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Glucuronidation, a New Metabolic Pathway for Pyrrolizidine Alkaloids

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Pyrrolizidine alkaloids (PAs) possess significant hepatotoxicity to humans and animals after metabolic activation by liver P450 enzymes. Metabolism pathways of PAs have been studied for several decades, including metabolic activation, hydroxylation, *N*-oxidation, and hydrolysis. However, the glucuronidation of intact PAs has not been investigated, although glucuronidation plays an important role in the elimination and detoxication of xenobiotics. In this study, PAs glucuronidation was investigated, and three important points were found. First, we demonstrated that senecionine (SEN)—a representative hepatotoxic PA—could be conjugated by glucuronic acid via an *N*-glucuronidation reaction catalyzed by uridine diphosphate glucuronosyl transferase in human liver microsomes. Second, glucuronidation of SEN was catalyzed not only by human but also other animal species and showed significant species differences. Rabbits, cattle, sheep, pigs, and humans showed the significantly higher glucuronidation activity than mice, rats, dogs, and guinea pigs on SEN. Kinetics of SEN glucuronidation in humans, pigs, and rabbits followed the one-site binding model of the Michaelis−Menten equation, while cattle and sheep followed the two-sites binding model of the Michaelis−Menten equation. Third, besides SEN, other hepatotoxic PAs including monocrotaline, adonifoline, and isoline also underwent *N*-glucuronidation in humans and several animal species such as rabbits, cattle, sheep, and pigs.

Introduction

Pyrrolizidine alkaloids (PAs) such as senecionine (SEN), adonifoline (ADO), monocrotaline (MON), and isoline (ISO) are common constituents of many plant species around the world (*1*). PAs are known to cause severe toxic effects, primarily to the hepatic system, and are thought to be the chemical basis of a natural, deterrent, defense mechanism developed by plants against animal attacks (*2*). Toxicity reports due to PAs have been described in different animal habitats—those in the wild, among domesticated livestock, and in experimental animals (*3*). PAs also are highly toxic to humans when ingested as food or in the form of traditional or herbal medicines, as described in numerous reports of PA-induced human poisoning (*4*). Even up to 1995, approximately 6000 people globally became the victims of PA-induced toxicity (*1*).

It has been demonstrated that metabolic activation of intact PAs is the key reason for PA-induced toxicity (*3*). P450 enzymes are responsible for most of the metabolic activation in which the nontoxic intact PAs are converted to the toxic reactive intermediate (dehydrogenated pyrrolizidine, DHP) (*3*). The reactive intermediate subsequently induces various toxic effects via covalent conjugation with proteins (*5*) and nucleic acids (*6*). Besides P450 enzymes, several other metabolizing enzyme systems including the esterase and flavin-containing monooxygenase (FMO) also participate in the metabolisms of PAs such as hydrolysis (*7*) and *N*-oxidation (*8*). In contrast to the activation of P450 enzymes on PAs, esterase and FMO may play a detoxication role via competitive consumption of intact PAs, resulting in a reduction of available PAs for P450 activation (*9*).

Besides the P450 enzymes, uridine diphosphate glucuronosyl transferases (UGTs) are considered as the other important metabolizing enzyme family (*10*). UGTs play an important role in the metabolism, excretion, and even detoxication of many xenobiotics, some of which possess a chemical structure similar to PAs (*11*). However, it has not been established if PAs can be metabolized by the enzymes of the UGT family.

The purpose of the present study is to investigate if the UGTs in human liver can metabolize PAs, if other species besides humans can catalyze PAs glucuronidation, and whether glucuronidation is an accidental phenomenon of a specified PA or a common metabolic process of most PAs. As SEN is one of the most widely investigated PAs, it was selected to be the model compound. Other toxic PAs such as ADO, MON, and ISO were also included in this study to confirm the results.

Materials and Methods

Materials. Uridine diphosphate glucuronic acid (UDPGA), β-glucuronidase, alamethicin, hecogenin, fluconazole, trifluoperazine, azidothymidine, glucose-6-phosephate (G-6-P), NADP Na_2, and G-6-P dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO). PAs (Figure 1) including SEN, ADO, MON, and ISO were isolated from *Senecio vulgaris*, *Senecio scandens*, *Crotalaria sessiliflora*, and *Ligularia duciformis*, respectively. Protocatechuic aldehyde (PAL) glucuronide was isolated from the in vitro

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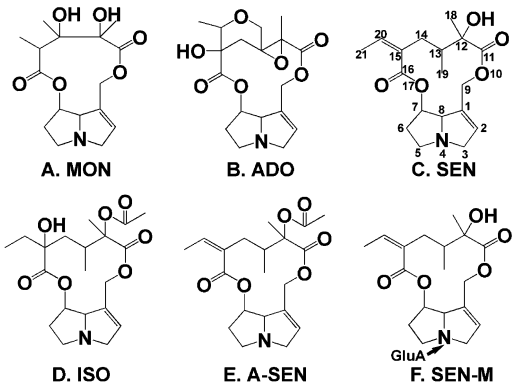

Figure 1. Structures of MON, ADO, SEN, ISO, A-SEN, and SEN-M.

microsomal incubation system for PAL glucuronidation. DHP was synthesized following the published method (*12*). Their structures were identified via NMR spectra, while the purities were more than 99% assayed via HPLC-DAD and HPLC-MS. SEN *N*-oxide was synthesized following the published method (*13*), and its structure was identified in our previous study (*14*) via NMR and MS analysis. Human liver microsomes were purchased from Rild Research Institute for Liver Diseases (Shanghai, China). Human intestinal microsomes were purchased from BD genest Corp. (Woburn, MA). Research involving human subjects was done under full compliance with government policies and the Helsinki Declaration. All other reagents were of HPLC grade or of the highest grade commercially available.

Preparation of Animal Liver Microsomes. Rat (SD), mouse (Swiss), guinea pig (DHP), rabbit (New Zealand white), and dog (beagle) livers were obtained from corresponding healthy experimental animals from the Experimental Animal Center of Shanghai University of Traditional Chinese Medicine (SUTCM, Shanghai, China). Pig, cattle, and sheep livers were freshly and legally obtained from animals in farms (Shanghai, China). The use of livers in the present study was conducted with the approval of the ethics committee of SUTCM. Liver samples of each species were from six individuals and were pooled for the preparation of microsomes. Liver specimens were stored in liquid nitrogen immediately after being harvested until the preparation of microsomes. Microsomes were prepared from pooled liver tissues by differential ultracentrifugation as previously described (*15*). The protein concentration was determined using bovine serum albumin as a reference (*16*). Liver microsomes were diluted to 10 mg/mL and stored at −80 °C.

Parameters for Ultra Performance Liquid Chromatography (UPLC) and Mass Spectrometry. A UPLC coupled with a triple-quadrupole mass spectrometry system (Acquity-Premier, Waters, Milford, MA) was introduced for analysis of glucuronidation metabolites of SEN, ADO, MON, and ISO. A Waters (Milford, MA) BEH C_{18} column (2.1 mm × 50 mm, 1.7 μm) was used for separation. Acetonitrile and formic acid (0.1% in water) were used as mobile phases A and B, respectively. The eluting gradient was set as follows: 0−1 min, 5% of A; 1−2.5 min, 5−17% of A; 2.5−3 min, 17−20% of A; 3−4 min, 20−35% of A; and 4−6 min, 35−50% of A. The flow rate was set at 0.3 mL/min. Mass spectrometry parameters were set as follows: capillary voltage, 4.2 kV; cone voltage, 45 V; extractor voltage, 3 V; RF lens, 0; source temperature, 120 °C; desolvation temperature, 300 °C; and cone and desolvation gas flow, 60 and 600 L/h, respectively.

Standard UGT Incubation System. A standard incubation system with total volume of 200 μL for UGT reactions contained liver microsomes (0.5 mg/mL total protein concentraion) of humans or animals including rats, mice, guinea pigs, pigs, dogs, sheep, cattle, or rabbits, alamethicin (25 μg/mg protein), UDPGA (5 mM), $MgCl_2$ (4 mM), Tris-HCl Buffer (50 mM, pH 7.4), and substrate (10 μL, dissolved in water phase). Reactions were started by adding UDPGA at 37 °C and stopped by adding ice-cold 10% trichloroacetic acid (200 μL) at 30 min. After the reaction was stopped, the incubation mixture was centrifuged at 20000*g* and 4 °C for 10 min. Aliquots of supernatant were subsequently transferred for analysis.

Mass Spectra Analysis of Metabolites. Three experimental groups were set to characterize the UDPGA-dependent product in human liver microsomes: a reaction group with all reaction factors described in standard UGT incubation system, a negative control group without UDPGA, and a blank group without SEN. A MS method for the full scan was used to obtain the molecular weight, and a MS/MS method for the daughter scan was used to obtain the MS/MS spectra. Molecular weight and MS/MS fragment ions of metabolites were compared with that of the parent compound to conclude the metabolite structure.

Enzymatic Hydrolysis of Glucuronidation Metabolite of SEN. To demonstrate whether the product was SEN *N*-glucuronide or *O*-glucuronide, the β-glucuronidase with a high efficiency on hydrolysis of *O*-glucuronide was used to hydrolyze the product. At first, SEN (100 μM) was incubated with human liver microsomes and UDPGA for the glucuronidation reaction at 37 °C. After 30 min, an aliquot of the UGT reaction mixture was mixed with the same volume of β-glucuronidase (1800 Fishman units) and incubated for 2 h at 37 °C and pH 7.4. Another group was set as the negative control in which the β-glucuronidase was replaced by the Tris-HCl buffer with the same volume. An incubation containing β-glucuronidase and PAL glucuronide was set as the positive control (*17*).

Acetylation of SEN. To distinguish the glucuronidation site between the free hydroxyl and the N-atom on SEN, the glucuronidation study was designed to be performed when the free hydroxyl was absent. To block the free hydroxyl, SEN (5 mM) was acetylated in the reaction system (10 mL) containing acetic anhydride and pyridine with a ratio of 1:1 at 50 °C. After 60 min, 50 mL of distilled water was added, and the pH value was adjusted to 10 by ammonia−water. Subsequently, the product was extracted by chloroform (100 mL × 3). The chloroform layers were combined and dried to obtain the synthesized product. The structure and purity of the synthesized product were analyzed by the UPLC-MS/MS.

Glucuronidation of Acetyl-SEN (A-SEN). A-SEN (100 μM) was incubated with human liver microsomes in a standard UGT incubation system. The reaction time was 60 min, and other incubation parameters were similar to those described in the standard UGT incubation system. The other two groups without A-SEN or UDPGA were set as blank and negative controls, respectively. Samples were analyzed using UPLC-MS/MS.

Deacetylation of A-SEN *N*-Glucuronide. It was demonstrated that rat liver microsomes contained an esterase with high efficiency on deacetylation of ethyl ester (*18*). In the present study, rat liver microsomes were used to deacetylate A-SEN *N*-glucuronide. After the glucuronidation reaction of A-SEN in human liver microsome incubation system, an aliquot of the microsomal reaction mixture was mixed with fresh rat liver microsomes (0.5 mg/mL) and incubated at 37 °C for 60 min. A group was set as the negative control in which rat liver microsomes were replaced by the same volume of Tris-HCl buffer. After the two-step reaction, the incubation mixture was prepared for analysis on UPLC-MS/MS system.

Glucuronidation of SEN *N*-Oxide. In the chemical structure of SEN *N*-oxide, the N-atom of the tertiary amine was blocked. To examine whether the molecule could be conjugated by glucuronic acid while the N-atom was blocked, SEN *N*-oxide (100, 500, and 1000 μM) was incubated with human liver microsomes in a standard UGT incubation system. The reaction time was 60 min, and other incubation parameters were similar to the process of SEN glucuronidation as described in the standard UGT incubation system. The other two groups without SEN *N*-oxide or UDPGA were set as blank or negative controls, respectively. A full scan and selective ion reaction monitor mode on UPLC-MS/MS were used to characterize the UDPGA-dependent product of SEN *N*-oxide.

Comparison of Chemically Synthesized SEN *N*-Glucuronide with Microsome-Generated Glucuronidation Metabolite. SEN *N*-glucuronide was prepared following the published method for the synthesis of quaternary glucuronide (*19*). The ^{1}H NMR spectrum

of this product was obtained from a Bruker NMR analyzer (AV-400, Bruker, Billerica, MA) with the solvent of deuterated methanol and the internal standard of tetramethylsilane. This product was also analyzed by the high-resolution mass spectrometry (quadrupole-TOF, Waters Corp., Milford, MA) to obtain its exact molecular weight. The NMR spectrum and exact molecular weight were used to identify the structure of the synthesized product. Subsequently, the chemically synthesized product was analyzed on a UPLC-MS/MS system to test its retention time, molecular weight, and *m*/*z* values of MS/MS fragment. These parameters would be compared with that of the microsome-generated metabolite.

Chemical Inhibition on SEN *N*-Glucuronidation in Human Liver Microsomal Incubation System. Selective inhibitors of UGT 1A4 (hecogenin) and UGT 2B7 (fluconazole) were used to inhibit the SEN *N*-glucuronidation in human liver microsome (0.5 mg/mL) incubation system. The SEN concentration was set at 500 μM. Inhibitor concentrations were set at 25 (*20*) and 1000 μM (*21*) for hecogenin and fluconazole, respectively. Inhibition of azidothymidine (500 μM) glucuronidation and trifluoperazine (200 μM) glucuronidation by fluconazole and hecogenin was set as the positive controls for UGT 2B7 and UGT 1A4, respectively. Other incubation parameters were similar to the standard UGT incubation system.

SEN Glucuronidation in Liver Microsomes of Humans and Eight Animal Species. To evaluate the species difference on SEN glucuronidation, SEN was incubated with liver microsomes (0.5 mg/mL) of humans and eight animals including rat, mouse, guinea pig, dog, pig, cattle, sheep, and rabbit. Concentrations of SEN used in incubation system were set at 100, 500, and 1000 μM. Other incubation factors were similar to the standard UGT incubation system described above. Parameters for animal incubations were completely the same as those for humans. Reaction velocities of SEN glucuronidation were used to evaluate the metabolic efficiency.

Metabolic Activation of SEN in Liver Microsomes of Humans and Eight Animal Species. For comparison to SEN glucuronidation, metabolic activation of SEN in humans and animals was investigated. Animal species included rat, mouse, guinea pig, dog, pig, cattle, sheep, and rabbit. Concentrations of SEN used in the incubation system were set at 100, 500, and 1000 μM. Other incubation factors were shown as follows: G-6-P (100 mM), G-6-P dehydrogenase (1 unit/mL), NADP Na_2 (1 mM), $MgCl_2$ (4 mM), Tris-HCl buffer (100 mM, pH 7.4), and liver microsomes (0.5 mg/mL). The reaction system volume was 200 μL. Reactions were initiated by adding NADP Na_2 at 37 °C for 30 min and terminated by adding acetonitrile (1000 μL). Reaction velocities were calculated based on the metabolite (DHP) formation and used to evaluate the metabolic efficiency of humans and animal species.

Kinetic Study of SEN Glucuronidation in Humans, Pigs, Cattle, Sheep, and Rabbits. Kinetics of SEN glucuronidation was performed at concentrations from 10 to 1000 μM of SEN in human, pig, cattle, sheep, and rabbit liver microsomes. The protein concentration of microsomes was set at 0.5 mg/mL, and the reaction time was set at 30 min, which was linear with metabolite formation. Eadie−Hofstee plots were carried out to determine the kinetic type. Nonlinear regressions were performed from Michaelis−Menten equation of the one-site binding or two-sites binding model to obtain the K_m and V_{max} values. The enzyme efficiency on SEN glucuronidation was evaluated by the ratio of V_{max} to K_m.

Glucuronidation of MON, ADO, and ISO. To confirm whether glucuronidation was a common metabolic process of PAs or only an accidental phenomenon of SEN, another three toxic PAs including MON (Figure 1A), ADO (Figure 1B), and ISO (Figure 1D) were adopted in our in vitro metabolism study conducted in the human liver microsomal incubation system. The substrate concentration was set at 100 μM for all three compounds. In the same manner as SEN glucuronidation, three experimental groups including a reaction group, negative control, and blank group were set to characterize the UDPGA-dependent products for each of the following: ADO, MON, and ISO. Besides human liver microsomes, liver microsomes of eight animal species including rat, mouse, guinea pig, dog, pig, cattle, sheep, and rabbit were also adopted to test the activity of MON, ADO, and ISO glucuronidation, respectively.

SEN Glucuronidation in Human Intestinal Microsomes. In vitro intestinal glucuronidation of SEN was performed at 37 °C for 60 min. Incubation parameters were as follows: human intestinal microsomes (0.5 mg/mL), alamethicin (25 μg/mg protein), UDPGA (5 mM), $MgCl_2$ (4 mM), Tris-HCl Buffer (50 mM, pH 7.4), and substrate (10 μL, dissolved in water phase). The system volume was 200 μL. Reactions were started by adding UDPGA at 37 °C and stopped by adding ice-cold 10% trichloroacetic acid (200 μL) at 30 min. After the reaction was stopped, the incubation mixture was centrifuged at 20000*g* and 4 °C for 10 min. An aliquot of supernatant was transferred for analysis. An incubation including all reaction factors was set as the reaction group. Another two incubations without SEN or UDPGA were set as the blank or negative control. PAL glucuronidation at the present incubation parameters was set as the positive control (*17*).

Determination of SEN *N*-Glucuronide in Vivo. Cystic duct cannula operations were performed on rats and rabbits. After the operations, SEN was given to rats and rabbits at the dosage of 30 mg/kg. Bile and urine were collected from 0 to 6 h postprocedure, and the blood was sampled at 0.5, 1, 2, 4, and 6 h after administration of SEN. One hundred microliters of ice-cold 10% trichloroacetic acid was added to 100 μL of each of serum, bile, and urine. After centrifugation at 4 °C and 20000*g* for 10 min, 2 μL of the supernatant was transferred for analysis.

Quantification Method. The formation of SEN *N*-glucuronide and DHP was used to calculate the reaction velocity of glucuronidation and metabolic activation of SEN, respectively. Multiple reaction monitoring (MRM) mode with an ion transition from *m*/*z* 512 to 336 was used to quantify SEN *N*-glucuronide, and an ion transition from *m*/*z* 154 to 136 was used to quantify the DHP. All of the experiments for quantification were performed in duplicate in different microsomal incubations.

Results

Analysis of Mass Spectra of SEN Glucuronide. In the standard UGT incubation system, one unique UDPGA-dependent metabolite (SEN-M) was detected for SEN in the reaction group but not in both the negative control and the blank group (Figure 2A) The mass spectrometer gave a M_r of 511 Da, which was 176 Da larger than that of SEN (Figure 3C′), for this metabolite. Meanwhile, in MS/MS spectrum of SEN-M, the $[M + H - GluA]^+$ ion at *m*/*z* 336 (Figure 3C′) indicated the loss of a glucuronide residue. All of these three pieces of evidence proved this metabolite to be a glucuronide. MS/MS fragmentation showed that SEN-M and SEN had some shared fragment ions such as ions at *m*/*z* 120 and 138, indicating that SEN-M was derived from SEN. Combining all of this evidence observed from mass spectrometry, SEN-M could be tentatively assigned as SEN glucuronide.

Enzymatic Hydrolysis of SEN Glucuronide. According to the reported method (*22*), the enzymatic hydrolysis of SEN glucuronide was performed. As a result, the quantity of SEN-M did not decrease after the enzymatic hydrolysis, indicating SEN-M more likely to be *N*-glucuronide rather than *O*-glucuronide. In contrast, PAL glucuronide, an *O*-glucuronide reported by Liu et al (*17*), was successfully hydrolyzed by β-glucuronidase (data not shown), demonstrating the β-glucuronidase used in the present study possessed positive activity on hydrolysis of *O*-glucuronide.

Acetylation of SEN. After acetylation of SEN and purification, the product was analyzed on UPLC-MS/MS. In the total ion chromatogram, the product was eluted out at 4.80 min (Figure 4B). The $[M + H]^+$ ion at *m*/*z* 378 of the synthesized

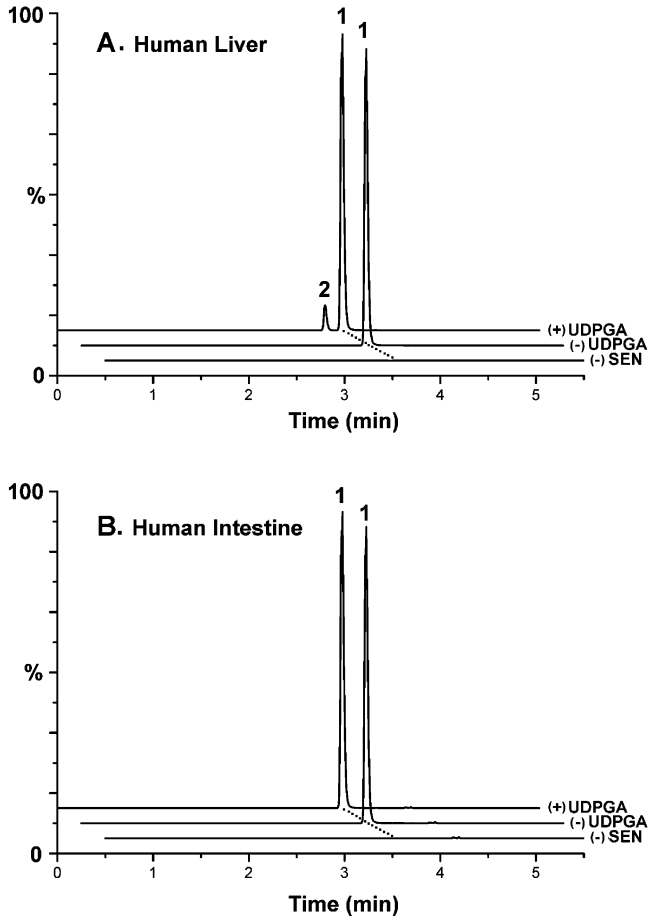

Figure 2. Microsomal formation of SEN glucuronide in human liver (A) and human intestine (B). One and two represent SEN and SEN *N*-glucuronide respectively. "(+) UDPGA" represents the reaction group with complete reaction factors, "(−) UDPGA" represents the negative control without UDPGA, and "(−) SEN" represents the blank group without SEN.

product (Figure 3E) corresponded with that of A-SEN, indicating that the SEN molecule was successfully acetylated. This was also demonstrated by the $[M + H - \text{acetyl}]^+$ ion at *m/z* 336. Meanwhile, the $[M + H - CH_3COOH]^+$ ion at *m/z* 318 indicated that the acetylation site was the free hydroxyl. Thus, the synthesized product was identified as 12-O-A-SEN (Figure 1E).

Glucuronidation of A-SEN. After incubation of A-SEN with human liver microsomes in the standard UGT incubation system, a unique new product was observed at 4.63 min of chromatogram (Figure 4C). The $[M + H]^+$ ion at *m/z* 554 and the $[M + H - \text{GluA}]^+$ ion at *m/z* 378 (Figure 3E′) indicated this product to be a glucuronide. Because the hydroxyl on SEN had been protected by acetyl, the N-atom of the tertiary amine in the pyrrole cycle was the only site that could be conjugated by the glucuronic acid. Thus, the glucuronidation product of A-SEN in human liver microsomes was identified as A-SEN *N*-glucuronide.

Deacetylation of A-SEN *N*-Glucuronide. After deacetylation of A-SEN *N*-glucuronide by rat liver microsomes, the peak area of A-SEN *N*-glucuronide decreased rapidly and a new product appeared at 3.28 min in the chromatogram (Figure 4D). This metabolite was given a molecular weight of 511, which is 42 Da smaller than A-SEN *N*-glucuronide, indicating that the deacetylation was successful and the SEN *N*-glucuronide was obtained. After comparison, the reference SEN *N*-glucuronide showed identical retention time, molecular weight, and MS/MS fragment ions to SEN-M (Figure 4E), proving that SEN-M was a *N*-glucuronidation product (Figure 1F).

Glucuronidation of SEN *N*-Oxide. SEN *N*-oxide was synthesized following the established method (*13*), and its structure was identified via ^{1}H NMR analysis in our previous study (*14*). The synthesized SEN *N*-oxide was incubated with human liver microsomes and other UGT reaction factors described in the standard UGT incubation system. As a result, none of new product for SEN *N*-oxide was observed in human liver microsomes (Figure 4G), indicating that the molecule of SEN would not undergo glucuronidation when the N-atom was blocked.

Comparison of Chemically Synthesized SEN *N*-Glucuronide with Microsome-Generated SEN Glucuronide. Following the published procedure, 1.6 mg of purified product was obtained. A high-resolution mass spectrometer gave an exact *m/z* value, for the protonated product, as 512.2136 (Table 1), which possessed a difference from the calculated value (512.2131) of SEN glucuronide as small as 0.97 ppm. To identify the conjugated site by glucuronic acid, the ^{1}H NMR spectrum of the synthesized product was compared to that of SEN. With this comparison, it was demonstrated that the signals of protons on the macrocycle (C9−C17, Figure 1C) of SEN were very close to that of the synthesized product (Table 1), but signals of protons on the pyrrole cycle (C1−C8) of SEN and the synthesized product showed significant difference, proving that the structural change occurred on the pyrrole cycle but not the macrocycle. This result indicated that the synthesized product was SEN *N*-glucuronide but not *O*-glucuronide. The assignment of the proton signals of SEN was obtained from the published data (*23*). The synthesized product was also analyzed on UPLC-MS/MS. The retention time, molecular weight, and the MS/MS fragment ions (Table 1) were completely the same as the microsome-generated glucuronidation metabolite of SEN. Thus, the glucuronidation product of SEN generated in human liver microsomal incubation system was identified as SEN *N*-glucuronide.

Chemical Inhibition on SEN *N*-Glucuronidation in Human Liver Microsomal Incubation System. Selective inhibitors of UGT 1A4 and UGT 2B7 were used to inhibit SEN *N*-glucuronidation in the human liver microsomal incubation system. It was found that hecogenin showed a significant inhibitory effect, but fluconazole did not inhibit SEN *N*-glucuronidation, proving that UGT 1A4 participated in the SEN *N*-glucuronidation but UGT 2B7 was not involved in this metabolic process (Figure 5). In positive controls, both UGT 1A4 and UGT 2B7 were significantly inhibited by their corresponding selective inhibitors, indicating that the result of chemical inhibition on SEN *N*-glucuronidation is credible. This result was consistent with the reports that UGT 1A4 is the main enzyme participating in the *N*-glucuronidation of compounds with tertiary amine groups (*24*).

SEN Glucuronidation in Humans and Eight Animal Species. To investigate whether SEN could be catalyzed by other species besides humans, SEN glucuronidation was studied in humans and eight animal species including rats, mice, guinea pigs, dogs, pigs, cattle, sheep, and rabbits. It was found that SEN glucuronidation could be observed in humans, pigs, cattle, sheep, and rabbits significantly but not significantly in guinea pigs, dogs, rats, and mice (Figure 6A). In rats and mice, even a near absence of activity was observed. Rabbits showed the highest metabolic activity (nearly two times greater than cattle and sheep) on SEN glucuronidation. To additionally understand the difference on SEN glucuronidation between various species,

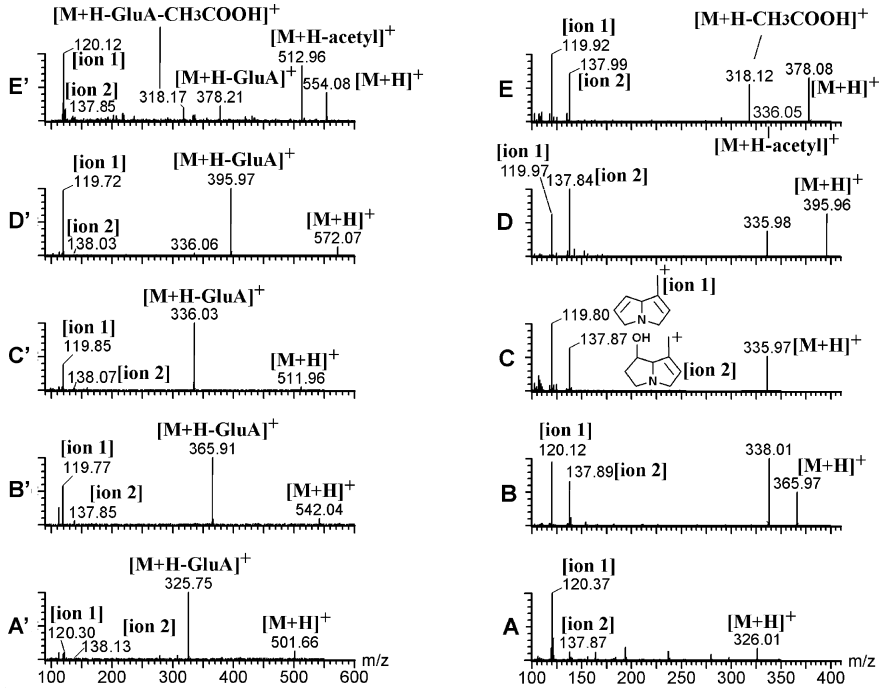

Figure 3. MS/MS spectrum of MON (A), ADO (B), SEN (C), ISO (D), and A-SEN (E) and their glucuronidation products including MON-M (A′), ADO-M (B′), SEN-M (C′), ISO-M (D′), and A-SEN-M (E′), respectively. The structures of ion 1 and ion 2 are shown in C.

the reaction velocities of SEN glucuronidation in each species were compared with each other. According to the comparison, the activity of SEN glucuronidation in all species investigated in the present study showed an order as follows: rabbits, humans, cattle, pigs and sheep > guinea pigs, dogs, rats, and mice.

Metabolic Activation of SEN in Humans and Eight Animal Species. P450-mediated metabolic activation of SEN was observed in human and all animal species (Figure 6B). Rodent animals including mice, rats, and guinea pigs showed the highest activity on metabolic activation of SEN among all species investigated in the present study. The activity on metabolic activation of SEN in dogs was lower than in rodent animals but significantly higher than in humans and other animal species. Pigs, cattle, sheep, and rabbits showed a similar activity to human. Activities on metabolic activation of SEN in all species showed an order as follows: rats, mice and guinea pigs > dogs > humans, pigs, cattle, sheep, and rabbits.

Kinetics of SEN Glucuronidation in Human, Pig, Cattle, Sheep, and Rabbit Liver Microsomes. Kinetic studies of SEN glucuronidation in humans, pigs, cattle, sheep, and rabbits were performed at a SEN concentration from 10 to 1000 μM (Figure 7). According to Eadie−Hofstee plots (Figure 7B), humans, pigs, and rabbits followed the typical one-site binding model of the Michaelis−Menten equation, while cattle and sheep followed the two-sites binding model of the Michaelis−Menten equation. Fitting to corresponding equations via nonlinear regressions, kinetic parameters for these five species were obtained (Table 2). To additionally evaluate the clearing capacity of these five species on SEN via glucuronidation pathway, the V_{max}/K_m values were calculated. It was found that cattle showed the highest enzyme efficiency followed by rabbits, although SEN glucuronidation showed higher reaction velocities in rabbits than in cattle at substrate concentrations of 100, 500, and 1000 μM. The clearing capacity of sheep was similar to humans. Rabbits and pigs showed the enzyme efficiency 5.5 and 2.5 times larger than humans, respectively. Thus, the capacity, clearing the SEN via glucuronidation pathway, of these five species showed an order as follows: cattle > rabbits > pigs > humans and sheep.

Glucuronidation of MON, ADO, and ISO. In the standard UGT incubation system in human liver microsomes, one unique UDPGA-dependent metabolite was detected for each of MON, ADO, and ISO, respectively (Figure 8). These metabolites were labeled as MON-M, ADO-M, and ISO-M, respectively. By mass spectrometer, M_r values 176 Da bigger than their parent compounds, were given for these metabolites (Figure 3), indicating that all of them were glucuronides. Meanwhile, $[M + H - GluA]^+$ ions at *m/z* 326, 366, and 396 were observed for ADO-M, MON-M, and ISO-M, respectively, additionally proving the existence of a glucuronic acid residue on each of them. Besides human, glucuronidation studies of these three compounds were also performed in eight animal species including rats, mouse, guinea pigs, dogs, pigs, cattle, sheep, and rabbits. As a result, all of the activities for MON, ADO, and ISO glucuronidation showed the same species differences as those of SEN glucuronidation (Table 3). These data indicated that the glucuronidation process was not an accidental phenomenon of SEN but a common metabolic pathway of other toxic PAs.

SEN Glucuronidation in Human Intestinal Microsomes. In the human intestinal microsome incubation system, the PAL glucuronide was detected, demonstrating the positive activity, on glucuronidation, of human intestinal microsomes used in the present study. However, the glucuronidation product of SEN was not observed, not only in negative control and blank control but also in the complete reaction samples (Figure 2B), indicating that SEN would not be catalyzed by human intestinal UGTs.

Determination of SEN *N*-Glucuronide in Vivo. The biosamples including serum, bile, and urine, obtained from the rabbits and rats fed with SEN, were assayed on UPLC-MS/MS. As a result, SEN *N*-glucuronide was detected in all of serum, bile, and urine samples of rabbits (Table 4), with the highest concentration in the bile. Conversely, in all of the in vivo samples of rats, there was no SEN *N*-glucuronide observed. These data were consistent with the species difference of SEN *N*-glucuronidation observed in the liver microsome incubations in the present study.

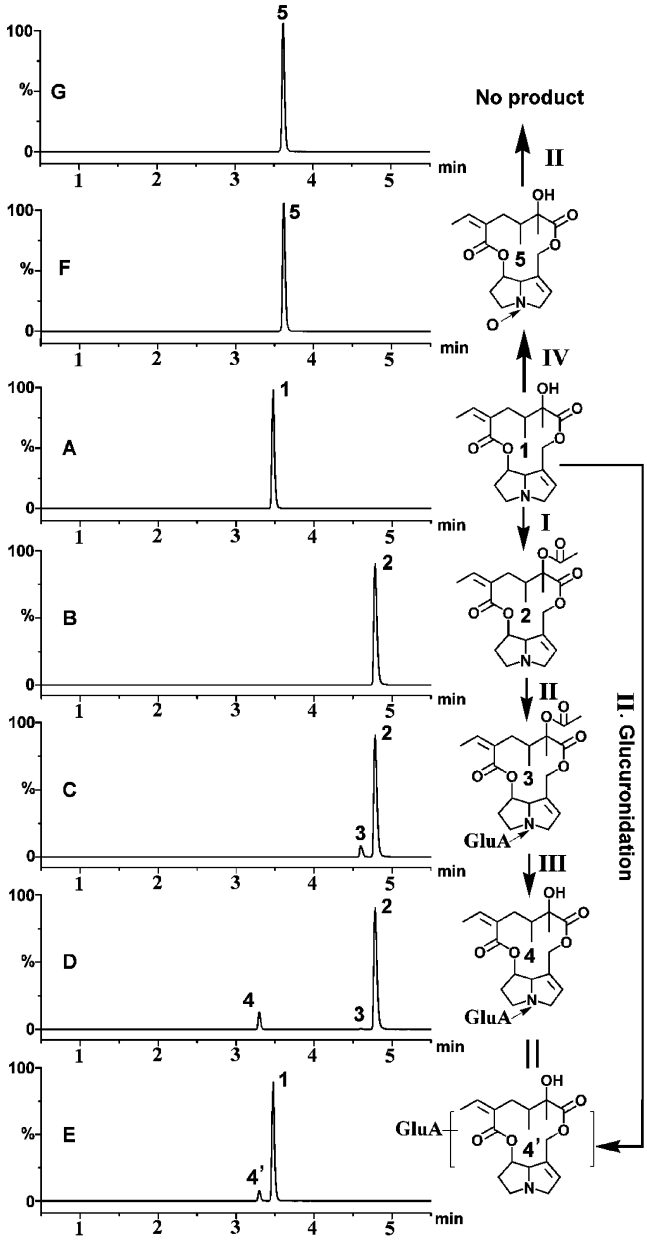

Figure 4. Process for identification of the glucuronidation site on SEN. I represents the acetylation, II represents the glucuronidation, III represents the hydrolysis, and IV represents the *N*-oxidation reaction. The Arabic numbers represent SEN (1), A-SEN (2), A-SEN *N*-glucuronide (3), SEN *N*-glucuronide (4), and SEN *N*-oxide (5), respectively. 4′ represents the microsome-formed glucuronidation product of SEN.

Table 1. ^{1}H-NMR, MS Spectrometry, and Chromatography Data of SEN, Synthesized Product, and Microsome-Generated Product[a]

position	SEN	synthesized product	microsome-generated product
	^{1}H NMR data of protons on pyrrole cycle		
2	6.19 (1H, br s)	6.15 (1H, br s)	
3a	3.92 (1H, dd)	4.90 (1H, d)	
3b	3.35 (1H, m)	4.33 (1H, m)	
5a	3.27 (1H, m)	4.27 (1H, m)	
5b	2.56 (1H, m)	3.71 (1H, m)	
6a	2.37 (1H, m)	2.69 (1H, m)	
6b	2.16 (1H, m)	2.37 (1H, m)	
7	5.08 (1H, m)	5.46 (1H, m)	
8	4.32 (1H, br s)	5.71 (1H, br s)	
	^{1}H NMR data of protons on macrocycle		
9a	5.49 (1H, d)	5.25 (1H, d)	
9b	4.15 (1H, d)	4.20 (1H, d)	
13	1.65 (1H, m)	1.61 (1H, m)	
14a	2.23 (1H, m)	2.15 (1H, m)	
14b	1.80 (1H, m)	1.80 (1H, m)	
18-Me	1.35 (3H, s)	1.24 (3H, s)	
19-Me	0.90 (3H, d)	0.83 (3H, d)	
20	5.81 (1H, br s)	5.87 (1H, br s)	
21-Me	1.82 (3H, dd)	1.82 (3H, dd)	
	high-resolution mass spectrometry		
$[M + H]^+$	336.1806	512.2136	512.2138
	MS/MS fragments		
$[M + H - GluA]^+$		336	336
ion 1	120	120	120
ion 2	138	138	138
	retention time		
	3.5 min	3.28 min	3.28 min

[a] Chemical shifts are expressed in parts per million. s, singlet; d, doublet; dd, double doublet; t, triplet; tt, triple triplet; q, quadruplet; br s, broad singlet; and m, multiplet. Structures of ion 1 and ion 2 are shown in Figure 3C.

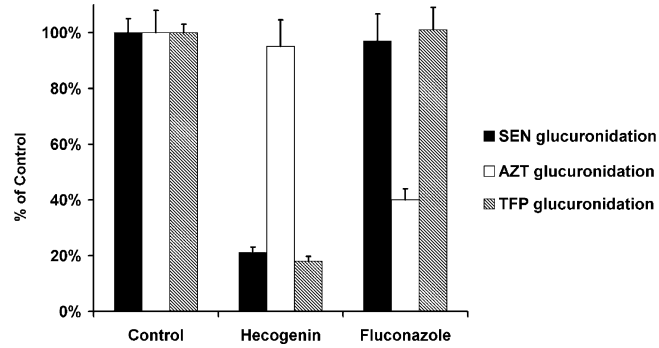

Figure 5. Chemical inhibition of SEN *N*-glucuronidation by fluconazole and trifluoperazine. Inhibition of azidothymidine (AZT) glucuronidation by fluconazole and trifluoperazine (TFP) glucuronidation by hecogenin are set as the positive controls. The metabolic activities for SEN, AZT, and TFP glucuronidation are compared with their correspondent solvent controls, which are normalized to 100%.

Discussion

Although both high-resolution mass spectrometer and the MS/MS fragment ions demonstrated that a glucuronic acid was added on SEN, the glucuronidation site could not be distinguished between the hydroxyl and the N-atom. To solve this problem, multiple identification pathways were adopted, and the metabolite was finally identified as *N*-glucuronide instead of *O*-glucuronide based on the following evidence from our experiments: (1) *N*-Oxidation but not the hydroxyl-protection could block SEN glucuronidation, which meant that the free tertiary amine was vital to this conjugation; (2) microsome-generated metabolite showed the same retention time, molecular weight, and MS/MS fragment ions as the reference of SEN *N*-glucuronide obtained from hydrolysis of A-SEN *N*-glucuronide; (3) as indirect evidence, β-glucuronidase with high activity on hydrolysis of *O*-glucuronide could not hydrolyze this metabolite to SEN; (4) direct evidence from the ^{1}H NMR analysis—signals of protons close to the N-atom but not hydroxyl of SEN glucuronide—showed significant changes from SEN, proving this metabolite to be a *N*-glucuronide.

As the N-atom of the tertiary amine is a shared structure of most PAs (*3*), the *N*-glucuronidation may be a common metabolic pathway for most PAs, which is essential to be confirmed because over 6000 PAs have been indentified occurring naturally in various plants (*25*). To confirm this, three more PAs including ADO, MON, and ISO were adopted for glucuronidation by liver microsomes of humans and various

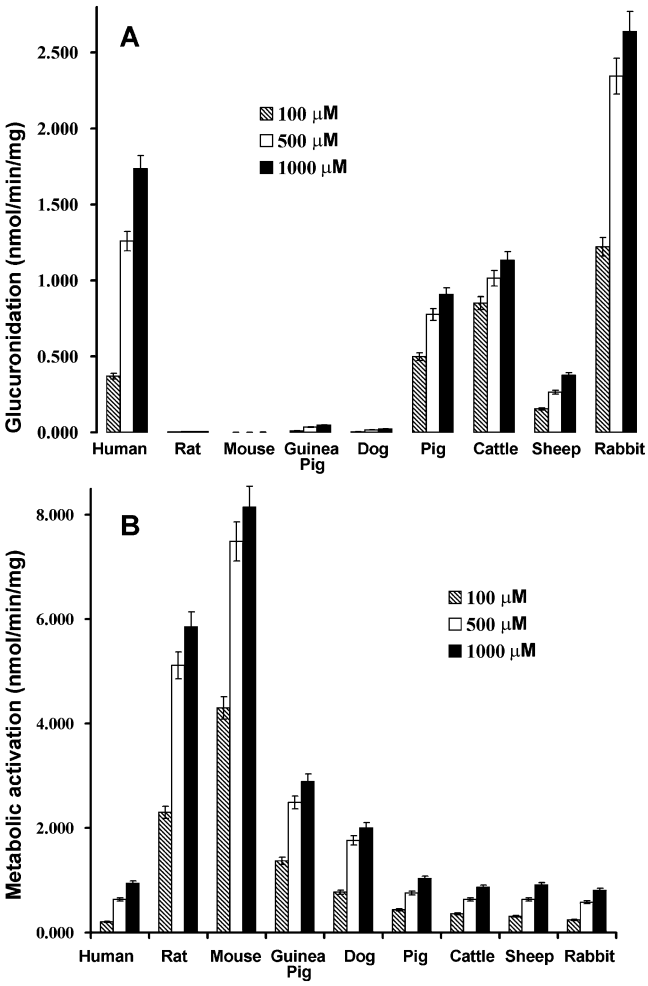

Figure 6. Activities for *N*-glucuronidation (A) and P450 activation (B) of SEN in human and eight animal species. Concentrations labeled in the figures represent the substrate (SEN) concentrations.

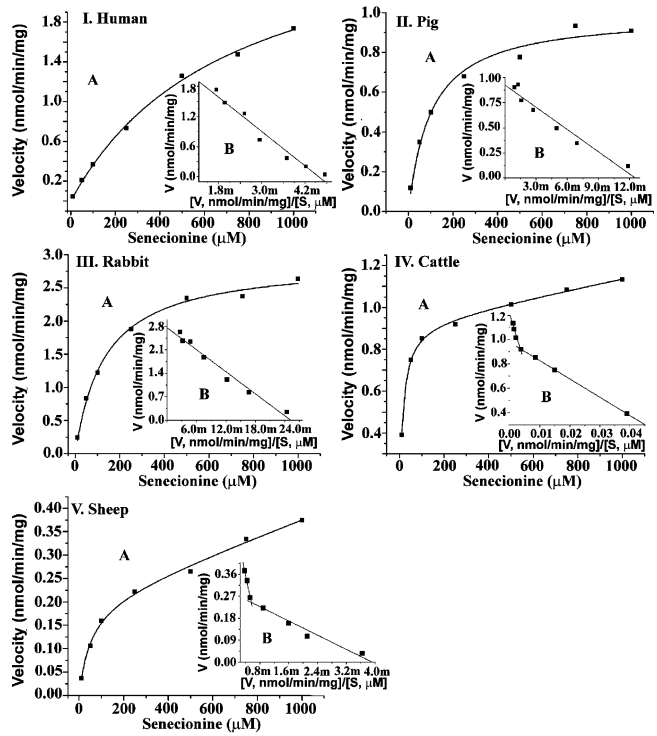

Figure 7. Kinetics of SEN glucuronidation in humans (I), pigs (II), rabbits (III), cattle (IV), and sheep (V). A represents the Michaelis−Menten plots, and B represents the Eadie−Hofstee plots. "m" on the coordinate axis represents "10^{-3}". All data were obtained from duplicate experiments in different microsome incubations.

Table 2. Kinetic Parameters of SEN Glucuronidation in Liver Microsomes of Humans, Pigs, Cattle, Sheep, and Rabbits

species	V_{max} (nmol/min/mg)	K_m (μM)	enzyme efficiency[b] (mL/min/mg)
human	$3.0 \times 10^{0} \pm 1.5 \times 10^{-1}$	$7.1 \times 10^{2} \pm 7.0 \times 10$	4.0×10^{-3}
pig	$9.9 \times 10^{-1} \pm 3.7 \times 10^{-2}$	$1.0 \times 10^{2} \pm 1.5 \times 10$	1.0×10^{-2}
cattle[a]	$9.8 \times 10^{-1} \pm 3.0 \times 10^{-3}$	$1.5 \times 10 \pm 2.0 \times 10^{-1}$	6.5×10^{-2}
	$1.2 \times 10^{0} \pm 2.9 \times 10^{-2}$	$8.3 \times 10 \pm 1.4 \times 10$	1.5×10^{-2}
sheep[a]	$3.1 \times 10^{-1} \pm 8.0 \times 10^{-3}$	$9.6 \times 10 \pm 7.5 \times 10^{0}$	3.0×10^{-3}
	$6.4 \times 10^{-1} \pm 3.1 \times 10^{-2}$	$6.9 \times 10^{2} \pm 7.2 \times 10$	1.0×10^{-3}
rabbit	$2.9 \times 10^{0} \pm 7.1 \times 10^{-2}$	$1.3 \times 10^{2} \pm 1.2 \times 10$	2.2×10^{-2}

[a] In cattle and sheep, there are two components that participate in the SEN *N*-glucuronidation; thus, parameters are calculated for these two components separately. [b] Enzyme efficiency is evaluated by V_{max}/K_m. Results are expressed as estimates ± SE.

animal species. ADO is the main constituent in *S. scandens*, which is used as the legal herb material of a traditional Chinese compound prescription (*26*). MON has ever been used as a compound in the treatment of skin cancer and demonstrated to have lung toxicity (*27*). ISO is a main constituent of *Ligularia duciforms* (*18*), a native herb of China. All of these three compounds possess an unsaturated structure between C1 and C2 on the pyrrole cycle and, together with PAs with this kind of structure, are considered to be potentially toxic and carcinogenic (*25*). In the present study, in addition to SEN, these three PAs were also found to be metabolized via the glucuronidation pathway in microsomes of humans and several animal species, indicating that glucuronidation is not an accidental phenomenon of SEN metabolism. This observation is consistent with our previous conclusion based on the glucuronidation site.

SEN glucuronidation was observed in humans and eight animal liver microsomes with significant species differences. In human, rabbit, cattle, sheep, and pig liver microsomes, SEN glucuronidation could be observed clearly; however, rats, mice, guinea pigs, and dogs failed to generate this metabolite significantly. This is very consistent with the report on the species difference for the formation of quaternary *N*-glucuronides via *N*-glucuronidation of tertiary amines (*24*). According to the reports, quaternary *N*-glucuronides could be observed in humans, nonhuman primates, and rabbits, but not in rats, mice, and guinea pigs (*24*). In dogs, only trace amounts of this type of product could be observed in urine (*24*). There is published evidence attributing this type of biotransformation to UGT 1A4 (*22, 24, 28*), an important enzyme responsible for *N*-glucuronidation of tertiary amines. Rats and mice have been demonstrated to be the species lacking expression of UGT 1A4 (*10*). Conversely, this enzyme is highly expressed in rabbits (*24*). That may be the reason the activity of SEN *N*-glucuronidation showed such significant differences between rabbits and rodent animals.

Although the liver plays the predominant role on most metabolisms of xenobiotics, other extrahepatic tissues may also be involved in metabolic processes such as glucuronidation. It is recognized that, in addition to the liver, the small intestine also participates in the first-pass process of some orally administrated drugs. Many xenobiotics such as PAL (*17*) and flavonoids (*29*) have been demonstrated to undergo *O*-glucuronidation in the small intestine. However, the activity of *N*-glucuronidation for tertiary amines has not been detected in human small intestines (*10, 30*), which was also proved by our present study (Figure 2B). For rats, mice, and guinea pigs, not only the livers but also the small intestines and other tissues

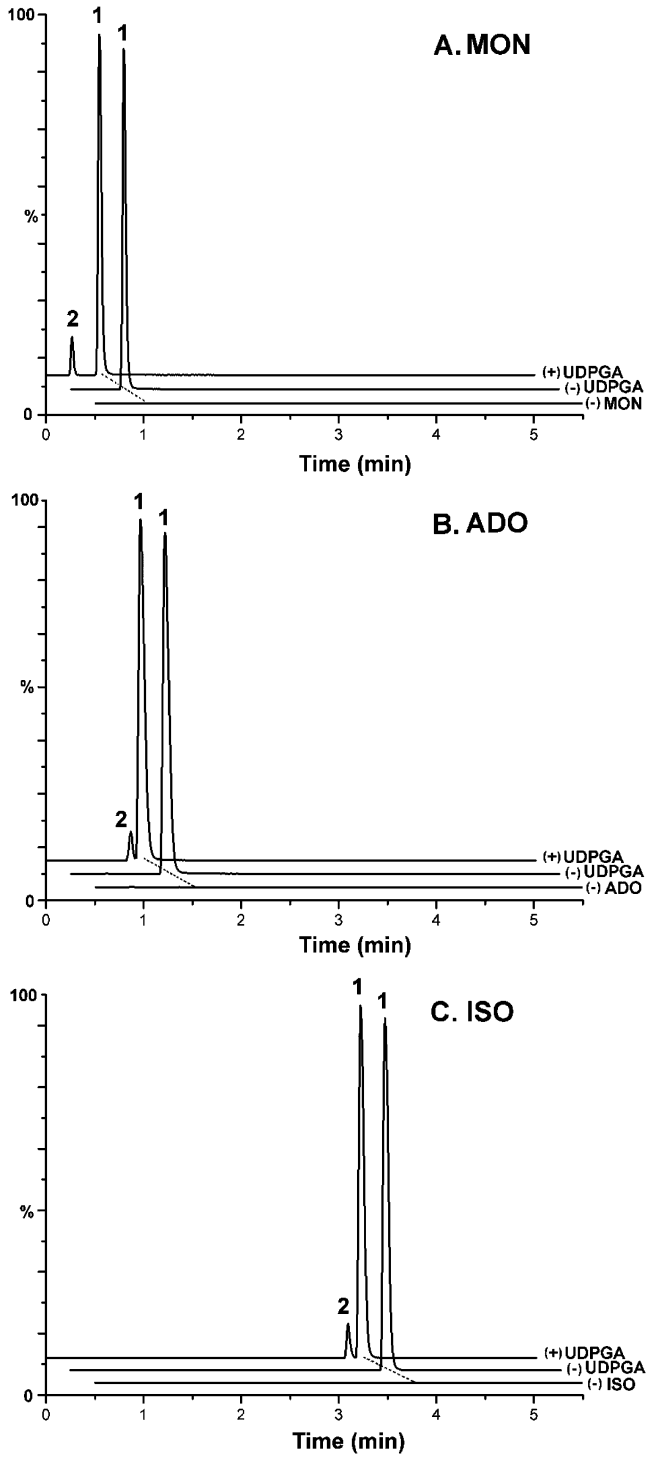

Figure 8. Human liver microsomal formation of glucuronidation products of MON (A), ADO (B), and ISO (C). "1" and "2" represent the parent compounds and metabolites, respectively. "(+) UDPGA" represents the reaction group, and "(−) UDPGA" represents the negative control. "(−) MO*N*", "(−) ADO", and "(−) ISO" represent the blank groups.

fail to catalyze *N*-glucuronidation of tertiary amines because of the lack of expression of UGT 1A4 (*31*−*33*). For rabbits, several UGTs have been detected in the small intestines; however, none of these enzymes could catalyze *N*-glucuronidation of tertiary amines (*33*). In summary, for SEN *N*-glucuronidation, the liver occupies the major metabolism position in humans, rats, mice, guinea pigs, and rabbits. In dogs, in vivo experiments indicated that only trace amounts of quaternary glucuronidation product could be detected (*24*), which implied

Table 3. Species Difference of Glucuronidation Activities on Four PAs among Human and Eight Animal Species[a]

	human	rat	mouse	guinea pig	dog	pig	cattle	sheep	rabbit
SEN	+	−*	−	L	L	+	+	+	+
ADO	+	−	−	−	−	+	+	+	+
MON	+	−	−	−	−	+	+	+	+
ISO	+	−	−	L	L	+	+	+	+

[a] "+" represents significant activity, "−" represents no activity, and "L" represents weak activity. *The limit of detection of PAs is 0.5 ng/mL.

Table 4. SEN *N*-Glucuronide in Biosamples of Rabbits and Rats[a]

	bile	serum	urine	liver microsome
rabbits	+	+	+	+
rats	−*	−	−	−

[a] "+" and "−" represent that SEN *N*-glucuronide was or was not detected, respectively. *The limit of detection of PAs is 0.5 ng/mL.

that the activities of both hepatic and intestinal *N*-glucuronidation of tertiary amines in dogs were low. For cattle, sheep, and pigs, there is no published report as yet on their intestinal activity of *N*-glucuronidation of tertiary amines. Thus, besides the livers, whether the small intestines of these three animal species participate in the SEN glucuronidation is still not clear. In the present study, we demonstrated that the catalytic capacity in livers of humans and eight animal species on SEN glucuronidation showed an order as follows: rabbits, humans, cattle, pigs and sheep > guinea pigs, dogs, rats, and mice. This order may not be changed even when both hepatic and intestinal metabolic capacity were considered, because of the following two reasons: (1) Humans and five animal species including rats, mice, guinea pigs, dogs, and rabbits showed no activity on SEN glucuronidation in their small intestines, and (2) despite the intestinal metabolic capacities, the hepatic *N*-glucuronidation activities of cattle, sheep, and pigs were found to be significantly and absolutely higher than rodent animals and dogs.

Although there was no tested toxicity data present in this study, the species differences on SEN *N*-glucuronidation were consistent with the known toxicity reports. For example, rabbits are considered as a resistant species to PA-induced toxicity (*3*), while this species shows high activity on SEN *N*-glucuronidation (Figure 6). Rats, mice, and dogs are susceptible species to PA-induced toxicity because of their high activity on metabolic activation of SEN (*8*), while they show nearly no activity on SEN *N*-glucuronidation (Figure 6). These phenomenons imply that the glucuronidation of SEN, to a certain degree, contributes to detoxication of PAs in some species. This observation can be drawn because the high activity of non-P450 enzymes can significantly consume the intact PAs and prevent PAs from metabolic activation by P450 enzymes (*9*). For guinea pigs, although nearly no glucuronidation activity on SEN was observed, this species was also found to be resistant to PA-induced toxicity (*3*). This resistance could be explained by the high activity of a special carboxyesterase named GPH1 in guinea pigs (*7*). This enzyme reduces the intact PAs via high efficient hydrolysis, preventing PAs from the metabolic activation by P450 enzymes. In humans, because of the considerable SEN *N*-glucuronidation activity observed, it would therefore be essential to evaluate, with further studies, if this metabolic pathway is directly, and significantly, related to SEN-induced toxicity. The role of glucuronidation of SEN in cattle and sheep can not be evaluated at present because of lack of data on their susceptibility to PA-induced toxicity.

In conclusion, a glucuronidation metabolite was discovered for SEN and was exactly identified as SEN *N*-glucuronide using multiple methods. Additionally, metabolic efficiency of SEN glucuronidation in various species was investigated in this study and showed significant species differences. This metabolic process was demonstrated to not be an accidental phenomenon of SEN metabolism; several other toxic PAs also could be catalyzed by UGTs in human, rabbit, cattle, sheep, and pig livers. For PAs, this is an important discovery because there is no published report on their glucuronidation metabolism, and in addition to P450-mediated metabolisms, the role of PAs glucuronidation should be given a notable consideration in the further toxicological studies.

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