0090-9556/10/3811-1907–1911$20.00
DRUG METABOLISM AND DISPOSITION
Vol. 38, No. 11
33829/3630350
Printed in U.S.A.

DMD 38:1907–1911, 2010

Short Communication

Bilirubin Glucuronidation Revisited: Proper Assay Conditions to Estimate Enzyme Kinetics with Recombinant UGT1A1

Received April 7, 2010; accepted July 28, 2010

ABSTRACT:

Bilirubin, an end product of heme catabolism, is primarily eliminated via glucuronic acid conjugation by UGT1A1. Impaired bilirubin conjugation, caused by inhibition of UGT1A1, can result in clinical consequences, including jaundice and kernicterus. Thus, evaluation of the ability of new drug candidates to inhibit UGT1A1-catalyzed bilirubin glucuronidation in vitro has become common practice. However, the instability of bilirubin and its glucuronides presents substantial technical challenges to conduct in vitro bilirubin glucuronidation assays. Furthermore, because bilirubin can be diglucuronidated through a sequential reaction, establishment of initial rate conditions can be problematic. To address these issues, a robust high-performance liquid chromatography assay to measure both bilirubin mono- and diglucuronide conjugates was developed, and the incubation conditions for bilirubin glucuronidation by human embryonic kidney 293-expressed UGT1A1 were carefully characterized. Our results indicated that bilirubin glucuronidation should be assessed at very low protein concentrations (0.05 mg/ml protein) and over a short incubation time (5 min) to assure initial rate conditions. Under these conditions, bilirubin total glucuronide formation exhibited a hyperbolic (Michaelis-Menten) kinetic profile with a K_m of ~0.2 μM. In addition, under these initial rate conditions, the relative proportions between the total monoglucuronide and the diglucuronide product were constant across the range of bilirubin concentration evaluated (0.05–2 μM), with the monoglucuronide being the predominant species (~70%). In conclusion, establishment of appropriate incubation conditions (i.e., very low protein concentrations and short incubation times) is necessary to properly characterize the kinetics of bilirubin glucuronidation in a recombinant UGT1A1 system.

Introduction

Bilirubin is a toxic waste product, formed from heme degradation. In humans, approximately 250 to 400 mg of bilirubin are produced each day (Brierley and Burchell, 1993). Glucuronidation of bilirubin, catalyzed primarily by UGT1A1, is an obligatory step for bilirubin elimination and takes place in the liver and intestine (Kadakol et al., 2000). In this reaction, a glucuronosyl moiety is conjugated to one of the propionic acid side chains, located on the C8 and C12 carbons of the two central pyrrole rings of bilirubin, resulting in two monoglucuronides (BMGs), which can be further glucuronidated, forming an 8,12-diglucuronide (BDG) (Crawford et al., 1992). Both BMGs and BDG are excreted into bile by an ATP-dependent transporter, multidrug resistance-associated protein 2 (MRP2) (Kamisako et al., 2000). BDG is the major bilirubin pigment (~80%) found in bile (Crawford et al., 1992).

Because glucuronidation by UGT1A1 is an essential step in bilirubin elimination, genetic polymorphisms resulting in partial or complete loss of UGT1A1 activity can lead to accumulation of unconjugated bilirubin in plasma (Kadakol et al., 2000). In general, bilirubin is highly bound to plasma albumin, but in hyperbilirubinemic situations, plasma albumin may become saturated. The unbound bilirubin is then free to cross the blood-brain barrier. Progressive accumulation of bilirubin in the brain can result in neurological damages, kernicterus, and eventually death (Brierley and Burchell, 1993). Type I and II Crigler-Najjar syndromes and Gilbert's syndrome represent three grades of unconjugated hyperbilirubinemia. Patients with type I Crigler-Najjar syndrome are unable to conjugate bilirubin and exhibit serum bilirubin concentration typically in the range of 20 to 50 mg/dl (normal serum bilirubin <0.9 mg/dl) (Brierley and Burchell, 1993). Type II Crigler-Najjar syndrome (serum bilirubin level ~7–20 mg/dl) is characterized as a severe but only partial loss of UGT1A1 activity and can be treated by administration of UGT1A1 inducers, such as phenobarbital (Brierley and Burchell, 1993). More than 50 genetic variants, mostly in the exons of UGT1A1, have been associated with Crigler-Najjar syndromes (Kadakol et al., 2000). The mildest form of unconjugated hyperbilirubinemia is Gilbert's syndrome, and the serum bilirubin levels in patients with Gilbert's syndrome typically fluctuate between normal to 5 mg/dl (Kamisako et al., 2000). In whites, Gilbert's syndrome is most frequently associated with a genetic mutation in the promoter region of UGT1A1 (UGT1A1*28). Characterized as an insertion of TA in the promoter A(TA)6TAA sequence, UGT1A1*28 has been observed to exhibit an allelic frequency of ~40% in whites (Strassburg, 2008). Genetic mutations associated with Gilbert's syndrome also include missense mutations, such as G71R in exon 1. This type of mutation is rare in white populations but is common in Asians. The allelic frequency for G7R

This work was supported in part by the National Institutes of Health National Institute of General Medical Sciences [Grant GM063215] (to T.S.T.); and an investigator-initiated grant from Bristol-Myers Squibb (to R.P.R.).

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org.

doi:10.1124/dmd.110.033829.

ABBREVIATIONS: BMG, bilirubin monoglucuronide; BDG, bilirubin diglucuronide; HEK, human embryonic kidney; UDPGA, uridine-diphosphate glucuronic acid; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; LOQ, limit of quantification.

in a Japanese population has been reported to be 24% (Kamisako, 2004; Takeuchi et al., 2004). Gilberts syndrome is also linked to a variant in the phenobartital responsive enhancer module (Matsui et al., 2010).

Xenobiotics that inhibit UGT1A1 may also cause an elevation of bilirubin in blood and further exacerbate the hyperbilirubinemia observed in patients with Gilbert's syndrome or Crigler-Najjar syndrome (Rotger et al., 2005; Gupta et al., 2007). Therefore, in drug discovery settings, the in vitro ability of new drug candidates to inhibit bilirubin glucuronidation is commonly evaluated. For this purpose, a simple and robust bilirubin glucuronidation assay is critical. However, the instability of bilirubin (Doumas et al., 1973) and its glucuronides (Jansen, 1973; Blanckaert et al., 1978; Adachi et al., 1985) makes in vitro bilirubin glucuronidation assays technically challenging. Furthermore, because the glucuronidation of bilirubin involves a sequential reaction that produces two monoglucuronides and a diglucuronide, establishment of initial rate conditions can be difficult, if not given particular attention. These challenges have been manifested in significant disparities in estimated kinetic parameters for bilirubin glucuronidation. One group has reported that substrate inhibition kinetics are operable (Udomuksorn et al., 2007), and others have reported hyperbolic (Michaelis-Menten) kinetics (Senafi et al., 1994; Seppen et al., 1994). Likewise, estimates of K_m have ranged from 0.26 μM (Udomuksorn et al., 2007) to as high as 24 μM (Senafi et al., 1994; Seppen et al., 1994). To more carefully characterize the necessary initial rate (linear) conditions for bilirubin glucuronidation by human embryonic kidney (HEK) 293-expressed UGT1A1, the present study evaluated the effect of incubation time and protein concentration on bilirubin glucuronidation over a broader range of values. In addition, a sensitive and robust assay was developed to measure the monoglucuronides and diglucuronide of bilirubin, providing the necessary analytical capabilities for evaluating these incubation conditions.

Materials and Methods

Materials. Bilirubin IXα (purchased from Frontier Scientific, Logan, UT) was used in this study. Uridine-diphosphate glucuronic acid (UDPGA), Trizma base, Trizma HCl, D-saccharic acid 1,4-lactone, and alamethicin were purchased from Sigma-Aldrich (St. Louis, MO). $MgCl_2$ was purchased from Mallinckrodt Corp. (Hazelwood, MO). All other chemicals used in the glucuronidation incubations, as well as the high-performance liquid chromatography (HPLC) solvents, were of HPLC grade and obtained from standard sources. Recombinant human UGT1A1 was expressed in HEK293 cells (gifts from Dr. Philip Lazarus, Penn State University, Hershey, PA). Cell lysates were prepared as previously reported (Zhou et al., 2010) and were added directly to the incubations as the enzyme source.

Incubation Conditions. Incubations were conducted at 37°C in a shaking water bath. Incubation mixtures (final volume = 0.2 ml) contained UGT1A1-HEK293 cell lysate, bilirubin, Tris-HCl buffer (0.1 M, pH 7.4 at 37°C), $MgCl_2$ (5 mM), D-saccharic acid 1,4-lactone (5 mM), UDPGA (3 mM), and alamethicin (50 μg/mg protein). Bilirubin was dissolved in 100% dimethyl sulfoxide (DMSO) just before addition to the incubation mixtures. The final DMSO concentration in the incubations was 1%. Cell lysate was pretreated with alamethicin on ice for 30 min, before addition to the incubations. The reaction was initiated by addition of UDPGA, after a 3-min preincubation at 37°C, and was terminated by addition of 0.2 ml of ice-cold methanol containing 200 mM ascorbic acid. Protein was precipitated by centrifugation at 13,000g for 5 min at 4°C, and 200 μl of supernatant were injected onto the HPLC system for quantification. To characterize initial rate (linear) conditions with respect to protein concentration and incubation time, 10 μM bilirubin was incubated with different concentrations of protein (0.05 to 1 mg/ml) for 5 or 6 min or with 0.5 mg/ml protein over the time range of 2 to 50 min. Based on the results of these experiments, the incubation conditions used to characterize UGT1A1-mediated bilirubin glucuronidation kinetics were 5 min (incubation time) and 0.05 mg/ml (protein concentration) or 6 min and 0.5 mg/ml. Rates of formation of bilirubin glucuronides under these incubation conditions and at different bilirubin concentrations (0.05–2 μM) were determined and expressed as pmol/(min · mg protein). Bilirubin and bilirubin glucuronide solutions were stored in amber vials and handled and processed under conditions of reduced light.

Quantification of Bilirubin Monoglucuronides and Diglucuronide. Quantification of bilirubin monoglucuronides and diglucuronide was conducted with a Shimadzu LC-10ADVP system (Shimadzu, Columbia, MD) coupled with a Shimadzu SPD-10ADVP UV-Vis detector. The mobile phase consisted of 0.1% formic acid in water (A) and 100% methanol (B) was delivered at a flow rate of 0.5 ml/min. A linear gradient elution program was used beginning with 60% of mobile phase B, then increasing mobile phase B linearly from 60 to 95% over 15 min, holding at 95% B for 7 min, and then re-equilibrating the column at initial conditions for 4 min. The HPLC column was a Zorbax Eclipse XDB-C18 column (150 × 3 mm, 5 μM; Agilent Technologies, Inc., Santa Clara, CA). Bilirubin glucuronides and bilirubin were detected at a visible wavelength of 450 nm. Figure 1 depicts representative chromatograms of the supernatant fractions from incubations conducted in the presence and absence of UDPGA. Three additional peaks were detected in the presence of UDPGA. The peaks at 9.7 and 10.2 min were assigned as the monoglucuronides, and the peak at 6.5 min was assigned as the bilirubin diglucuronide (Fig. 1). Peak assignment was based on lipophilicities of the glucuronides and retention times reported by others (Adachi et al., 1985; Udomuksorn et al., 2007). The combined peak areas from the products eluting at 9.7 and 10.5 min were used to estimate the concentration of total monoglucuronides. Because bilirubin glucuronides were not commercially available, the UV absorbance response for bilirubin was used to estimate bilirubin glucuronide concentration, assuming that bilirubin and bilirubin glucuronides have the same molar extinction coefficient. Because the added glucuronic acid moiety does not absorb at the 450-nm wavelength, the molar extinction coefficient of the parent compound is not affected, upon which the assumption of equal extinction coefficients is based. Bilirubin standards were prepared just before analysis. Each standard also contained the same concentrations of Tris-HCl buffer, $MgCl_2$, D-saccharic acid 1,4-lactone, and HEK293 cell lysate as the incubation reactions. Protein in the standards was precipitated by addition of 100% ice-cold MeOH instead of ascorbic acid-MeOH solution due to the potential precipitation of bilirubin under acidic conditions. Supernatants (200 μl) were injected onto the HPLC column. The limit of quantification (LOQ) for bilirubin was set at the lowest concentration in the linear standard curve and was equal to 3.1 nM. For accuracy and precision determinations, standard curves were constructed on eight separate days. The average accuracy and between-day precision at LOQ were 109.4 and 18.6%, respectively. Accuracy at LOQ was calculated as the measured value divided by the true value × 100. The precision is calculated as the S.D. divided by the mean concentrations × 100.

Data Analysis. Kinetic data were analyzed by fitting the Michaelis-Menten equation (eq. 1) or the Hill equation (eq. 2) to the kinetic data with Sigma Plot 9.0 (Systat Software Inc., San Jose, CA). The parameters V_{max} and K_m in eq. 1 are defined as the maximum velocity (V_{max}) and the substrate concentration at which velocity equals to half of the maximum velocity (K_m). The parameter V_{max} in eq. 2 has the same definition as in eq. 1. S_{50} and n are defined as substrate concentration at which velocity equals to half of the maximum velocity and the Hill coefficient, respectively. Determination of model appropriateness was determined by visual inspection of the Eadie-Hofstee plots, comparison of the Second-Order Akaike Information Criterion, and the residual sum of squares.

$$V_0 = \frac{V_{max} \times [S]}{K_m + [S]} \quad (1)$$

$$V_0 = \frac{V_{max} \times [S]^n}{S_{50}^n + [S]^n} \quad (2)$$

Results and Discussion

Several factors contribute to the substantial technical challenges in the quantitation of bilirubin glucuronidation assay. Bilirubin itself is highly water insoluble at pH values below 8 (Heirwegh et al., 1972). Thus, to achieve desired concentrations for in vitro assays, bilirubin is commonly dissolved in alkaline solutions, followed by pH adjustment

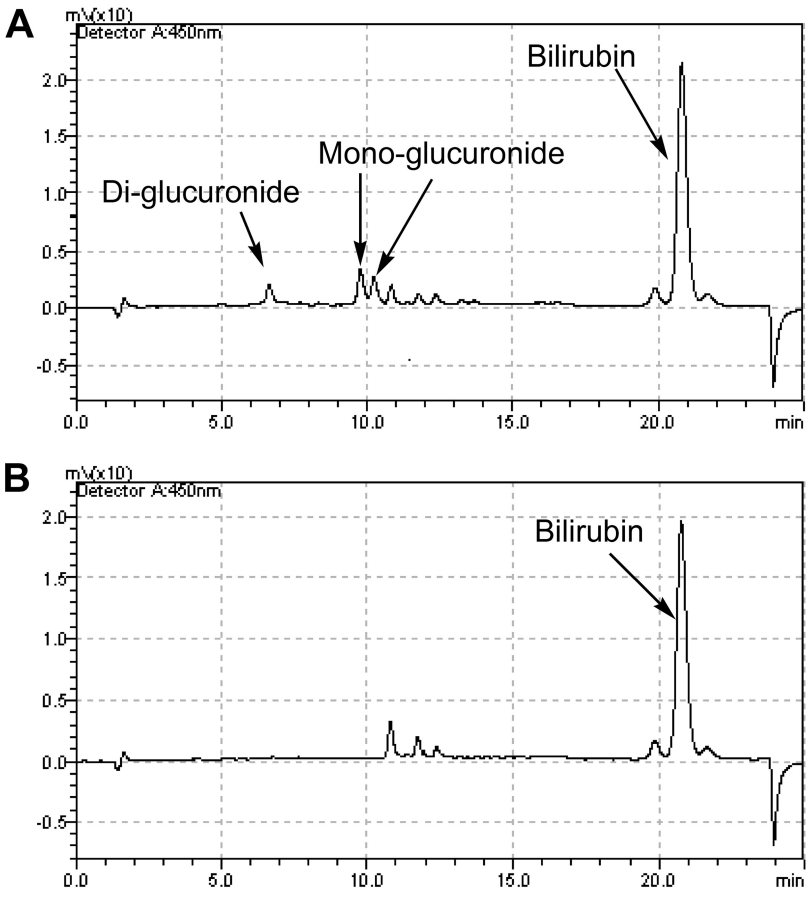

FIG. 1. Chromatograms for bilirubin glucuronidation in the presence (A) or absence (B) of UDPGA. Incubations were conducted with 50 μM bilirubin at 0.5 mg/ml protein for 30 min in the presence or absence of UDPGA. Twenty microliters of the incubation supernatant were injected onto HPLC for analysis.

(Gordon et al., 1983; Seppen et al., 1994), or in 100% organic solvents (Ciotti et al., 1998; Zhang et al., 2005; Udomuksorn et al., 2007). Due to its instability under these conditions, alkaline solutions of bilirubin must be prepared immediately before addition to the incubations (De Ewenson et al., 1966; Doumas et al., 1973), and one must be concerned about the potential precipitation of bilirubin after adjusting the pH to the neutral range. To address this issue, addition of albumin has been used to stabilize bilirubin solutions prepared in this manner (Black et al., 1970; Crawford et al., 1992). However, because bilirubin extensively binds to albumin, decreased rates of glucuronidation (Crawford et al., 1992) and non-Michaelis-Menten kinetics (Heirwegh et al., 1972) may be observed. In the present study, bilirubin was dissolved in 100% DMSO and stability was established for at least 8.5 h at room temperature and at least 7 days at −80°C.

Equally as problematic is the instability of bilirubin glucuronides, especially the monoglucuronides (Jansen, 1973; Blanckaert et al., 1978; Adachi et al., 1985). In aqueous solutions, the monoglucuronides rapidly disproportionate to diglucuronide and parent bilirubin (Adachi et al., 1985). This nonenzymatic conversion confounds kinetic determinations, due to loss of one molecule of total glucuronide (two molecules of monoglucuronide transform to one molecule of diglucuronide and one molecule of bilirubin). However, this conversion can be strongly inhibited by high concentrations of reducing agents, bovine serum albumin, or rat liver cytosolic proteins (Adachi et al., 1985). Thus, to eliminate the conversion of BMGs to BDG during sample analysis, a high concentration of ascorbic acid in methanol (200 mM final concentration) was used to terminate the reactions. Under these conditions, BMGs and BDG were stable in the final incubation supernatant for at least 17 h at room temperature.

Because of the disparate kinetic profiles obtained previously by others (Senafi et al., 1994; Seppen et al., 1994; Udomuksorn et al., 2007), the effects of protein concentration and incubation time on bilirubin glucuronidation were carefully examined to accurately establish linear (initial rate) conditions. Formation of BDG or BMGs was linear at protein concentrations up to 0.5 mg/ml. However, linearity with respect to incubation time was different for BDG and BMGs. Formation of BDG was linear at incubation times up to 30 min, whereas for the BMGs, linearity was only observed up to 6 min. These results are consistent with a sequential formation process for BMG and BDG. Based on these results, experiments were initially conducted at a high protein concentration (0.5 mg/ml) for 6 min.

Because the K_m for bilirubin glucuronidation with recombinant UGT1A1 has previously been reported to range from 0.26 to 24 μM (Senafi et al., 1994; Seppen et al., 1994; Zhang et al., 2005; Udomuksorn et al., 2007), experiments were initially conducted over the concentration range of 1.25 to 80 μM bilirubin. However, under the conditions used in the present study, no change in bilirubin glucuronidation rate was noted over this concentration range, suggesting that the reaction was already at V_{max}, even at 1.25 μM bilirubin (data not shown). Thus, even lower bilirubin concentrations (0.05–2 μM) were evaluated to allow estimation of K_m and V_{max}. As depicted in Fig. 2B, the kinetic profile for total bilirubin formation deviated from the classic hyperbolic (Michaelis-Menten) profile and was best described by the Hill equation (eq. 2). The derived S_{50}, V_{max}, and n values were 0.30 ± 0.02 μM, 199 ± 6.1 pmol/(min · mg protein), and 1.47 ± 0.09, respectively. However, amounts of the total glucuronides formed in this experiment were, on a molar basis, greater than 90% of the amount of bilirubin added to the incubation mixtures at low bilirubin concentrations, indicating this experi-

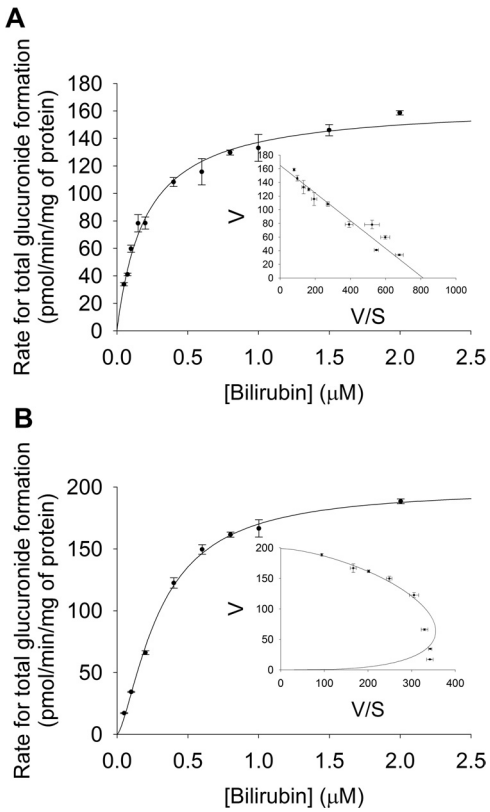

FIG. 2. Substrate-concentration versus rate plots for total bilirubin glucuronide formation. A, incubations with 0.05 mg/ml protein for 5 min; B, incubations with 0.5 mg/ml protein for 6 min. The bars indicate the range of triplicate measurements. The embedded figures are Eadie-Hofstee plots for the same data. The Michaelis-Menten equation was fit to data in A, and the Hill equation was fit to data in B.

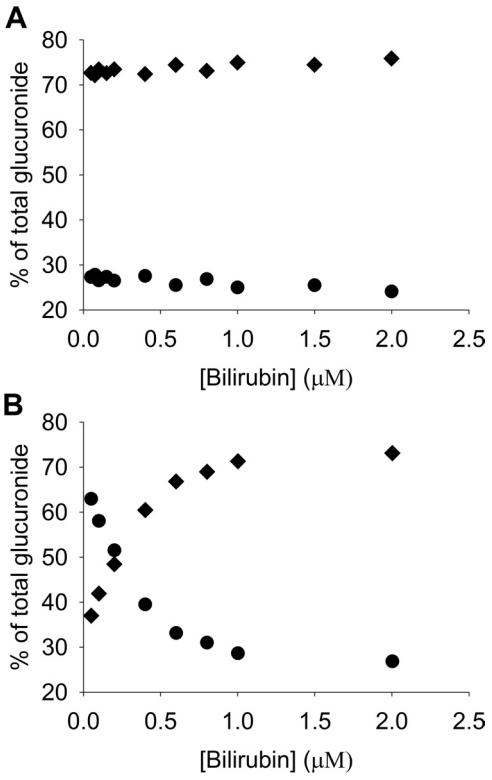

FIG. 3. Effects of bilirubin concentration on the proportions of BMGs or BDG. A, incubations with 0.05 mg/ml protein for 5 min; B, incubations with 0.5 mg/ml protein for 6 min. ● represents BDG and ◆ represents BMG.

ment was not conducted under initial rate conditions. To address this issue, 0.05 mg/ml protein and a 5-min incubation time were then evaluated. Under these conditions (Fig. 2A), formation of bilirubin glucuronides followed hyperbolic (Michaelis-Menten) kinetics with estimates of K_m = 0.20 ± 0.018 μM and V_{max} = 165 ± 4.3 pmol/(min · mg protein). Substrate consumption under these conditions was <20% at all substrate concentrations. It is interesting to note that although a different kinetic model was used by Udomuksorn et al. (2007) compared to our study, the K_m for total bilirubin glucuronide formation was very similar to the value we obtained. The low K_m observed in the present study and also reported by Udomuksorn (2007) indicates that saturation of glucuronidation occurs at relatively low substrate concentrations in vivo.

To assure that the saturation of velocity observed in the present study (the plateau in the hyperbolic kinetic profile) truly represents the V_{max} of the enzyme and is not due to saturation of bilirubin solubility in the incubation solution, we compared the peak areas of bilirubin in buffer solutions, where different concentrations of bilirubin in DMSO were added. A linear increase in bilirubin peak area was observed up to 10 μM final bilirubin concentration. Thus, the saturation of velocity observed at approximately ~2 μM (Fig. 2A) is a reflection of achieving maximal velocity of the enzyme and not artificially due to limits on bilirubin solubility. However, the peak areas observed from measuring bilirubin in buffer solutions were lower than the same concentrations of bilirubin in 100% acetonitrile. Over the range of 0.05 to 2 μM bilirubin, the peak areas of bilirubin in buffer solutions were approximately 60 to 80% of the peak areas in acetonitrile. This is probably due to the nonspecific binding of bilirubin to walls of the test tubes that occurs when bilirubin is in aqueous environment. In vivo, bilirubin is also highly bound to albumin in the systemic circulation and to cytosolic proteins (ligandin: a dimer of GSTA1 and/or GSTA2 and Z class proteins) present in hepatocytes (Kamisako et al., 2000). It is likely, under the present incubation conditions, that a portion of bilirubin nonspecifically binds to the proteins in the incubation, although the protein concentration in the present study was low (0.05 mg/ml), which would serve to minimize this binding. In addition, because bilirubin is highly lipophilic, it may also partition into the membranes of the cell lysate. For the above reasons, the K_m observed in the present study (0.2 μM) probably represents an apparent K_m, and the actual K_m of bilirubin glucuronidation is likely lower than 0.2 μM.

Several groups have reported that their proportions of BDG and BMGs formed in incubation were bilirubin-concentration dependent (Blanckaert et al., 1979; Gordon and Goresky, 1980; Gordon et al., 1983; Senafi et al., 1994). At low bilirubin concentrations, BDG was reported to be the dominant species formed, whereas BMG formation predominated at high bilirubin concentrations (Blanckaert et al., 1979; Gordon and Goresky, 1980; Gordon et al., 1983; Senafi et al., 1994). Senafi et al. (1994) conjectured that this kinetic phenomenon might be the reason that BDG is the predominant species found in bile because the free concentration of bilirubin in plasma is extremely low. Only under noninitial rate conditions were our results congruent with these reports (Fig. 3B). When true initial rate conditions were used, the proportions of BMGs and BDG formed were constant, with BMGs being the predominant species formed (~70%) (Fig. 3A). Thus, in the previously reported work (Senafi et al., 1994), the higher percentages of BDG formed at low bilirubin concentrations were most likely due to depletion of bilirubin and subsequent accumulation of BMGs in the in vitro incubations. In addition, it is well established that bilirubin glucuronidation by UGT1A1 is a sequential reaction (Peters and Jansen, 1986; Crawford et al., 1992; Senafi et al., 1994), and the kinetic model can be simplified as shown in Fig. 4. In this model,

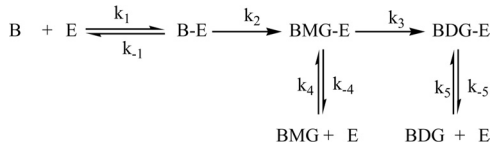

FIG. 4. Kinetic scheme for bilirubin glucuronidation. k_1, k_{-1}, k_2, k_3, k_4, k_{-4}, k_5, and k_{-5} refer to the rate constants of the corresponding reaction steps.

BMG can either be directly converted to BDG without releasing from the enzyme or it is released from the enzyme, but BMG can quickly rebound to the enzyme and then be converted to BDG. Based on the kinetic model, the ratio between initial rates for BMG and BDG formation equates to k_4/k_3, independent of bilirubin concentration. Thus, the observed constant proportion between BMGs and BDG across the range of bilirubin concentrations under true linear conditions (Fig. 3A) is consistent with the sequential kinetic model in Fig. 4.

In summary, the present study established the proper conditions to conduct bilirubin glucuronidation with HEK293-expressed UGT1A1. Under true linear conditions, bilirubin glucuronidation displayed Michaelis-Menten kinetics with a K_m of 0.2 μM, and the ratios of BMGs and BDG formation were constant across the range of bilirubin concentration evaluated. In addition, a robust assay that possessed sufficient sensitivity to permit characterization of low amounts of glucuronide formation was developed in the present study. We are currently evaluating a battery of UGT1A1 inhibitors on bilirubin glucuronidation with this assay.

Acknowledgments. We thank Dr. Philip Lazarus at Penn State University for providing transfected HEK293 cells that express UGT1A1.

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