the K_m values obtained on the off-line system by 14 and 12%, respectively, Table 1. However, these differences were not significant. The data indicate that the ability of the UDPGT to bind substrates was not affected by the entrapment of the microsomal proteins within the sieving gel or by the fact that the proteins were placed within an electric field. It is of interest to note that the covalent immobilization of microsomal proteins on silica beads, to produce a UDPGT-IMER, reduced the binding affinity, i.e., increased K_m value, associated with the glucuronidation of 4Me7OHC by 18% relative to nonimmobilized microsomes.[10] However, a comparison of the standard deviations and statistical analysis using Student's t test demonstrated that these differences were not statistically significant.

The calculated kinetic rate constant, expressed as V_{max}, obtained on the on-line system for 4Me7OHC was 12% less than the corresponding values obtained with the nonimmobilized microsomes, while the on-line V_{max} for 4-NP was 15% greater than the value obtained using the off-line system, respectively, Table 1. Neither difference was significant. The data indicate that the ability of the UDPGT to catalyze the glucuronidation of the substrates does not appear to be significantly affected by the entrapment of the microsomal proteins within the sieving gel or by the fact that the proteins were placed within an electric field.

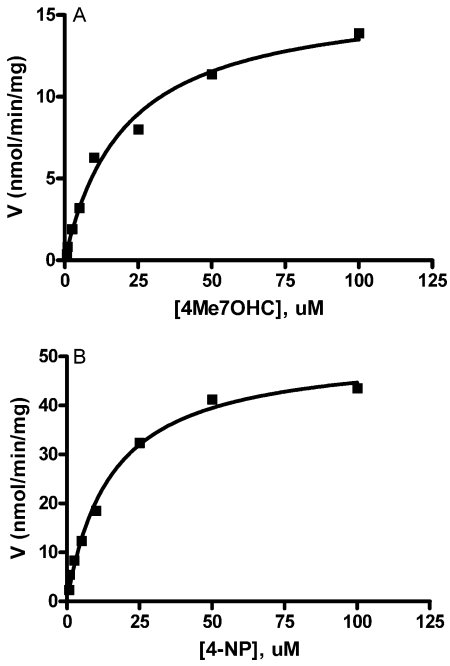

Figure 4. Michaelis−Menten plots for the glucuronidation of (A) 4Me7OHC and (B) 4NP obtained from on-line CE experiments.

Table 1. Michaelis−Menten Kinetic Parameters, K_m and V_{max}, Determined for the Glucuronidation of 4Me7OHC and 4-NP Obtained from On-Line and Off-Line CE Experiments[a]

substrates	K_m (μM)	V_{max} (nmol min^{-1} mg of protein^{-1})
4Me7OHC (on-line)	20.4 (±4.7)	16.3 (±4.5)
4Me7OHC (off-line)	23.7 (±2.1)	18.6 (±3.4)
4-NP (on-line)	15.2 (±3.0)	51.5 (±2.7)
4-NP (off-line)	17.2 (±2.3)	43.9 (±5.3)

[a] The values in parentheses represent a range of ±1 SD, where n = 3.

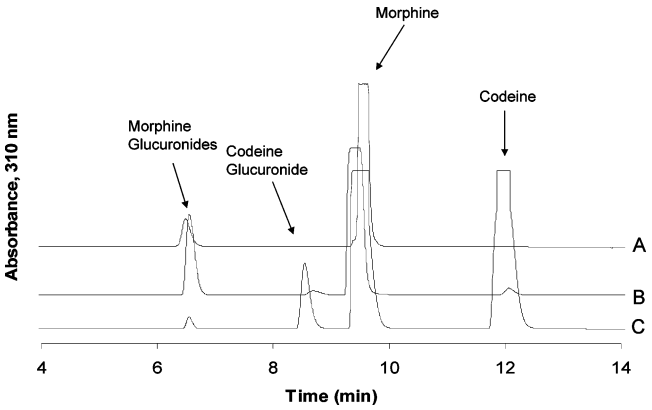

Figure 5. Electropherograms on competitive inhibition of morphine by different amount of codeine. (A) Morphine (10 mM) only, (B) morphine (100 mM) and codeine (2 mM), and (C) morphine (100 mM) and codeine (100 mM) in reaction mixture.

When the glucuronidation of 4Me7OHC and acetaminophen were studied on the UGDPT-IMER, the observed V_{max} values were significantly increased ($p < 0.05$, Student's t test) by 115 and 182%, relative to the V_{max} values calculated using nonimmobilized microsomes.[10] The increase in V_{max} was consistent with the data from other IMER studies, which utilized different enzymes.[25−27] The IMER experiments were conducted using liquid chromatography, and it is possible that the observed effect may arise from the different chromatographic formats, i.e., LC versu CE, and from the presence or absence of a chromatographic stationary phase. For example, placing an immobilized enzyme in a flowing system affects the kinetics of the distribution of the substrate from the mobile phase to the stationary system and produces shearing forces that affect the enzyme−substrate complexes. In addition, the presence of the solid support places the protein in a new microenvironment that can alter the rate at which the substrate reaches the active site of the enzyme. Both of these situations are not found in the CE system containing entrapped enzymes as the UDPGT, substrates, and cofactors are all solubilized in a flowing mobile system.

While both the IMER and CE approaches can be used to study glucuronidation, it appears that the data from the CE technique using entrapped microsomes are in better agreement with the results obtained using standard off-line microsomal incubations. The source of the difference in V_{max} between the CE and IMER formats is under investigation, and the results will be reported elsewhere.

Morphine and Codeine Inhibition Study. The ability of the on-line CE system to identify and quantify competitive inhibition of UDGPT activity was investigated using the inhibition of the glucuronidation of morphine by codeine. Morphine and codeine are primarily cleared from the body as glucuronides, which are produced by UDPGT 2B7.[28,29] Morphine and codeine are metabolized as morphine-3-glucuronide, morphine-6-glucuronide, and codeine-6-glucuronide, respectively.

When morphine was injected alone onto the on-line CE system, the resulting electropherogram contained a peak corresponding to morphine and a second peak that corresponded to morphine-3-glucuronide and morphine-6-glucuronide, as these compounds were not separated under the experimental conditions, Figure 5A. When codeine was coadministered with morphine, the electropherogram contained additional peaks corresponding to codeine and codeine-6-glucuronide, Figure 5B. When the morphine concentration was held constant and the codeine concentration increased, the relative area of the peak representing the morphine glucuronides decreased, indicating a competitive inhibition of morphine glucuronidation, cf. Figure 5C.

The effect of codeine concentration on the glucuronidation of morphine, as measured by the relative decrease in the concentrations of morphine-3-glucuronide and morphine-6-glucuronide, was used to construct dose−response curves, Figure 6A and to calculate the inhibition constant, IC_{50} value, corresponding to the ability of codeine to inhibit the glucuronidation of morphine. The correlation of variance (r^2) values of dose−response curve obtained from the on-line CE system was 0.989 and the calculated IC_{50} value was 0.17 (±0.03) mM.

The inhibition experiments were repeated using standard off-line incubation techniques with the same microsomal mixture and morphine and codeine concentrations. The effect of codeine concentration on the glucuronidation of morphine, as measured by the relative decrease in the concentrations of morphine-3-glucuronide and morphine-6-glucuronide, was used to construct dose−response curves, Figure 6B, and to calculate the inhibition constant, IC_{50} value, corresponding to the ability of codeine to inhibit the glucuronidation of morphine. The correlation of variance (r^2) values of dose−response curve obtained from the on-line CE system was 0.991, and the calculated IC_{50} value was 0.58 (±0.02) mM. This value was similar to the previously reported IC_{50} value, 0.60 (±0.16) mM, for the inhibition of morphine glucuronidation by codeine.[30]

Unlike the on-line and off-line determination of UDPGT activity, there was a 3-fold difference in the IC_{50} values, with the CE approach reflecting a more potent inhibitory effect. The reason for this is not currently understood and may reflect the fact that the on-line study reflects a flow equilibrium while the off-line data are obtained from a static equilibrium. This issue will be investigated and the results reported elsewhere. Nevertheless, the on-line CE system used in this study, demonstrated the ability to screen compounds for potential inhibitory activities toward drug-metabolizing enzymes.

CONCLUSION

The results from this study demonstrate the on-line CE system containing entrapped microsomal proteins can be used to monitor phase II metabolic conversions. The data obtained by this technique were equivalent to that obtained using standard off-line incubations, and it appears that both approaches can be used interchangeably. The CE system is easy to construct, can be used with standard CE equipment, takes 15 min for the formation and separation of the substrates and products, and does not require

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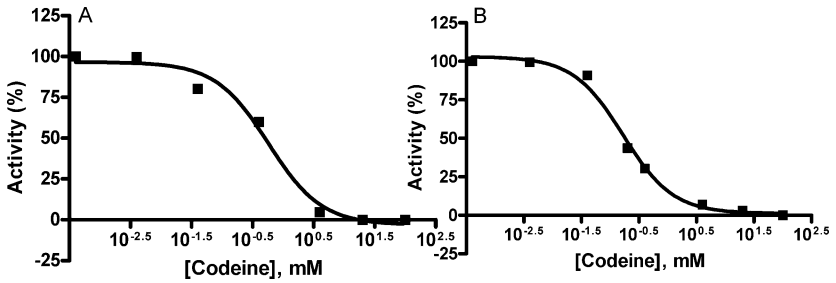

Figure 6. Off-line (A) and on-line (B) competitive inhibition of codeine (inhibitor) on glucuronidation of morphine. Experimental conditions are in text.

sample preparation steps such as protein precipitation or centrifugation. The data also indicate that carrying out the conjugation reactions within an applied electronic field had no effect on enzymatic activity. Thus, it is likely that this approach can be used to study phase I microsomal metabolism as well as the activities of other membrane-bound enzymes.

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