Contribution of UDP-Glucuronosyltransferase 1A1 and 1A8 to Morphine-6-Glucuronidation and Its Kinetic Properties

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ABSTRACT:
The metabolic conversion of morphine to morphine-6-glucuronide (M6G) seems to play a significant role in mediation of the clinical effect of morphine because of the superior analgesic effect of M6G. Therefore, it would be of great interest to clarify the specificity of morphine-6-glucuronidation by UDP glucuronosyltransferase (UGT) isozymes. We investigated the specificity of morphine-6-glucuronidation catalyzed by recombinant human UGT isozymes in microsomes from baculovirus-infected insect cells. The morphine glucuronidation activity of recombinant human UGT isozymes incubated with morphine and UDP-glucuronic acid was determined by high-performance liquid chromatography with a fluorescence detector. Not only UGT2B7, which is well known to catalyze morphine-6-glucuronidation, but also UGT1A1 and 1A8 effectively catalyzed morphine-6-glucuronidation at relatively low morphine concentrations (<100 μM). The kinetics of both isozymes at the low substrate concentrations showed hyperbolic Michaelis-Menten kinetics. However, as the morphine concentration approached 100 μM, morphine-6-glucuronidation activity gradually decreased, and the kinetics closely resembled substrate inhibition Michaelis-Menten kinetic behavior. The Km values were 67.9 and 68.1 μM and the Kmax values were 218.9 and 88.0 μM for UGT1A1 and 1A8, respectively. These kinetics are basically different from that of morphine-6-glucuronidation by UGT2B7, which suggested biphasic Michaelis-Menten kinetic behavior. Furthermore, to estimate the contribution of these UGT isozymes in M6G formation in vivo, the expression levels of UGT1A1 and 1A8 mRNA in human liver and intestine were determined by reverse transcription real-time polymerase chain reaction. The results strongly suggest that UGT1A1 and UGT1A8 are isozymes involved in morphine-6-glucuronidation in vivo, as is UGT2B7 in humans.

Morphine, the typical opioid analgesic, has been the most important and widely used drug for the relief of acute and chronic pain, especially in cancer patients with severe pain. Morphine is metabolized in vivo primarily to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). In humans, 60% of morphine is glucuronidated to M3G, whereas 5 to 10% of morphine is glucuronidated to M6G (Osborne et al., 1990; Hasselström et al., 1991; Hasselström and Sawe, 1993; Lötch et al., 1996; Christrup, 1997). M6G has been shown to be a more potent opioid agonist than morphine, whereas M3G is not. However, the potency of M6G depends greatly on the study design used to assess the drug effect. The relative analgesic potency (M6G:morphine) in rodents has been reported to range from 1:1 to 678:1, and the range in humans is reported consistently to be 2.6:1 to 4:5:1 (Lötch and Geisslinger, 2001). These findings strongly suggest that the metabolic conversion of morphine to M6G may play a significant role in mediation of the clinical effect of morphine. In fact, it has been reported that M6G contributes to morphine analgesia in patients with cancer pain (Portenoy et al., 1992; Faura et al., 1996; Dennis et al., 1999; Tighe et al., 1999; Klepstad et al., 2000).

Glucuronidation reactions are catalyzed by enzymes in the UDP-glucuronosyltransferase (UGT) superfamily. In humans, 31 genes, including some pseudo-genes, have been identified to date (Tukey and Strassburg, 2000; Mackenzie et al., 2005). However, 15 isozymes belonging to either subfamily UGT1 or UGT2, such as UGT1A1, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9, 1A10, 2A1, 2B4, 2B7, 2B15, 2B17, and 2B28, seem to be the catalytically active isozymes. One of them, UGT2B7, is believed to be the major isofrom involved in the glucuronidation of morphine in humans and has been shown to catalyze the conversion of morphine to both M3G and M6G (Coffman et al., 1997, 1998). It was reported that many isozymes, such as UGT2B7, 1A1, 1A3, 1A6, 1A8, 1A9, and 1A10, are involved in the glucuronidation to M3G, but only UGT2B7 is involved in the glucuronidation to M6G (Stone et al., 2003). To the best of our knowledge, there are no reports that have thoroughly investigated the isozymes involved in M6G formation, with the exception of UGT2B7. In general, a high concentration of morphine, at the millimolar order, is used as the substrate for determination of UGT activity. Plasma morphine concentrations typically observed in humans at 24 to 48 h after administration of therapeutic dosages were reported to reach the micromolar order (McQuay et al., 1990; Tiseo et al., 1995; Klepstad et al., 2003, 2004). It is worthy to consider determining the specificity

ABBREVIATIONS: M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; UGT, UDP-glucuronosyltransferase; HPLC, high-performance liquid chromatography; KPB, potassium phosphate buffer; LC, liquid chromatography; MS, mass spectrometry; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
of the glucuronidation of morphine by UGT isozymes, in vitro, using relatively low morphine (substrate) concentrations reflecting the therapeutic dosage in clinical situations. Furthermore, the metabolic conversion of morphine to morphine-6-glucuronide seems to play a significant role in mediation of the clinical effect of morphine because of its own superior analgesic effect. It would be of great interest to clarify the specificity of morphine-6-glucuronidation by UGT isozymes.

In this article, we investigated the isozyme specificity of M6G formation catalyzed by recombinant human UGT isozymes in microsomes from baculovirus-infected insect cells at relatively low concentrations of morphine (>10 μM). Consequently, we found that the UGT1A1 and 1A8 isozymes contribute to M6G formation at low substrate concentrations (<100 μM) and herein describe their kinetic properties.

Materials and Methods

Chemicals and Reagents. Morphine hydrochloride and dihydrocodeine phosphate were purchased from Daiichi Sankyo Healthcare Co., Ltd. (Tokyo, Japan). Morphine-6-β-glucuronide was purchased from Sigma-Aldrich UK (Dorset, England). Morphine-3-β-glucuronide was purchased from Cerilliant (Round Rock, TX). Recombinant human UGT Supersomes, such as 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17, expressed in the reaction mixture consisting of 50 mM Tris-HCl buffer (pH 7.5) containing 8 mM MgCl₂, 25 μg/ml amphotericin, 2 mM UDP-glucuronic acid and 200 μg/ml Supersomes. After adding the Supersomes, incubation was performed for 3 h at 37°C. The reaction was terminated by addition of 0.16 ml of ice-cold acetonitrile containing 6% acetic acid and then adding dihydrocodeine phosphate (60 pmol) as an internal standard to calibrate the correction rate of M6G and M3G for determination by HPLC. After vigorous shaking the mixture was chilled in an ice bath for 30 min followed by centrifugation at 15,000 rpm for 5 min. The supernatant was collected, 360 μl of 0.5 M potassium phosphate buffer (KPB) (pH 9.5) and 80 μl of 2 M sodium hydroxide solution were added, and then the mixture was passed through a Bond elute C18 HF cartridge (50 mg/ml; Varian Inc., Palo Alto, CA) that had been activated by prewashing with 2 ml each of methanol and 10 mM KPB (pH 9.5). The solid-phase extraction methods and HPLC analysis were performed according to the reports of Meng et al. (2000) and Hartley et al. (1993) with some modification. Briefly, the cartridge was washed twice with 1 ml of the same phosphate buffer and eluted with 1 ml of methanol-0.5% triethylamine. After evaporation, the sample was dissolved in 30 μl of 30% acetonitrile-2.5 mM SDS-50 mM KPB (pH 2.1). This purified sample was centrifuged at 15,000 rpm for 20 min and then subjected to HPLC analysis. The HPLC system consisted of LC6A with a RF-10AXL fluorescent detector (Shimadzu, Kyoto, Japan), which was set at excitation and emission wavelengths of 283 and 350 nm, respectively, and an Inertil ODS-3 column (4 μm, 250 × 4.6 mm i.d.) attached to a Guard column (5 × 4.6 mm i.d.) (GL Sciences, Tokyo, Japan). The mobile phase consisted of 30% acetonitrile-2.5 mM SDS-50 mM KPB (pH 2.1) at a flow rate of 1 ml/min. Analyte retention times, confirmed by authentic standards, were 6.0 min for M3G, 7.2 min for M6G, 14.0 min for morphine, and 20.5 min for dihydromorphone as the internal standard. Under these conditions, the standard curves for M3G (5–1000 pmol) and M6G (5–500 pmol) were linear with correlation coefficients greater than 0.9930. Our HPLC method with fluorescence detection at an excitation wavelength of 283 nm has relatively high detection efficiency without hindrance of fluorescence by solvent.

Identification of Morphine Glucuronide by LC-MS. The morphine glucuronide metabolites catalyzed by recombinant human UGTs were further identified by LC-MS if necessary. The LC-MS system consisted of an LCMS-2010A (Shimadzu, Kyoto, Japan) with a Shim-pack VP-ODS column (150 × 2.0 mm i.d.; Shimadzu) as the chromatographic column for HPLC. The mobile phase was acetonitrile-1 mM ammonium formate (6/94, v/v) with 1% formic acid with a flow rate of 0.3 ml/min at a column temperature of 40°C. Electrospray ionization (positive ion mode) MS was performed at 250°C with a nitrogen gas stream (1.5 liters/min) at a potential of +4.5 kV. The m/z 462, 462, and 286 ions were detected for assignment of the [M + H]+ ions of M6G, M3G, and morphine, respectively, with a fragmentor voltage of 1.75 kV.

Kinetic Analysis. The kinetic analyses were performed using recombinant human 1A1, 1A8, and 2B7 expressed in baculovirus-infected insect cells. To determine the kinetic parameters, the concentration range of morphine as a substrate was 10 μM to 10 mM. The kinetic parameters were estimated from the fitted curves using Prism nonlinear least-square regression analysis software (version 4.0; GraphPad Software Inc., San Diego, CA) and are presented as a regression parameter estimate ± the S.E. of the estimate. The following equations were used in the determination of the kinetic parameters:

Hyperbolic Michaelis-Menten model,

\[ V = V_{max} \cdot S \left( \frac{K_m + S}{K_m + S} \right) \] (1)

Substrate inhibition Michaelis-Menten model (Houston and Kenworthy, 2000),

\[ V = V_{max} \cdot S \left( \frac{K_m + S + S^2/K_m}{K_m + S} \right) \] (2)

Biphasic Michaelis-Menten model (Kobayashi et al., 1999),

\[ V = V_{max1} \cdot S \left( \frac{K_m1 + S}{K_m1 + S} + V_{max2} \cdot S \left( \frac{K_m2 + S}{K_m2 + S} \right) \right) \] (3)

where \( K_m \) is a Michaelis-Menten constant, \( V_{max} \) is the maximum velocity, \( K_{mi} \) is the substrate inhibition constant, and \( K_{mi} \) and \( K_{mi} \) are Michaelis-Menten constants for the high- and low-affinity components, respectively, and \( V_{max} \) and \( V_{max} \) are the maximum velocities for the high- and low-affinity components, respectively.

Reverse Transcription Real-Time Polymerase Chain Reaction of UGT1A1, 1A8, and 2B7. Two micrograms of total RNA from human liver or small intestine was reverse transcribed by Ready-To-Go reverse transcription-PCR beads (GE Healthcare UK Ltd., Buckinghamshire, UK) with 0.5 μg of oligo(dT)₁₂₋₁₈ as the reverse transcription primer at 42°C for 30 min and followed by at 95°C for 5 min. Certain parts of human UGT1A1 and UGT2B7 cDNA were amplified by PCR from a reverse-transcribed human liver cDNA

<table>
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TABLE 1 Human total RNA donor information
library using 5'-AATAAAAAAGGACTCTGCTATGCT-3' (sense) and 5'-ATTCACATGCTCCAGAAGCA-3' (antisense) for UGT1A1 and 5'-GGGAATTTCATCATGCAACAGA-3' (sense) and 5'-ATTCCATGTTCTCCAGAAGCA-3' (antisense) for UGT2B7 as specific primers. Human UGT1A8 cDNA was amplified from a small intestine cDNA library using 5'-GAAAGCACAAGTACGAAGTTTG-3' (sense) and 5'-ATTCCATGTTCTCCAGAAGCA-3' (antisense) as the specific primers. Under these conditions, the amplicon was detected as a single band in an agarose gel in all PCR reactions and the lengths of the amplicons were 562, 123, and 603 base pairs for UGT1A1, UGT2B7, and UGT1A8, respectively. Each amplicon was subcloned into pGEM-T Easy (Promega, Madison, WI) for sequencing and was identified as each subtype. Real-time PCR was performed using the SYBR Green method with GAPDH as the internal standard using a Light Cycler 480 (Roche Diagnostics K.K., Tokyo, Japan) according to the instructions of the manufacturer. Standard curves for quantification were prepared in parallel with each real-time PCR analysis using diluted subcloned pGEM-T Easy vectors, described above, as the template. The primers for human GAPDH were 5'-TCCACTGGCCTCCACC-3' (sense) and 5'-GGCAGATGATGACCTTTT-3' (antisense) and the expected amplicon length was 78 base pairs. The PCR reaction conditions were as follows: total 45 cycles of 95°C for 12 s, 60°C for 12 s, and 72°C for 40 s.

Results

Morphine Glucuronidation Catalyzed by Recombinant UGT Isozymes. Twelve recombinant UGT isozymes (1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17) expressed in baculovirus-infected insect cells were used to determine the activities of M3G and M6G formation at a relatively low concentration of morphine (50 μM) and also at a relatively high concentration of morphine (2.5 mM) to compare them. As shown in Fig. 1A, UGT2B7 exhibited high catalytic activity with respect to M3G formation. Other UGT isoforms, specifically 1A1, 1A3, 1A8, 1A9, 2B4, and 2B15, also exhibited significant catalytic activity for M3G formation. As a whole, in contrast to the UGT1A family isozymes, M3G formation by UGT2B family isozymes exhibited high activity. Their catalytic activities appeared to be dependent on the morphine concentration. In addition, low catalytic activities for M3G formation were also observed for UGT1A6, 1A10, and 2B17 without any dependence on the substrate concentration. M6G formation for each UGT isozyme is shown in Fig. 1B. At a morphine (substrate) concentration of 50 μM, UGT2B7 exhibited catalytic activity with respect to M6G formation. M6G formation by UGT2B7 has already been demonstrated by Stone et al. (2003) and even indicated to be a selective probe for this enzyme. However, interestingly, UGT1A1 and 1A8 also significantly catalyzed M6G formation, and the rates were higher than those for M3G formation for both. However, at a substrate concentration of 2.5 mM, M6G formation catalyzed by UGT1A1 and 1A8 was considerably decreased although the catalytic activity of M6G formation by UGT2B7 was notably increased. UGT2B4 also catalyzed the M6G formation at a morphine concentration of 2.5 mM, although it was not detectable at 50 μM.

Identification of Morphine Glucuronides. Two glucuronidation products, M3G and M6G, from morphine catalyzed by UGT1A1 and 1A8 were identified by the retention times of the authentic standards.
using HPLC with a fluorescence detector. Figure 2A shows a typical chromatogram of the metabolites from morphine catalyzed by UGT1A8. Furthermore, the metabolites were analyzed by LC-MS for accurate identification. Figure 2B shows representative LC-MS chromatograms of the glucuronide from morphine catalyzed by UGT1A8, which was measured by selected ion monitoring at m/z 462, a molecular ion ([M + H]⁺) of M3G or M6G (Naidong et al., 1999). The metabolites from morphine catalyzed by UGT1A1 were also identified to be M3G and M6G (data not shown). The results obtained here demonstrated that UGT1A1 and 1A8 catalyzed not only M3G formation but also M6G formation.

Kinetics of Morphine-6-Glucuronidation Catalyzed by UGT1A1 and UGT1A8. We observed that M6G formation was catalyzed by UGT1A1 and 1A8 at a morphine concentration of 50 μM. Therefore, kinetic analysis of M6G formation by recombinant UGT1A1 and 1A8 was performed. As shown in Fig. 3A, the kinetics of M6G formation by UGT1A1 at 10 to 100 μM morphine exhibited characteristics of Michaelis-Menten kinetics. The apparent kinetic constants estimated by fitting to the hyperbolic Michaelis-Menten model (eq. 1) were 40.4 ± 9.4 μM (Kₘ) and 7.2 ± 0.9 pmol/min/mg (Vₘₐₓ). However, when the morphine concentration exceeded 100 μM, M6G formation gradually decreased. The kinetics with UGT1A1 indicated substrate inhibition behavior and exhibited characteristics of the substrate inhibition Michaelis-Menten model (eq. 2). The apparent kinetic constants estimated by fitting to eq. 2 at morphine concentrations of 10 to 1000 μM were 67.9 ± 25.6 μM (Kₘ), 10.5 ± 2.4 pmol/min/mg (Vₘₐₓ), and 218.9 ± 84.8 μM (Kₗ). Next, the kinetics of M6G formation by recombinant UGT1A8 at morphine concentrations of 10 to 1000 μM were examined (Fig. 3B). The kinetics with UGT1A8 also indicated substrate inhibition behavior. The apparent kinetic constants estimated by fitting to the hyperbolic Michaelis-Menten model (eq. 1) at morphine concentrations of 10 to 80 μM were 35.3 ± 6.9 μM (Kₘ) and 28.9 ± 2.4 pmol/min/mg (Vₘₐₓ). Furthermore, the apparent kinetic constants estimated by fitting to the substrate inhibition Michaelis-Menten model (eq. 2) at 10 to 1000 μM morphine were 68.1 ± 26.0 μM (Kₘ), 49.8 ± 12.5 pmol/min/mg (Vₘₐₓ), and 88.0 ± 31.4 μM (Kₗ). The kinetic parameters are summarized in Table 2. As indicated in Fig. 3, although the kinetic analysis was performed using 10 to 1000 μM morphine, M6G formation catalyzed by UGT1A1 and 1A8 was determined up to 10 mM morphine. The activity at 1 to 10
mM morphine decreased further in a dose-dependent manner and almost reached 0 at 10 mM morphine (data not shown except for the activity at 2.5 mM morphine, shown in Fig. 1).

**Kinetics of Morphine-6-Glucuronidation Catalyzed by UGT2B7.** UGT2B7 is well known to catalyze M6G formation. With the aim of comparing the kinetics of M6G formation by UGT1A1 or UGT1A8 with that of M6G formation catalyzed by UGT2B7, we analyzed the kinetics of M6G formation catalyzed by recombinant UGT2B7. As shown in Fig. 4, the kinetics of M6G formation by UGT2B7 indicated biphasic Michaelis-Menten model (eq. 3) behavior. The Eadie-Hofstee plots presented as an inset in Fig. 4 also exhibited atypical kinetics behavior. The apparent kinetic constants for the high-affinity component estimated by fitting to eq. 3 were 99.6 ± 67.6 μM (K_m) and 39.4 ± 10.3 pmol/min/mg (V_max), whereas those for the low-affinity component were 13.3 ± 8.3 mM (K_m) and 250.5 ± 76.6 pmol/min/mg (V_max). The kinetic parameters are summarized in Table 2.

**Expression Levels of UGT1A1, 1A8, and 2B7 in Human Liver and Intestine.** We demonstrated the contribution of UGT1A1 and 1A8 to M6G formation in vitro at comparatively low morphine concentrations (<100 μM). To estimate the contributions of these UGT isoforms to M6G formation in vivo, the expression levels of UGT1A1 and 1A8 in human liver and intestine were quantitatively determined by reverse transcription real-time PCR and were compared with that of UGT2B7, which is known to be highly expressed in the liver. As shown in Fig. 5, UGT1A1 was expressed in liver, the level of which was 30.6% that of UGT2B7, whereas UGT1A8 was hardly expressed in the liver. In the intestine, UGT 1A1 was also expressed, and the level was 30.2% compared with intestinal UGT2B7. UGT1A8 was expressed in the intestine, with the level being 3.3% that of intestinal UGT2B7. Intestinal UGT2B7 expression was 63% of that expressed in liver.

**Discussion**

The UGTs, membrane-bound enzymes on endoplasmic reticulum that catalyze glucuronidation reactions, are important enzymes in the human phase II metabolic system. Glucuronidation is involved in the formation of inactive water-soluble products from many substrates, such as steroids, bile acid, bilirubin, hormones, dietary constituents, and many xenobiotics including drugs and environmental toxicants (Tukey and Strassburg, 2000). Although 31 genes, including some pseudo-genes, which can be divided into four families, UGT1, UGT2, UGT3 and UGT8, have been identified (Tukey and Strassburg, 2000; Mackenzie et al., 2005), only 17 human UGT cDNAs and their respective gene products have been characterized to date. However, two gene products, UGT2B10 and 2B11, have been found to have no glucuronidation activity (Jin et al., 1993; Beaulieu et al., 1998). Accordingly, 15 isozymes belonging to either subfamily UGT1 or UGT2, such as UGT1A1, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9, 1A10, 2A1, 2B4, 2B7, 2B15, 2B17, and 2B28, are catalytically active. These isozymes have overlapping but distinct substrate specificity (Tukey and Strassburg, 2000).

Morphine is metabolized in vivo primarily to M3G and M6G. UGT2B7 that is mainly expressed in liver is the major isoform involved in this morphine glucuronidation. Some isozymes, including UGT2B7, are also involved in the glucuronidation to M3G, but only UGT2B7 is involved in M6G formation (Stone et al., 2003). M6G shows superior analgesic activity to morphine and appears to have a superior side effect profile in terms of reduced liability to induce nausea and vomiting and respiratory depression (Kilpatrick and Smith, 2005). The metabolic conversion of morphine to M6G has a significant role in mediation of the clinical effect of morphine because M6G has these high-integrity opioid agonistic activities. Accordingly, characterization of the UGT isoforms involved in M6G formation is believed to be very important, and thus it would be of great interest to clarify the specificity of M6G formation by UGT isozymes.

The ranges of plasma concentrations of morphine typically observed in humans with therapeutic dosages have been reported to be 213 to 2390 nM (Tiseo et al., 1995), 46 to 2520 nM (Klepstad et al., 2004), 1 to 2560 nM (Klepstad et al., 2003), and 2 to 3497 nM (McQuay et al., 1999). Most elevated concentrations of plasma morphine in each population were in the micromolar order. These values are the concentration at 24 to 48 h after administration; hence it seems that the peak concentration of morphine is higher than the concentration at 24 to 48 h after administration of therapeutic dosages of morphine, as indicated by Naidong et al. (1999). In this article, to clarify the specificity of the morphine-6-glucuronidation catalyzed by UGT isozymes, we examined morphine glucuronide formation by human recombinant UGT isozymes at relatively low concentrations of morphine that reflect therapeutic dosages. Consequently, M6G formation was catalyzed by UGT2B7, which is well known to catalyze M6G formation, but interestingly we observed that UGT1A1 and 1A8 also catalyzed M6G formation at a morphine concentration of 50 μM, which is the concentration used in the screening process. This rate of M6G formation by UGT1A1 or 1A8 was higher than the rate of M3G.
The kinetic parameters are summarized in Table 2.

All kinetic parameters are presented as a regression parameter with S.E.M.

**TABLE 2**

<table>
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<th>Isozyme</th>
<th>Hyperbolic Michaelis-Menten Model</th>
<th>Substrate Inhibition Michaelis-Menten Model</th>
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<td>$V_{max}$ (pmol/min/mg)</td>
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<td>UGT1A1</td>
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<td>UGT1A8</td>
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<td>UGT2B7</td>
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The present findings have clearly demonstrated the contribution of UGT1A1 and 1A8 to morphine-6-glucuronidation at comparatively low morphine concentrations (<100 μM) by kinetic analysis. We also compared the kinetics to the kinetic properties of morphine-6-glucuronidation by UGT2B7, an isozyme widely recognized as mediating morphine-6-glucuronidation, appear to be biphasic. Stone et al. (2003) described the biphasic kinetic properties based on a biphasic Michaelis-Menten model or a two-site model and apparent $K_m$ values of 0.97 and 13.7 mM for the high- and low-affinity components, respectively, using UGT2B7 expressed in HK293 cells. In this article, using the isozyme from baculovirus-infected insect cells, we also demonstrated that M6G formation by UGT2B7 exhibited biphasic Michaelis-Menten kinetics. The apparent $K_m$ values from fitting to the biphasic Michaelis-Menten model are 99.6 μM for the high-affinity component and 13.3 mM for the low-affinity component. There is a slight difference between the $K_m$ values reported by Stone et al. and by us. This difference remains to be clarified. As pointed out by Stone et al. (2003), previous studies concerning M6G formation by UGT2B7 assumed that the kinetic properties were not biphasic. Apparent $K_m$ values reported were 1.3 mM (Coffman et al., 1997), 0.43 to 0.67 mM (Coffman et al., 1998), 0.24 mM (Soars et al., 2003), and 0.63 mM (Court et al., 2003).

The kinetics of M6G formation by UGT2B7, an isozyme widely recognized as mediating morphine-6-glucuronidation, appear to be biphasic. Stone et al. (2003) described the biphasic kinetic properties based on a biphasic Michaelis-Menten model or a two-site model and apparent $K_m$ values of 0.97 and 13.7 mM for the high- and low-affinity components, respectively, using UGT2B7 expressed in HK293 cells. In this article, using the isozyme from baculovirus-infected insect cells, we also demonstrated that M6G formation by UGT2B7 exhibited biphasic Michaelis-Menten kinetics. The apparent $K_m$ values from fitting to the biphasic Michaelis-Menten model are 99.6 μM for the high-affinity component and 13.3 mM for the low-affinity component. There is a slight difference between the $K_m$ values reported by Stone et al. and by us. This difference remains to be clarified. As pointed out by Stone et al. (2003), previous studies concerning M6G formation by UGT2B7 assumed that the kinetic properties were not biphasic. Apparent $K_m$ values reported were 1.3 mM (Coffman et al., 1997), 0.43 to 0.67 mM (Coffman et al., 1998), 0.24 mM (Soars et al., 2003), and 0.63 mM (Court et al., 2003).

The present findings have clearly demonstrated the contribution of UGT1A1 and 1A8 to morphine-6-glucuronidation at comparatively low morphine concentrations (<100 μM) by kinetic analysis. We also compared the kinetics to the kinetic properties of morphine-6-glucuronidation by UGT2B7, an isozyme widely recognized as mediating morphine-6-glucuronidation, appear to be biphasic. Stone et al. (2003) described the biphasic kinetic properties based on a biphasic Michaelis-Menten model or a two-site model and apparent $K_m$ values of 0.97 and 13.7 mM for the high- and low-affinity components, respectively, using UGT2B7 expressed in HK293 cells. In this article, using the isozyme from baculovirus-infected insect cells, we also demonstrated that M6G formation by UGT2B7 exhibited biphasic Michaelis-Menten kinetics. The apparent $K_m$ values from fitting to the biphasic Michaelis-Menten model are 99.6 μM for the high-affinity component and 13.3 mM for the low-affinity component. There is a slight difference between the $K_m$ values reported by Stone et al. and by us. This difference remains to be clarified. As pointed out by Stone et al. (2003), previous studies concerning M6G formation by UGT2B7 assumed that the kinetic properties were not biphasic. Apparent $K_m$ values reported were 1.3 mM (Coffman et al., 1997), 0.43 to 0.67 mM (Coffman et al., 1998), 0.24 mM (Soars et al., 2003), and 0.63 mM (Court et al., 2003).
ronidation catalyzed by UGT2B7. UGT1A1 is one of the major isozymes expressed in human liver and is also expressed in stomach, intestine, and colon (Tukey and Strassburg, 2000). UGT1A8 was found to be expressed in human intestine and colon but not in the liver (Cheng et al., 1998; Tukey and Strassburg, 2000). However, there is no report concerning their quantitative determination. The gastrointestinal tract has important barrier functions, and UGT1A1 and 1A8 may play important roles in the detoxification of xenobiotics in the human intestine. To estimate the contributions of these UGT isozymes to M6G formation in vivo, we quantitatively determined the expression levels of UGT1A1 and 1A8 in human liver and intestine using a reverse transcription real-time PCR method. We have established a reverse transcription real-time PCR method for selective and specific determination of the expression of UGT isozymes (S. Ohno and S. Nakajin, manuscript in preparation). Consequently, UGT2B7 expression, which was used for comparison, in the intestine was 63% of the level in the liver. UGT 1A1 was expressed in the intestine, the level of which was 30.2% compared with intestinal UGT2B7. UGT1A8 was also expressed in the intestine, and the level was 3.3% that of intestinal UGT2B7. UGT1A1 expression in the liver was 30.6% that of UGT2B7, whereas UGT1A8 was hardly expressed in the liver.

When morphine is administered orally, a portion of it may be metabolized to M6G in the intestinal tract in the initial phase of the metabolism. Taking into consideration the in vivo kinetics of morphine used clinically, it is conceivable that millimolar concentrations of morphine will be reached during its metabolism. Viewed in this light, we speculate that UGT1A1 and also 1A8 expressed in the intestine may play a critical role in the formation of M6G before the first-pass effect in the liver. Furthermore, some of the morphine may be metabolized in the liver by not only UGT2B7 but also UGT1A1 which was also expressed in the liver. For this reason, M6G formation catalyzed by UGT1A1 or 1A8 at relatively low morphine concentrations is very interesting in terms of mediation of morphine actions and mechanisms. Med Res Rev. 25:521–544.

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