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Research Paper

Metabolism and Renal Elimination of Gaboxadol in Humans: Role of UDP-Glucuronosyltransferases and Transporters

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Purpose. Gaboxadol, a selective extrasynaptic agonist of the delta-containing γ-aminobutyric acid type A ($GABA_A$) receptor, is excreted in humans into the urine as parent drug and glucuronide conjugate. The goal of this study was to identify the UDP-Glucuronosyltransferase (UGT) enzymes and the transporters involved in the metabolism and active renal secretion of gaboxadol and its metabolite in humans.
Methods. The structure of the glucuronide conjugate of gaboxadol in human urine was identified by LC/MS/MS. Human recombinant UGT isoforms were used to identify the enzymes responsible for the glucuronidation of gaboxadol. Transport of gaboxadol and its glucuronide was evaluated using cell lines and membrane vesicles expressing human organic anion transporters hOAT1 and hOAT3, organic cation transporter hOCT2, and the multidrug resistance proteins MRP2 and MRP4.
Results. Our study indicated that the gaboxadol-*O*-glucuronide was the major metabolite excreted in human urine. UGT1A9, and to a lesser extent UGT1A6, UGT1A7 and UGT1A8, catalyzed the *O*-glucuronidation of gaboxadol *in vitro*. Gaboxadol was transported by hOAT1, but not by hOCT2, hOAT3, MRP2, and MRP4. Gaboxadol-*O*-glucuronide was transported by MRP4, but not MRP2.
Conlusion. Gaboxadol could be taken up into the kidney by hOAT1 followed by glucuronidation and efflux of the conjugate into urine via MRP4.

KEY WORDS: gaboxadol; glucuronidation; kidney; transporters.

INTRODUCTION

γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system. The $GABA_A$ receptor agonists are known to exert hypnotic properties by reducing sleep latency, increase sleep continuity, and promoting non-rapid-eye-movement (NREM) sleep as well as the occurrence of spindles (1–3). Gaboxadol, (also known as THIP, 4,5,6,7-tetrahydroisoxazolopyridin-3-ol, Fig. 1A), a heterocyclic analogue of GABA and a selective extrasynaptic agonist of the delta-containing $GABA_A$ receptor (4,5), has been in development for the treatment of insomnia.

Earlier pharmacokinetic studies in humans and animals using [^{14}C]-gaboxadol suggested a rapid and complete absorption of gaboxadol after oral administration (6). Highest concentrations of radioactivity were found in the kidneys, although radioactivity was seen in all tissues investigated, including brain. The majority of radioactivity was excreted in the urine (84–93%) in the form of unchanged drug and a glucuronic acid conjugate (6).

Andersen *et al.* (7) demonstrated that the urinary metabolite of gaboxadol in rats was an *O*-glucuronide. However, the chemical structure of the glucuronide metabolite and the UDP-Glucuronosyltransferase (UGT) enzymes responsible for the glucuronidation of gaboxadol in humans were not identified. Considering the significant metabolic disposition of gaboxadol through conjugation with glucuronic acid in humans, it is important to understand the nature of human gaboxadol glucuronic acid conjugates and the UGT enzymes involved in conjugation.

After a single oral dose of gaboxadol (10 mg) in healthy subjects, renal clearance of gaboxadol averaged ~450 ml/min (8), which is markedly higher than the typical glomerular filtration rate in young healthy males (125 ml/min). Therefore, although the plasma protein binding of gaboxadol in rodents (9) and human is low (<2%; unpublished observation), active renal secretion seems to contribute to the renal elimination of gaboxadol in humans.

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ABBREVIATIONS: hOAT1, human organic anion transporter 1; hOAT3, human organic anion transporter 3; hOCT2, human organic cation transporter 2; MDR, multidrug resistance; MRP2, multidrug resistance protein 2; MRP4, multidrug resistance protein 4; PAH, *p*-aminohippuric acid; Pgp, P-glycoprotein; TEA, tetraethylammonium; UGT, UDP-Glucuronosyltransferase.

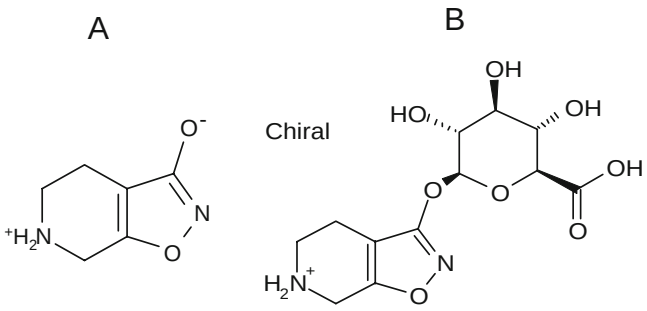

Fig. 1. Chemical structure of gaboxadol (**A**) and gaboxadol-*O*-glucuronide (**B**).

Multiple transporters are expressed in the renal proximal tubules of animals and humans and play a key role in active secretion of many endogenous and xenobiotic compounds by the kidney (10,11). Among the transporters identified in humans, organic anion transporters OAT1 (*SLC22A6*), OAT3 (*SLC22A8*), and organic cation transporter OCT2 (*SLC22A2*), are predominantly expressed in the kidney and localized in the basolateral membrane of the renal proximal tubule cells. They transport organic anions and cations, and some amphipathic compounds from the blood into cells (11–14). In addition, several efflux transporters are localized on the brush border membrane and transport substrates into the urine. The multidrug resistance proteins MRP2 (*ABCC2*) and MRP4 (*ABCC4*) are involved in the efflux of a wide variety of conjugated and unconjugated organic anions (11,15–17), while MDR1 P-glycoprotein (Pgp; *ABCB1*) and the recently identified multidrug and toxin extrusion transporters (*MATE1/2*) predominantly mediate the efflux of organic cations (18–20). These transporters have been shown to directly transport or interact with a number of clinically used drugs (21–22).

The main objectives of our studies were to identify (1) the structure of the human gaboxadol glucuronic acid conjugate, (2) the UGT enzymes involved in conjugate formation, and (3) the renal transporters potentially contributing to the active renal secretion of gaboxadol and its metabolite in humans.

MATERIAL AND METHODS

Materials

[^{14}C] Gaboxadol (25 mCi/mmol) was synthesized by Amersham Biosciences, UK. Unlabeled gaboxadol was purchased from MP Biomedicals (St. Louis, MO). Gaboxadol *N*- and *O*-glucuronide conjugates were prepared by the Drug Metabolism Labeled Compound Synthesis group, Merck Research Laboratories (Rahway, NJ) and their identity were confirmed by NMR. [^{3}H]Aminohippuric acid, *p*-[glycyl-2-^{3}H] (PAH; 4.3 Ci/mmol) and [^{3}H]estrone sulfate (57.3 Ci/mmol) were purchased from PerkinElmer Life Sciences, Inc. (Boston, MA). [^{14}C]Tetraethylammonium (TEA; 55 mCi/mmol) was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). [^{3}H]Folic acid (35 Ci/mmol) was purchased from Moravec Biochemicals, Inc (Brea CA). [^{14}C]Ethacrynic acid glutathione conjugate (EA-SG; 57.15 mCi/mmol) was synthesized by the Merck Labeled Compound Synthesis Group, Rahway, NJ. UDP-glucuronic acid was obtained from Sigma (St. Louis, MO). All other reagents were commercially obtained with the highest analytical purity grade.

Supersomes and Microsomes

Recombinant human UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15 and 2B17 and control supersome; and pooled human liver microsomes were purchased from BD Gentest (Woburn, MA). Pooled human intestinal and kidney microsomes were obtained from Xenotech, LLC (Lenexa, KS) and *In Vitro* Technologies, Inc. (Baltimore, MD), respectively.

Urine Sample Preparation and Analysis

Urine samples were from six healthy subjects originating from a clinical study with oral dosing of 10 mg gaboxadol. The samples from 6 subjects were pooled with equal volume (1-mL each) at pre-dose, 0–4, 4–8 and 8–12 h periods, respectively. Urine samples (200 μL) were diluted with equal volume of Milli-Q water and 75 μL of acetonitrile, vortexed and centrifuged for 3 min at 14,000 rpm. The supernatants were then transferred to HPLC vials and analyzed by LC-MS/MS. HPLC was conducted on a Hewlett-Packard HP1100 gradient HPLC system (Agilent Technologies, Palo Alto, CA). Separation was achieved on a Shodex Asahipak NH2P-50 4D column (5 μm, 4.6×150 mm, Thermo Electron, Bellefonte, PA) using a mobile phase consisting of 20 mM ammonium acetate (pH ~4.5) in water (solvent A) and acetonitrile (solvent B) at a constant flow rate of 0.5 mL/min. The gradient was as follows: 0 min, 70%B; 10 min, 50%B; 15 min, 20%B; 16–30 min, 5%B and 31 min 70%B. The system was equilibrated for 4 min at 70%B prior to the next injection. The HPLC system was interfaced to a Thermo Electron's TSQ Quantum tandem mass spectrometer (Palm Beach, CA). Selective reaction monitoring (SRM) and product ion scan were carried out using electrospray ionization (ESI) in the positive ion mode. The temperature of the ion transfer tube was 320°C and the ESI ionizing voltage was maintained at 4.1 kV. The collision gas was at a pressure of 1.5 mtorr and a collision energy at 15 eV were used for SRM and parent product ion scan. The product ion scan at *m*/*z* 317 was monitored for structural elucidation of the of gaboxadol glucuronide. The SRM of *m*/*z* 317 → 141 was used for quantitative measurement of the gaboxadol-*O*-glucuronide.

Gaboxadol Glucuronidation Assay

All incubations were conducted at 37°C in a final volume of 0.25 or 0.5 ml in a shaking water bath. The incubation mixture contained 0.1 M potassium phosphate buffer (pH=7.4), 5 mM $MgCl_2$, 5 mM D-saccharic acid-1,4-lactone, 50 μg alamethincin/mg microsomal protein, 0.4 mg/ml human microsomes or individual UGTs, and 25 to 1,000 μM gaboxadol (dissolved in Milli-Q water). The final volume of acetonitrile in the incubation was 1%. After a short period of pre-incubation, reaction was initiated by the addition of UDPGA (4 mM), and allowed to proceed for 30 min. The reaction was terminated by the addition of

cooled acetonitrile containing internal standard for quantitation. The samples were vortexed and centrifuged at 3,000 rpm for 10 min. The supernatants were transferred to HPLC vials and aliquots were analyzed by LC/MS-MS. The quantitation of *O*-glucuronide of gaboxadol from incubations were determined with the LC-MS/MS conditions identical to that described above except with a different gradient (0 min, 70%B; 3 min, 70% B; 10 min, 5%B; 15 min, 5%B; 16 min 70%B). The system was equilibrated for 4 min at 70%B prior to the next injection.

Transfection and Cell Culture

hOAT1 cDNA was amplified from total human kidney cDNA (Clontech, Palo Alto, CA) by PCR as described by Chu *et al.* (23). hOCT2 and hOAT3 cDNAs were provided by Dr. Richard Kim (Department of Medicine, University of Western Ontario, London, Ontario, Canada). hOCT2 and hOAT1 cDNA was cloned into the expression vector pCEP4 and pcDNA3.1 (Invitrogen, Carlsbad, CA), respectively. hOAT3 cDNA was cloned into the pcDNA5/FRT vector (Invitrogen). hOAT1/pcDNA3.1 and hOCT2/pCEP4 construct was transiently transfected into CHO-K1 cells (American Type Culture Collection (ATCC) Manassas, VA) using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. CHO-K1 cells stably transfected with hOAT3 cDNA were generated by using the Flp-In™ system (Invitrogen). The transport activity by each cell line was confirmed by examining the uptake of [^{3}H]PAH (1 μM), [^{3}H]estrone sulfate (1 μM), and [^{14}C]TEA (5 μM) in hOAT1, hOAT3, and hOCT2 transfected cells, respectively.

CHO-K1 cells were cultured in F-12K medium supplemented with 10% fetal bovine serum, 2 mM glutamax, penicillin (100 U/mL), and streptomycin (100 μg/mL), and were maintained at 37°C in an atmosphere of 95% air/5% CO_2 and 90% relative humidity.

Uptake Studies in CHO-K1 Cells Transfected with hOAT1, hOAT3, or hOCT2 cDNA

CHO-K1 cells were seeded onto Costar 24 well cell culture plates (Corning Incorporated, Corning, NY) at a density of 1.8×10^5 cells/well and transiently transfected with cDNAs encoding hOAT1, hOCT2 or vector only, as described above. CHO-K1 cells stably transfected with hOAT3 cDNA were cultured with 10 mM sodium butyrate for 24 h before the uptake study. Uptake was initiated by the addition of radiolabeled test compounds. Cells were then incubated for the indicated time at room temperature. Uptake times for kinetic analyses were conducted within the linear range of uptake for the test compounds in hOAT1 transfected cells. Uptake was stopped by the addition of ice-cold PBS. After washing the cells with PBS, the cells were solubilized in 500 μL of Triton X-100 (1%) and transferred to scintillation vials containing 4 mL scintillation cocktail and the radioactivity was determined by liquid scintillation counting in a LS 6500 Multipurpose Scintillation Counter (Beckman Coulter, Fullerton, CA).

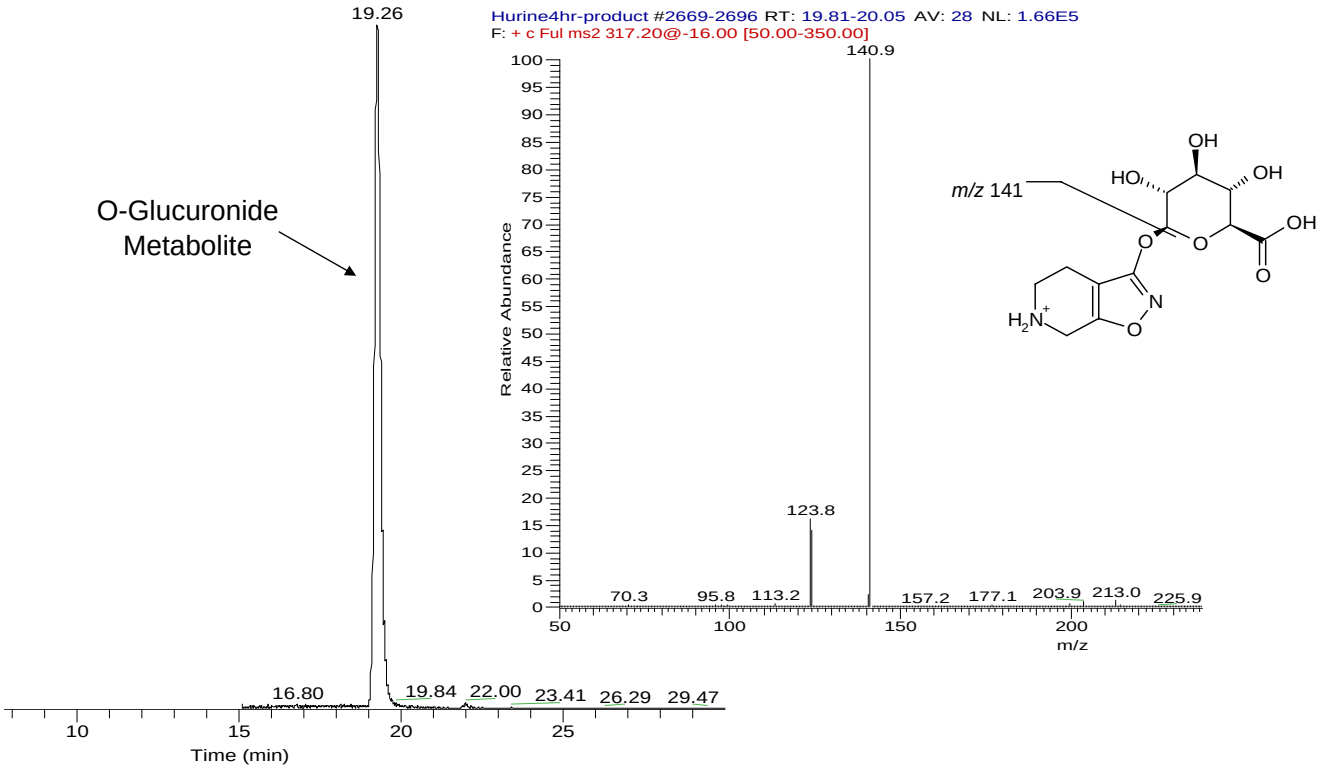

Fig. 2. Representative SRM chromatogram from the injections of pooled human urine samples (n=6, 0–4 h) in subjects receiving gaboxadol 10 mg PO. The peak eluted at 19.2 min has the same retention time as the gaboxadol-*O*-glucuronide standard. The product ion spectrum of the metabolite (m/z 317.2) showed a peak at m/z 141 corresponding to the loss of UDPGA moiety (mass: 176 Da).

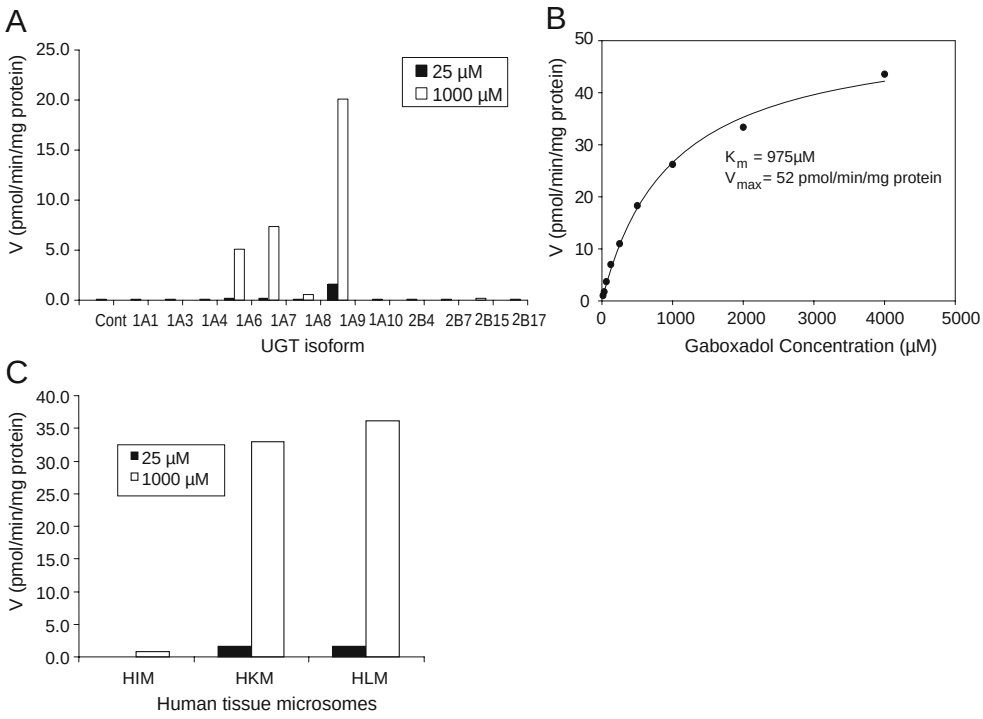

Fig. 3. Gaboxadol glucuronosyltransferase activity by recombinant human UGTs and in human liver, kidney and intestinal microsomes. In **A**, human UGTs (0.4 mg/mL) were incubated with 25 μM (closed columns) and 1,000 μM (open columns) gaboxadol as described in the "Materials and Methods". **B** showed kinetics of gaboxadol glucuronidation by human UGT1A9. The data were fitted to the Michaelis–Menten equation and the K_m value of 975 μM and V_{max} of 52 pmol/mg protein/min were obtained. In **C**, human tissue microsomes (0.4 mg/mL) were incubated with 25 and 1,000 μM gaboxadol as described in the "Materials and Methods". Each *bar* represents the mean of duplicate determinations.

Vesicular Uptake Studies in Baculovirus Infected Sf9 Cells Containing Human MRP2 or MRP4

Membrane vesicles isolated from baculovirus infected Spodoptera frugiperda (Sf9) cells containing human MRP2, MRP4 or β-galactosidase were purchased from Solvo Biotechnology (Szeged, Hungary) and GenoMembrane (Yokohuma, Japan), respectively. Vesicular uptake studies were performed using the rapid filtration technique as reported previously (24). The transport medium contained test compound or positive control substrate [^{14}C]EA-SG (2 μM) for MRP2 and [^{3}H]folic acid (10 μM) for MRP4, 250 mM sucrose, 10 mM Tris/HCl (pH 7.4), 10 mM $MgCl_2$, 5 mM adenosine 5′ triphosphate (ATP), or 5 mM adenosine monophosphate (AMP), and an ATP-regenerating system (10 mM creatine phosphate and 100 μg/mL creatine phosphokinase). The uptake study was performed at 37°C. After preincubation for 3 min at 37°C, the uptake study was started by the addition of vesicle suspension (20 μg protein). The final incubation volume was 40 μL. At designated time, transport was terminated by adding 1 mL of ice-cold stop solution containing 10 mM Tris/HCl (pH 7.4), 250 mM sucrose, and 100 mM NaCl. The stopped reaction mixture was filtered through 0.45 μm HA Millipore filters (Millipore Corporation, Bedford, MA) and subsequently washed twice with 5 mL ice-cold stop solution. For uptake of [^{14}C] gaboxadol, the radioactivity retained on the filter and in the reaction mixture was measured in a liquid scintillation counter (LS 6500 Beckman Coulter, Fullerton, CA). For uptake of gaboxadol-*O*-glucuronide, the amount of gaboxadol-*O*-glucuronide in the filter and the reaction mixture was analyzed by LC/MS-MS as described previously.

Kinetic Analysis

Substrate concentration (S) and velocity (V) data were fitted to the Michaelis-Menten model (Eq. 1) by a nonlinear least-squares method using *Kaleida Graph* (Synergy Software, Reading, PA) or Sigmaplot (SPSS, Chicago, IL) to derive the enzyme kinetic parameters V_{max} (maximal velocity) and K_m (substrate concentration at half-maximal velocity).

$$V_o = V_{max} \times S/(Km + S) \quad (1)$$

RESULTS

Glucuronide Metabolite of Gaboxadol in Humans

To identify the structure of the glucuronide conjugate of gaboxadol in human urine, the potential gaboxadol glucuronide metabolites, gaboxadol *N*- and *O*-glucuronide, were chemically synthesized. In theory, it is anticipated that glucuronic acid reacts with the hydroxyl group of the gaboxadol molecule and/or the nitrogen on the isoxazol ring. An LC/MS/MS method with a specific selected reaction

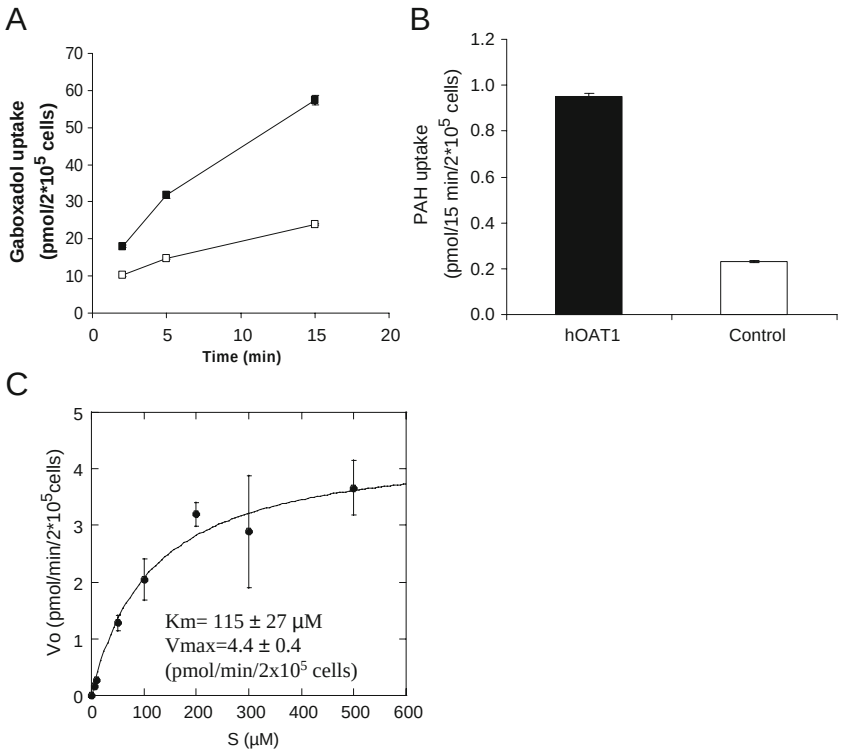

Fig. 4. Uptake of Gaboxadol by hOAT1 transfected cells. Time-dependent uptake of [^{14}C] Gaboxadol (50 µM) (**A**) into CHO-K1 cells transiently transfected with hOAT1 cDNA (*closed squares*) or vector only (*open squares*). **B** Uptake of [^{3}H] PAH (1 µM) into CHO-K1 cells transiently transfected with hOAT1 cDNA (*closed column*) or vector only (*open column*) at 15 min. **C** Concentration dependence of the uptake of gaboxadol in hOAT1 transiently transfected CHO-K1 cells. The hOAT1-mediated transport (*closed circles*) was calculated by subtracting the transport velocity in vector control cells from that in hOAT1 transfected cells. Values shown are mean±SE of experiments performed in triplicate.

monitoring (SRM) transition of *m*/*z* 317 → 141 was developed to analyze the gaboxadol glucuronide metabolites. The assay had a very good resolution and achieved baseline separation of the *O*- and *N*-glucuronide metabolites at retention times of 19.2 and 21.9 min, respectively. The representative SRM (*m*/*z* 317 → 141) chromatogram from pooled human urine samples at 0–4 h after oral administration of gaboxadol (10 mg) is shown in Fig. 2. A single peak of metabolite with an *m*/*z* 317.2 eluted at 19.2 min, identical to the mass and retention time of the *O*-glucuronide standard spiked in blank urine samples. The product ion scan of the metabolite showed a major fragment ion at *m*/*z* 141 resulting from the neutral loss of glucuronic acid (176 Da) from the tetrahydroisoxazolo[5,4-c] pyridin-3-ol moiety. The SRM chromatograms of the metabolite among all urine samples at different collection times were identical.

Gaboxadol Glucuronidation by Recombinant Human UGT Isoforms

UGT reaction phenotyping was conducted using recombinant human UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17, expressed in baculovirus-infected insect cells. Of all UGTs examined (Fig. 3A), UGT 1A9 showed the highest activity (20.1 pmol/min/mg protein) at 1,000 µM gaboxadol, followed by UGT1A7 (7.4 pmol/min/mg protein), UGT1A6 (5.1 pmol/min/mg protein) and UGT1A8 (0.5 pmol/min/mg protein). Kinetic analysis of gaboxadol *O*-glucuronidation by UGT1A9 showed the best fit to the Michaelis-Menten model with a K_m of 975 µM and V_{max} of 52 pmol/min/mg protein, respectively (Fig. 3B). Gaboxadol glucuronidation by human UGT1A6 followed non-Michaelis–Menten kinetics consistent with homotropic activation (Hill coefficient, n=1.2), with an apparent K_m value greater than 5 mM (data not shown).

Gaboxadol Glucuronidation by Human Tissue Microsomes

Gaboxadol glucuronosyltransferase activity was also studied in pooled human liver, kidney, and intestinal microsomes (Fig. 3C). Both human liver and kidney microsomes were capable of catalyzing glucuronidation of gaboxadol (33.0–36.2 pmol/min/mg protein at 1,000 µM gaboxadol). Human intestinal microsomes showed very low turnover toward gaboxadol *O*-glucuronide (0.75 pmol/min/mg protein at 1,000 µM gaboxadol), while the 4-methylumbelliferone glucuronidation activity was high (3,500 pmol/min/mg protein).

Uptake of Gaboxadol by hOAT1-Expressing CHO-K1 Cells

To evaluate whether gaboxadol was transported by the renal uptake transporter hOAT1, the time dependent uptake

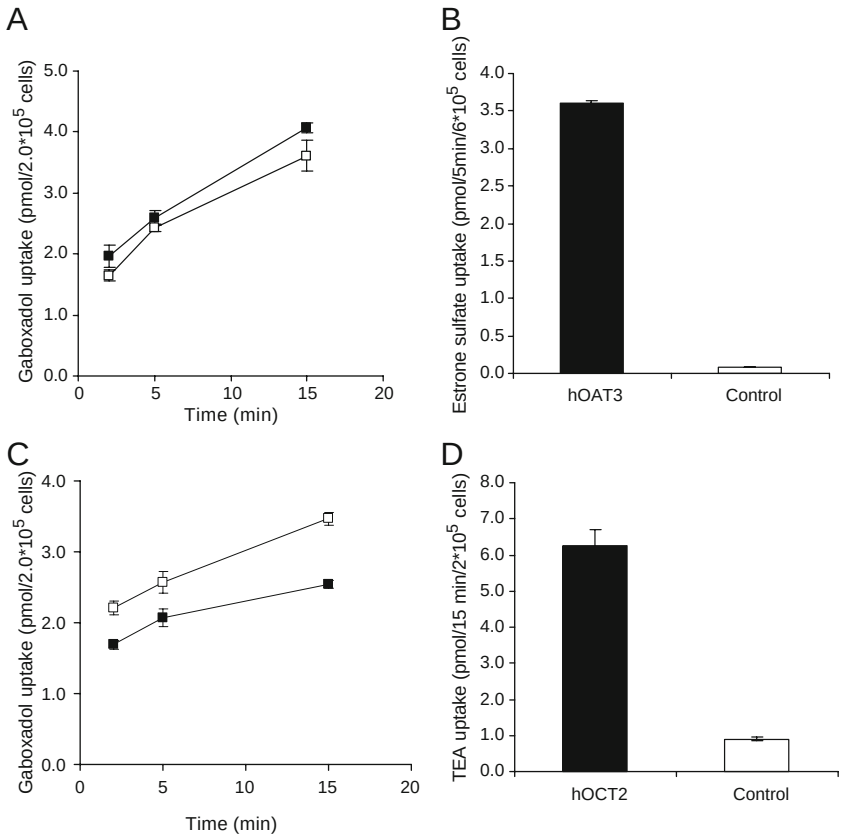

Fig. 5. Uptake of gaboxadol by hOAT3 and hOCT2 transfected cells. Time-dependent uptake of [^{14}C] gaboxadol (10 μM) was evaluated in hOAT3 stably transfected CHO-K1 cells (**A**) and hOCT2 transiently transfected CHO-K1 cells (**C**). **B** and **D** show the uptake of the prototypical substrates [^{3}H] Estrone sulfate (1 μM) and [^{14}C] TEA (10 μM) in hOAT3 or hOCT2 transfected cells (*closed columns*) or control cells (*open columns*) at 5 and 15 min, respectively. Values shown are means±SE of experiments performed in triplicate.

of [^{14}C] gaboxadol into CHO-K1 cells transiently transfected with hOAT1 cDNA was measured. The uptake of [^{14}C] gaboxadol (50 μM) into CHO-hOAT1 cells was time-dependent and significantly higher than in control cells (Fig. 4A). The uptake of [^{3}H] PAH (1 μM), the positive control for hOAT1-mediated transport, was comparable to that reported by Chu *et al.* (23) (Fig. 4B). hOAT1-mediated gaboxadol uptake, obtained by subtracting the transport velocity in vector control cells from that in hOAT1 transfected cells, was saturable with a K_m of 115±27 μM and a V_{max} of 4.4±0.4 pmol/min/2×10^5 cells (Fig. 4C).

Uptake of Gaboxadol by hOAT3- and hOCT2-Expressing CHO-K1 Cells

To examine whether gaboxadol was transported by the renal uptake transporters hOAT3 and hOCT2, uptake into CHO-K1 cells stably or transiently transfected with hOAT3 or hOCT2 cDNAs was measured. The uptake of [^{14}C] gaboxadol (5 μM) in hOAT3 and hOCT2 transfected cells was similar or slightly lower compared to that in control cells (Fig. 5A, C), while the uptake of [^{3}H] Estrone sulfate (1 μM) and [^{14}C] TEA (1 μM), positive controls for hOAT3 and hOCT2, respectively, was high in hOAT3 or hOCT2 transfected cells.

Uptake of Gaboxadol by MRP2- and MRP4-Containing Membrane Vesicles

Uptake of [^{14}C] gaboxadol (15 μM) into MRP2 or MRP4 containing vesicles was not time- and ATP-dependent and the uptake was comparable to that in control membrane vesicles containing β-galactosidase (Fig. 6A, C). Uptake of [^{14}C] EA-SG (2 μM), the positive control for MRP2, was comparable to literature data (Fig. 6B) (26). The uptake of [^{3}H] folic acid (10 μM), the positive control for MRP4, showed a significant time- and ATP-dependent uptake in MRP4 containing vesicles, but not in control vesicles (Fig. 6D).

Uptake of Gaboxadol-*O*-glucuronide by MRP2- and MRP4-Containing Membrane Vesicles

No uptake of gaboxadol-*O*-glucuronide (20 μM) into MRP2 containing vesicles was detectable (Fig. 7A). In contrast, uptake of gaboxadol-*O*-glucuronide (20 μM) into MRP4 containing vesicles was time- and ATP-dependent, and significantly higher than in control vesicles (Fig. 7B). Kinetic analysis demonstrated that ATP-dependent uptake in MRP4 containing vesicles was saturable with a K_m of 590±91 μM and a V_{max} of 1,675±164 pmol/min/mg protein (Fig. 7C).

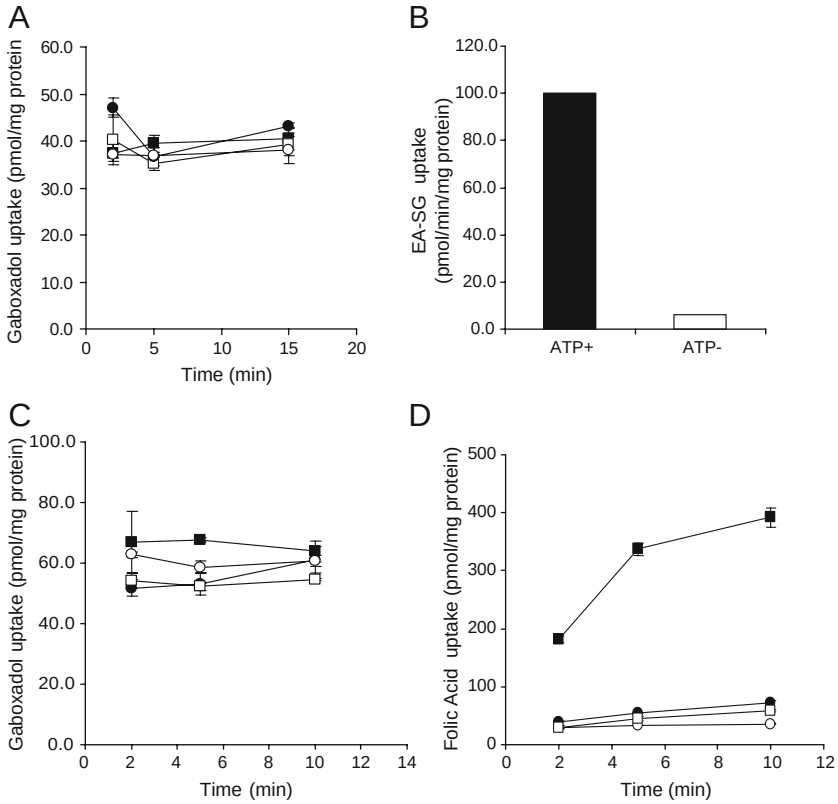

Fig. 6. ATP-dependent uptake of [^{14}C] gaboxadol (15 μM) by membrane vesicles containing MRP2 and MRP4. Membrane vesicles (20 μg of protein) prepared from Sf9 cells expressing β-galactosidase (*circles*; **A**, **C**, and **D**), MRP2 (*squares*; **A**, **B**), or MRP4 (*squares*; **C** and **D**) were incubated at 37 °C in transport buffer containing [^{14}C] gaboxadol (15 μM) (**A** and **C**) or [^{14}C] EA-SG (2 μM) (**B**), and [^{3}H] Folic acid (10 μM) (**D**) in the presence of 5 mM ATP (*closed symbols*) or 5 mM AMP (*open symbols*) and an ATP-regenerating system. Uptake of [^{14}C] EA-SG (2 μM) in MRP2 containing vesicles was performed at 5 min (**B**). Values shown are means±SE of experiments performed in triplicate.

DISCUSSION

In the present study, we identified that the *O*-glucuronide conjugate of gaboxadol is the only major metabolite of gaboxadol formed in humans. Our *in vitro* studies also suggested that UGT1A9 could be involved in the *O*-glucuronidation of gaboxadol in humans. The potential contribution of a range of transporters to the active renal secretion of gaboxadol and its glucuronic acid conjugate in humans was investigated. Our data indicated that gaboxadol was a substrate for the renal uptake transporter hOAT1, but not for hOAT3 and hOCT2, or the renal efflux transporters MRP2 and MRP4. We also found that gaboxadol-*O*-glucuronide, the only metabolite excreted in human urine, was a substrate of MRP4.

In healthy subjects, approximately 34% of an oral dose of gaboxadol (15 mg) was excreted as gaboxadol-*O*-glucuronide in urine (25). This confirmed that the major route of gaboxadol metabolism in human is similar to that of rats (7). UGT1A6, UGT1A7, UGT1A8 and UGT1A9 were capable of catalyzing the *O*-glucuronidation of gaboxadol, with UGT1A9 showing the highest activity followed by UGT1A7 and UGT1A6 (Fig. 2). Since UGT1A7 and UGT1A8 are not expressed in liver and kidney (27), these isoforms were not analyzed in more detail. The pooled human liver and kidney microsomes showed a high level of gaboxadol glucuronidation activity while very low activity was observed with pooled human intestinal microsomes (Fig. 3). This is consistent with the highest rate of gaboxadol *O*-glucuronidation by recombinant UGT1A9 as several reports have indicated significant expression of UGT1A9 in liver and kidney but not intestine (27,28). The K_m for UGT1A9-mediated gaboxadol glucuronidation was 975 μM, while for UGT1A6 the K_m value was greater than 5 mM. Given that the low affinity of UGT1A6 in catalyzing gaboxadol glucuronidation and low therapeutic plasma concentration of gaboxadol (<50 μM), this isoform likely does not play a significant role in the conjugation of gaboxadol *in vivo*. Thus from the list of UGT isoforms that showed significant activity towards gaboxadol *O*-glucuronidation, UGT1A9 appears to be the strongest candidate involved in the metabolism of gaboxadol in kidney and liver.

Gaboxadol has a low molecular weight (MW 145) and contains both a basic moiety (pKa ~8) and an acidic carboxylic functional group (pKa ~4.3), and presents as a zwitterion at physiological pH. Currently, limited information is available regarding renal active uptake mechanism of

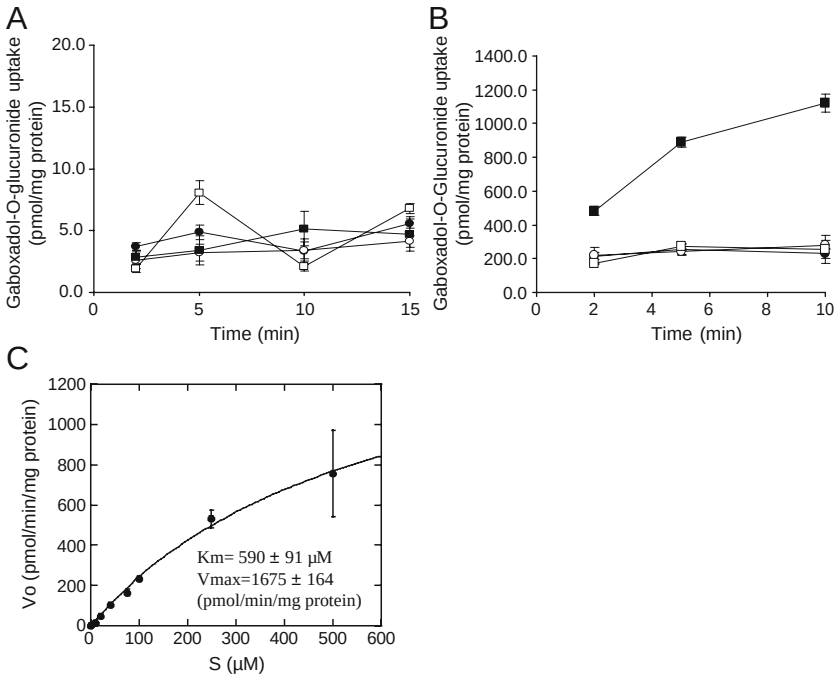

Fig. 7. ATP-dependent uptake of gaboxadol-*O*-glucuronide (20 μM) by membrane vesicles containing MRP2 and MRP4. Membrane vesicles (20 μg of protein) prepared from Sf9 cells expressing β-galactosidase (*circles*; **A** and **B**) or MRP2 (*squares*; **A**) and MRP4 (*squares*; **B**) were incubated at 37°C in transport buffer containing gaboxadol-*O*-glucuronide (20 μM) (**A** and **B**) in the presence of 5 mM ATP (*closed symbols*) or 5 mM AMP (*open symbols*) and an ATP-regenerating system. **C** Concentration dependence of the uptake of gaboxadol-*O*-Glucuronide in MRP4 containing membrane vesicles. The ATP-dependent uptake was calculated by subtracting the uptake in the presence of 5 mM AMP from that in the presence of 5 mM ATP. Values shown are means±SE of experiments performed in triplicate.

zwitterionic compounds compared to anionic and cationic compounds. As shown in Fig. 4A, uptake of gaboxadol in hOAT1 transfected cells was time-dependent and significantly higher than in control cells. Kinetic analysis showed that hOAT1-mediated gaboxadol uptake was saturable (K_m 115 μM; Fig. 4C) and also completely inhibited by probenecid at 1 mM, an inhibitor of organic anion transporters (29) (data not shown). Therefore, it is likely that hOAT1 is involved in the active uptake of gaboxadol into renal proximal tubule cells. Our observation provides an example that zwitterionic compounds can be substrates for OAT1.

Vectorial transport across the renal tubule is achieved by the interplay between uptake and efflux transporters at the basolateral and brush border membrane. To identify the transporters responsible for the luminal efflux of gaboxadol we investigated if gaboxadol is a substrate of the renal efflux transporters MRP2 and MRP4. Our results indicated that uptake of gaboxadol in MRP2 and MRP4 containing vesicles was not time- and ATP-dependent and comparable to that in control vesicles. In addition, uptake of gaboxadol in MRP2 or MRP4 vesicles was not stimulated by reduced glutathione (data not shown), which was known to co-transport or stimulate the uptake of some MRP2 and MRP4 substrates (30,31). Gaboxadol was also unlikely to be a substrate of MDR1 Pgp, based on our transcellular transport study in human MDR1 Pgp transfected cells (data not shown). The mechanism of gaboxadol efflux into the urine therefore remains to be clarified. Potential candidates are MATE1 and -2, but we currently have no assays available for these transporters.

Interestingly, we found that gaboxadol-*O*-glucuronide, was a substrate of MRP4. MRP4 transports a variety of organic anions, such as cAMP, cGMP, *p*-aminohippuric acid, and 17β-estradiol-17β-D-glucuronide (32). MRP4 is also responsible for the luminal efflux of some clinically used drugs, including diuretics, hydrochlorothiazide and furosemide, antiviral drugs, adefovir and tenofovir (15,16), and several cephalosporins (33). Gaboxadol-*O*-glucuronide is a low affinity substrate of MRP4 (K_m 590 μM, V_{max} 1,675 pmol/min/mg protein). Similarly, Adefovir and tenofovir are also low affinity substrates of MRP4 *in vitro* (K_m>1 mM) (16). Studies in Mrp4 knockout mice confirmed that Mrp4 is involved in the luminal efflux of both adefovir and tenofovir *in vivo* (16). Given that approximately 34% of the administered dose of gaboxadol was excreted into urine as gaboxadol-*O*-glucuronide, followed by the glucuronidation in kidney and liver, MRP4 possibly contributes to the luminal efflux of gaboxadol-*O*-glucuronide.

Renal failure could potentially alter the renal elimination of drugs by affecting their transport and metabolism. As reported by Sun *et al.* (34) and Enomoto *et al.* (35), the renal secretion of organic ions mediated by organic anion or

cation transporters decreases with the development of renal failure. For instance, some organic anionic uremic toxins may directly inhibit the renal excretion of various drugs and endogenous organic acids by competitively inhibiting OATs (34). In addition, the expression of OAT1 and OCT2 was reduced in rats with chronic renal failure (36,37). In an unpublished study, renal clearance of both gaboxadol and gaboxadol *O*-glucuronide in subjects with severe renal impairment decreased to 34% and 50%, respectively, of that observed in healthy subjects. Interestingly, plasma AUC of gaboxadol was increased nearly 5-fold. These data suggest that severe renal impairment impacts not only renal excretion of gaboxadol and its glucuronide conjugate but also the glucuronidation by the kidney. This is in line with our *in vitro* data, which suggest that the glucuronidation of gaboxadol could occur in the kidney. The decrease in renal clearance of gaboxadol observed in severely renal impaired patients could be caused in part by reduced renal uptake of gaboxadol by OAT1. Currently, it is unknown if renal failure would result in a reduced activity of MRP4 and UGT in the kidney.

In summary, our data suggest that hOAT1 and MRP4 are involved in the renal uptake of gaboxadol and luminal efflux of gaboxadol-*O*-glucuronide, and that UGT1A9 contributes to the glucuronidation of gaboxadol in both kidney and liver.

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