Isolation and Identification of Diglucuronides of Some Endogenous Steroids in Dogs

Takahiro Murai, Naotoshi Yamamura, Takashi Nitanai, Naozumi Samata, Makoto Takei, Haruo Iwabuchi, Kohji Tanaka, Kei Mikamoto, and Toshihiko Ikeda

Drug Metabolism and Pharmacokinetics Research Laboratories (T.M., N.Y., T.N., M.T., H.I.) and Medicinal Safety Research Laboratories (K.T., K.M.), Daiichi Sankyo Co., Ltd., Tokyo, Japan; Research Department II, Daiichi Sankyo RD Associe Co., Ltd., Tokyo, Japan (N.S.); and Association for Promoting Drug Development, Tokyo, Japan (T.I.)

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ABSTRACT:

Diglucuronidation is a novel glucuronidation reaction where the second glucuronosyl moiety is attached at the C2’ position of the first glucuronosyl moiety. To examine whether diglucuronidation takes place in endogenous substrates in vivo, control urine and bile samples were collected from male Crl:CD(SD) IGS rats, beagle dogs, and cynomolgus monkeys, and analyzed by liquid chromatography-mass spectrometry (LC-MS) after solid phase extraction. Several diglucuronides of C19 steroids, including M1 (C31H46O14) and M2 (C31H44O14), were detected in the urine and bile of the dogs but not in the excreta of the rats and monkeys. A milligram quantity of M1 was successfully isolated from the pooled dog urine and analyzed by nuclear magnetic resonance (NMR) spectroscopy. M1 was unambiguously identified as epiandrosterone 3-O-diglucuronide by comparing the LC-MS and two-dimensional NMR data of M1 with those of the biosynthesized epiandrosterone 3-O-diglucuronide. M2 was identified as dehydroepiandrosterone 3-O-diglucuronide. According to these findings, the diglucuronidation reaction was proven to be occurring on steroid hormones in vivo in dogs.

The numerous isoforms of UDP-glucuronosyltransferase (UGT) constitute a superfamily of enzymes that catalyze the transfer of the glucuronosyl moiety from the cofactor uridine 5’-diphosphoglucuronic acid (UDPGA) to a suitable functional group, such as an amino, carboxyl, hydroxyl, or sulfhydryl group, as well as an acidic carbon on a lipophilic substrate (Radominska-Pandya et al., 1999; King et al., 2000; Tukey and Strassburg, 2000; Fisher et al., 2001). A glucuronidation reaction is regarded as one of the most important phase II metabolic reactions not only for xenobiotics but also for endogenous substances such as steroids and bile acids. UGTs are classified into one of two families, UGT1 or UGT2, based on their evolutionary divergence (Mackenzie et al., 1997). UGTs are distributed widely in several tissues, with the liver possessing the highest levels of activity (Tukey and Strassburg, 2001).

As a result of a hydrophilic glucuronosyl moiety being introduced, glucuronides are generally hydrophilic enough to be excreted readily in either the bile or the urine. However, when the glucuronidation does not supply enough hydrophilicity to a molecule or when the glucuronide is still recognized as a good substrate by UGTs, subsequent glucuronidation may occasionally take place on the glucuronides. Glucuronides carrying two glucuronosyl moieties are classified into two groups, namely bisglucuronides and diglucuronides (Murai et al., 2005). Bisglucuronides are defined as glucuronides where two separate functional groups on the parent molecule are conjugated at the same time. Bisglucuronides are relatively common phase II metabolites of both endogenous and exogenous compounds (Gordon et al., 1976; Bock et al., 1992). On the other hand, diglucuronides are defined as particular glucuronides where a single functional group on the parent molecule is repeatedly conjugated by two glucuronosyl groups in tandem (Murai et al., 2005).

The first diglucuronide identified was naloxone 3-O-diglucuronide, which was found in dog urine as a chromatographically more polar glucuronide than naloxone glucuronide (Dixon et al., 1989; Murthy et al., 1996). After the discovery of naloxone diglucuronide, we reported that diglucuronides could be produced in vitro from 4-hydroxybiphenyl, a phenolic compound used commonly as a model substrate for glucuronidation (Murai et al., 2002). Moreover, we found that endogenous sex steroids, namely androstenedione, dihydrotestosterone (DHT), estradiol, estriol, estrone, and testosterone, also undergo diglucuronidation in vitro (Murai et al., 2005). Significant species differences were observed in the production of diglucuronides in vitro. Dog liver microsomes exhibited the highest activity for diglucuronidation, whereas monkey and human liver microsomes exhibited detectable diglucuronidation activity for particular steroids (Murai et al., 2005). Activity producing DHT diglucuronide from the corresponding monoglucuronide in recombinant human UGT isoforms was proven.

ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; UDPGA, uridine 5’-diphosphoglucuronic acid; NMR, nuclear magnetic resonance; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; CID, collision-induced dissociation; HSQC, heteronuclear single quantum correlation spectroscopy; HMBC, heteronuclear multiple bond correlation spectroscopy; DMSO, dimethyl sulfoxide; LC-MS, liquid chromatography-mass spectrometry; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; CID, collision-induced dissociation; HSQC, heteronuclear single quantum correlation spectroscopy; HMBC, heteronuclear multiple bond correlation spectroscopy; DMSO, dimethyl sulfoxide.
forms was found in UGT1A8, UGT1A1, and UGT1A9, in descending order (Murai et al., 2006).

Steroids are included in a class of lipophilic small molecules that act as ligands of their nuclear receptors, and their circulating levels are regulated by a balance of steroidogenesis and metabolic inactivation by cytochrome P450, UGT, and sulfotransferase (You, 2004). The fact that androgens and estrogens are good substrates for diglucuronidation in vitro using dog liver microsomes (Murai et al., 2005) does not necessarily indicate that the diglucuronidation reaction is occurring under in vivo conditions. To date, no diglucuronides produced from endogenous compounds have been identified in vivo.

The objective of the present study was to determine whether the diglucuronidation of endogenous substances takes place in vivo. In this study, control bile and urine samples obtained from male rats, dogs, and monkeys were cleaned up using mixed mode anion exchange solid phase extraction cartridges, and the extracts were analyzed using liquid chromatography-mass spectrometry (LC-MS) to detect any endogenous diglucuronides. We were successful in detecting the [M-H] ions of C19 steroid diglucuronides in the bile and urine of dogs but not in those of rats and monkeys. To identify the endogenous diglucuronides detected in dog bile and urine, several diglucuronides were biosynthesized using dog liver microsomes and commercially available androgens (C19 steroids). The present report also describes in detail the isolation and structural elucidation of an endogenous steroid diglucuronide detected in dog urine, which has been unambiguously identified as epiandrosterone 3-0-diglucuronide, in which the second glucuronosyl moiety is attached at the C2’ position of the first glucuronosyl moiety, leading to the diglucuronosyl conjugation of a single hydroxyl group of epiandrosterone at the C3 position.

**Materials and Methods**

**Materials.** Androsterone, epiandrosterone, dehydroepiandrosterone (DHEA), DHT, and testosterone were purchased from Sigma-Aldrich Co. (St. Louis, MO). Alamethicin, D-saccharic acid 1,4-lactone, and UDPGA were also from Sigma. Deuterated dimethyl sulfoxide (DMSO-d6, 99.9% D) was purchased from Isotec, Inc. (Miamisburg, OH). Other reagents and solvents used were of the highest grade available. Pooled rat, dog, monkey, and human liver microsomes were obtained from BD Gentest (Woburn, MA) and were stored frozen at approximately –80°C until use.

**Urine and Bile Samples for Analysis of Diglucuronides.** Male Crl CD(SD) IGS rats (N = 3; body weight: approximately 200 g; age: 8 weeks old) were housed in a cage with free access to food (FR-2; Funabashi Farms Co., Ltd, Chiba, Japan) and water. Male beagle dogs (N = 3; body weight: 11–13 kg; age: 2 and 3 years old) were individually housed in cages with free access to water and were given 250 g of solid food daily (DS-A; Oriental Yeast Co., Ltd, Tokyo, Japan). Male cynomolgus monkeys (N = 3; body weight: 2.8–4.3 kg; age: 3 and 5 years old) were individually housed in cages with free access to water and were given 80 g of solid food daily (PS; Oriental Yeast Co., Ltd.). Urine samples were collected overnight prior to collecting bile samples. The rat bile samples were collected for 6 h via a polyethylene tube cannulated into the bile duct. The dog and monkey bile samples were collected from the gallbladder with syringes under anesthesia by pentobarbital for dogs and ketamine hydrochloride and xylazine hydrochloride for monkeys. The samples collected were stored frozen at or below –20°C until analysis. The protocols for all of the animal experiments were approved by the Institutional Ethical Committee for Animal Experiments.

**Solid Phase Extraction of Bile and Urine Samples.** The bile and urine samples were subjected to solid phase extraction using Waters Oasis MAX cartridges (6 cc, 150 mg; Waters Corp., Milford, MA). Urine samples (1 ml) were diluted with water (4 ml), and bile samples (0.1 ml) were diluted with water (4.9 ml). Each diluted sample (5 ml) was loaded onto the cartridge, which had been preconditioned by washing successively with 5 ml of methanol and 5 ml of water. The cartridge was washed with 10 ml of methanol in 50 mM sodium acetate (pH 5.95) followed by 10 ml of methanol. Then, the substances retained by the column were eluted with 10 ml of formic acid in methanol (2 in 98). The eluate was evaporated to dryness under a nitrogen gas stream and reconstituted in 500 µl of acetonitrile in water (20 in 80). The samples were filtered by centrifugation using 0.22-µm centrifugal filters (Millipore, Billerica, MA), and 10-µl aliquots were subjected to LC-MS analysis.

**In Vitro Glucuronidation of Steroid Hormones.** Androsterone, DHEA, DHT, epiandrosterone, and testosterone were incubated with liver microsomes from rats, dogs, monkeys, and humans in the presence of UDPGA to generate steroid diglucuronides. The incubation mixture contained Tris-HCl buffer (100 mM, pH 7.5), magnesium chloride (10 mM), D-saccharic acid 1,4-lactone (5 mM), steroid substrate (200 µM), liver microsomes (1 mg protein/ml), and UDPGA (5 mM) in a final volume of 200 µl. The substrates were added as a solution in DMSO (final: 0.5% DMSO). Before starting the incubation, the microsomes were treated with alamethicin (50 µg/mg protein) for 15 min (Fisher et al., 2000). A reaction was started by the addition of UDPGA, allowed to proceed for 2 h at 37°C on a shaking incubator, and terminated by the addition of 200 µl of acetonitrile. After centrifugation, a 100-µl aliquot of the supernatant was collected and evaporated to dryness using a centrifugal evaporator. The residue was reconstituted in 100 µl of acetonitrile in water (20 in 80), and the 10-µl aliquot was subjected to LC-MS analysis.

**LC-MS Analysis for Detecting the Steroid Diglucuronides Produced in Vivo and in Vitro.** The mass spectrometry was performed on a LTQ Orbitrap (Thermo Fisher Scientific, Inc., Waltham, MA). Because electrospray ionization can generate intense and stable [M-H] ions from the steroid glucuronides (Kuarrane et al., 2000) and diglucuronides (Murai et al., 2005), the instrument was operated in the negative ion electrospray ionization mode. The capillary temperature was set at 275°C. Full scan LC-MS data were acquired using an Orbitrap detector at a resolution of 30,000 in a mass range from m/z 100 to 1000. The collision-induced dissociation (CID) was performed with a relative collision energy of 35%, and the fragment ions generated were detected with the Orbitrap detector. The LC system used was a Waters Alliance 2695. Chromatographic separations were carried out on a Capcell Pak C18 MGII column (5-µm particle; Nacalai Tesque, Inc., Kyoto, Japan) maintained at 30°C in a column oven. The mobile phase, which was used at a flow rate of 0.2 ml/min, was a mixture of formic acid in water (0.1 in 100, solvent A) and formic acid in acetonitrile (0.1 in 100, solvent B), and the initial mobile phase consisted of 20% of solvent B in 80% of solvent A. After the sample injection, the proportion of solvent B was increased linearly from 20 to 60% over 20 min and 60 to 80% over 0.1 min, maintained at 80% for 4.9 min, and decreased linearly from 80 to 20% over 1 min. Thereafter, the column was re-equilibrated with the initial mobile phase for 15 min. To minimize contamination of the ion source, the flow of the first 2 min and that after 20 min of each run was diverted to a waste line using a switching valve. Taking advantage of the high mass accuracy, the [M-H] ions of steroid glucuronides were searched for using reconstructed ion chromatograms with a mass chromatogram window of 4 mDa (theoretical exact mass ± 2 mDa). In this study, we concentrated on detecting the diglucuronides of androgens and estrogens having the chemical formulas C36H34O14, C28H28O14, C31H30O15, C32H28O15, C16H14O3, and C31H30O6.

**Isolation of M1 from Dog Urine.** Dog urine was collected for 2 days from 16 male beagle dogs, each of which received a normal diet and water, as described previously. The isolation procedures of M1 from the urine are summarized in Fig. 1. The urine samples were concentrated as a pool (4.0 liters), acidified (pH 4) by formic acid, and loaded onto an open column of 500 g of Amberlite XAD-2 (Rohm and Haas Co., Philadelphia, PA), which was packed in a size of 8 cm in internal diameter and approximately 20 cm in length and preconditioned with methanol followed with formic acid in water (0.1 in 100). After the sample loading, the column was washed with 3 liters of formic acid in water (0.1 in 100), and the substances adsorbed to the column were eluted with 2 liters of methanol. The methanol extracts were concentrated using a rotary evaporator to remove the methanol, and the resulting aseptically solution was supplemented with water to 500 ml and adjusted to pH 9 with ammonium hydroxide. The solution was extracted twice with 500 ml of ethyl acetate to remove any nonpolar impurities. The aqueous phase was evaporated in vacuo to a dark brown oil and was dissolved in 100 ml of formic acid in water (0.5 in 100). The solution was loaded onto an open column of 40 g of Cosmosil 75C218 (75-µm particle; Nacalai Tesque, Inc., Kyoto, Japan), which was packed in a size of 3.7 cm in internal diameter and approximately 7 cm in length and preconditioned with acetonitrile followed with formic acid in water (0.1 in 100).
Control Dog Urine (4.0 L)

1) Acidified (pH 4)
2) Amberlite XAD-2 column

Water extract

Methanol extract

1) Concentrated
2) Supplemented with water and adjusted to pH 9
3) Extracted twice with ethyl acetate

Ethyl acetate phase

Water phase

1) Cosmosil 75 C(18) column
2) LC-MS analysis

Waste

Fractions containing M1

1) Adjusted to pH 9
2) Bondesil SAX column
3) LC-MS analysis

Waste

Fractions containing M1

Purification by HPLC on
1) Inertsil ODS-3 column (7.6 x 50 mm)
2) Inertsil ODS-3 column (4.6 x 250 mm)
3) Atlantis HILIC silica column (4.6 x 150 mm)
4) Inertsil ODS-3 column (4.6 x 250 mm)
5) Carbopak Pak C18 MGII column (4.6 x 150 mm)

M1 (1.6 mg)

Results and Discussion

Bile and urine from rats, dogs, and monkeys were extracted by a mixed mode anion exchange solid-phase extraction cartridge, and the

Fig. 1. Isolation procedure for M1 from control dog urine. a) Mobile phase: mixture of formic acid in water (0.1 in 100, solvent A) and formic acid in acetonitrile (0.1 in 100, solvent B); linear gradient, 25 to 65% solvent B (0–20 min); flow rate, 1 ml/min.
b) Mobile phase: mixture of water in acetonitrile (45 in 55) containing 10 mM ammonium formate (solvent C) and water in acetonitrile (15 in 85) containing 10 mM ammonium formate (solvent D); linear gradient, 100 to 0% (0–15 min) solvent D; flow rate, 1 ml/min. c) Mobile phase: mixture of solvent A and solvent B; linear gradient, 25 to 65% (0–15 min) solvent B; flow rate, 1 ml/min.
extracts were subjected to the LC-MS analysis. The clean-up process using the solid phase extraction cartridge was necessary for the successful detection of the diglucuronides present in biological fluids at low concentrations because it effectively concentrated acidic compounds, such as diglucuronides carrying two carboxylic acids in the molecule, while eliminating neutral and basic compounds.

Taking advantage of the high mass accuracy of the LC-MS instrument, we searched for steroid diglucuronides in bile and urine samples based on the theoretical accurate masses of their [M-H]$^{-}$ ions. Based on our previous observations in vitro (Murai et al., 2005), we concentrated on detecting the diglucuronides produced from androgens and estrogens that possess 18 or 19 carbons in their molecules. As shown in the accurate mass-based reconstructed ion chromatograms of m/z 641 (m/z 641.2789 to 641.2829) and m/z 639 (m/z 639.2633 to 639.2673), several diglucuronides of C$_{19}$ steroids, including M1 and M2 as major components, were detected in both the dog bile and urine samples (Fig. 2). On the other hand, no diglucuronides were detected in the bile and urine samples from rats and monkeys (data not shown). Because the chemical structures of steroid diglucuronides resemble each other, the LC-MS sensitivity of steroid diglucuronides could be considered similar. Therefore, M1 and M2 would be regarded as major components in the dog bile and urine in comparison to the other diglucuronides.

M1, M2, and other small peaks in the dog bile and urine (Fig. 2)
were characterized as diglucuronides because they all exhibited the typical fragment ions for diglucuronides at m/z 351 in their CID spectra. Based on the observed accurate mass and mass fragmentation schemes (Table 1), M1 was proposed to be a diglucuronide of C₁₉ steroid and to have the molecular formula of C₃₁H₄₆O₁₄. In a similar manner, M2 was proposed to be a diglucuronide of C₁₉ steroid and to have the molecular formula of C₃₁H₄₄O₁₄. The structure of the aglycone of these diglucuronides was difficult to identify using only LC-MS because the mass fragmentation provided only limited structural information.

For the identification of diglucuronides of C₁₉ steroids detected in dog bile and urine, androsterone, DHEA, DHT, epiandrosterone, and testosterone were incubated with liver microsomes to generate steroid diglucuronides, and the samples were analyzed by LC-MS. The dog liver microsomes generated diglucuronides in varying degrees for all the steroid hormones used in this study; however, rat, monkey, and

Table 1

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<td>641.28131</td>
<td>0.27</td>
<td>623.2701, 579.2802, 465.2490, 351.0565, 289.0562, 193.0352</td>
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<tr>
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<td>C₃₁H₄₆O₁₄</td>
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<td>641.28101</td>
<td>0.73</td>
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<td>639.26569</td>
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<td>0.22</td>
<td>621.2545, 577.2647, 463.2333, 351.0563, 289.0562, 193.0351</td>
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Fig. 3. HSQC spectrum of M1 that was isolated from dog urine. The HSQC spectrum of epiandrosterone 3-O-diglucuronide, which was biosynthesized using dog liver microsomes, exhibited exactly the same spectrum.
human liver microsomes produced no detectable diglucuronides under the incubation conditions and analytical methods used in this study (data not shown). The HPLC retention times of the steroid diglucuronides biosynthesized by dog liver microsomes were as follows: androsterone 3-O-diglucuronide, 11.5 min; epiandrosterone 3-O-diglucuronide, 11.5 min; DHEA 3-O-diglucuronide, 11.0 min; DHT 170-O-diglucuronide, 12.3 min; and testosterone 17-O-diglucuronide, 10.5 min.

The HPLC retention time and the CID spectrum of M1 were identical to those of both androsterone 3-O-diglucuronide and epiandrosterone 3-O-diglucuronide obtained in vitro (Table 1). Biosynthesized androsterone 3-O-diglucuronide and epiandrosterone 3-O-diglucuronide exhibited the same HPLC retention time, and they were not distinguished from each other by their CID spectra. Therefore, structure analysis by NMR spectroscopy was necessary for the unambiguous identification of M1. For this purpose, approximately 1.6 mg of M1 was isolated from 4.0 liters of pooled dog urine that was collected from 16 male dogs (Fig. 1). Separately, approximately 6.0 mg of epianandrosterone 3-O-diglucuronide was biosynthesized in large scale in vitro incubation with dog liver microsomes. Isolated M1 and biosynthesized epianandrosterone 3-O-diglucuronide were subjected to two-dimensional NMR analysis, and it was found that they exhibit exactly the same NMR spectra (Fig. 3). Because androsterone and epianandrosterone are diastereomers that differ in the configuration of the hydroxyl group at the C3 position and therefore exhibit different 1H and 13C NMR spectra (data not shown), the complete coincidence of the NMR spectra of M1 with that of epianandrosterone 3-O-diglucuronide is synonymous with the identification of M1 as epianandrosterone 3-O-diglucuronide and not as androsterone 3-O-diglucuronide. The full assignment of the 1H and 13C signals was accomplished by a detailed analysis of the correlated spectroscopy, HSQC, and HMBC spectral data (Fig. 4; Table 2). The first glucuronosyl moiety was attached to the C3 position of epianandrosterone, as confirmed by the key HMBC correlation peak from the H1’ proton (4.51 ppm) to the C3 carbon (77.6 ppm). The second glucuronidation occurred at the C2’ position of the first glucuronosyl moiety, which was determined by the key HMBC correlation peak from the H2’ proton (3.16 ppm) to the C1’ carbon (104.6 ppm).

M2 was identified as DHEA 3-O-diglucuronide, since M2 exhibited the same HPLC retention time and CID spectrum as those of DHEA 3-O-diglucuronide obtained in vitro (Table 1). To assess the chemical structure of DHEA 3-O-diglucuronide produced by dog liver microsomes, biosynthesized DHEA 3-O-diglucuronide was subjected to NMR analysis. Two-dimensional NMR data enabled us to completely characterize the chemical structure of the biosynthesized DHEA 3-O-diglucuronide (Fig. 4; Table 2). The first glucuronosyl moiety was attached to the C3 position of DHEA, as confirmed by the key HMBC correlation peak from the H1’ proton (4.53 ppm) to the C3 carbon (78.1 ppm). In the same manner as epianandrosterone 3-O-diglucuronide, the second glucuronidation in DHEA 3-O-diglucuronide occurred at the C2’ position of the first glucuronosyl moiety, which was determined by two key HMBC correlation peaks from the H2’ proton (3.16 ppm) to the C1’ carbon (104.6 ppm).

The regiochemistry of the diglucuronosyl moiety in both epianandrosterone 3-O-diglucuronide and DHEA 3-O-diglucuronide is attached at the C2’ position of the first glucuronosyl moiety (Fig. 4), which is common among the glucuronidases characterized previously (Dixon et al., 1989; Murai et al., 2002, 2005, and 2007). The hydroxyl group at the C2’ position of the glucuronosyl group in a specific monoglucuronide was recognized as a functional group for further glucuronidation by unique dog UGT isofoms, which would have...
similar enzymatic properties to human UGT1A8, as demonstrated in our previous study in vitro (Murai et al., 2006).

Epiandrosterone is a metabolite of testosterone that possesses less androgenic activity (Dorfman, 1948). DHEA is the principal androgen secreted by the adrenal gland and circulates in plasma mainly as the sulfate form. DHEA plays an important role as the primary precursor of many important sex steroids (Regelson et al., 1994). In humans, these androgens are excreted in the urine as sulfates or glucuronides after metabolism in the liver (Bongiovanni and Cohn, 1970). Our finding of epiandrosterone, DHEA, and other C19 steroids being excreted as diglucuronides in the bile and urine of dogs suggests that the levels of circulating steroid hormones in dogs are regulated differently from other species. However, the physiological significance of the diglucuronidation pathways for steroid sex hormones in comparison to monoglucuronidation, sulfation, and the other metabolic pathways remains to be clarified. The age, sex, and pathological conditions would be possible factors that affect the level of diglucuronides in dogs, just as 17-ketosteroid sulfates and glucuronides do in humans (Kroboth et al., 1999; Jia et al., 2001).

In conclusion, we first identified the endogenous diglucuronides of steroid hormones excreted in the bile and urine of male dogs as novel metabolic pathways for the disposition of steroids in dogs. Further in vivo studies using human bile and urine should be conducted in the future to investigate whether the diglucuronidation pathway also exists in humans and whether this has any physiological significance.

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References


Address correspondence to: Takahiro Murai, Drug Metabolism and Pharmacokinetics Research Laboratories, Daiichi Sankyo Co., Ltd., 1-2-58, Hiromachi, Shinagawa-ku, Tokyo 140-8710, Japan. E-mail: murai.takahiro.a4@daichisankyo.co.jp