The Absorption, Metabolism, and Excretion of the Novel Neuromodulator RWJ-333369 (1,2-Ethanediol, [1-2-Chlorophenyl]-, 2-carbamate, [S]-) in Humans

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ABSTRACT:

RWJ-333369 (1,2-ethanediol, [1-2-chlorophenyl]-, 2-carbamate, [S]-; CAS Registry Number 194085-75-1) is a novel neuromodulator currently under clinical investigation for the treatment of epilepsy. To study the disposition of RWJ-333369, eight healthy male subjects received a single oral dose of 500 mg of 14C-RWJ-333369. Urine, feces, and plasma were collected for analysis for up to 1 week after dosing. Radioactivity was mainly excreted in urine (93.8 ± 6.6%) and much less in feces (2.5 ± 1.6%). RWJ-333369 was extensively metabolized in humans, since only low amounts of parent drug were excreted in urine (1.7% on average) and feces (trace amounts). The major biotransformation pathways were direct O-glucuronidation (44% of the dose), and hydrolysis of the carbamate ester followed by oxidation to 2-chloromandelic acid, which was subsequently metabolized in parallel to 2-chlorophenyl glycine and 2-chlorobenzoic acid (mean percentage of the dose for the three acids together was 36%). Other routes were chiral inversion followed by O-glucuronidation (11%), and aromatic hydroxylation in combination with sulfate conjugation (5%). In plasma, unchanged drug accounted for 76.5% of the total radioactivity, with the R-enantiomer and the O-glucuronide of the parent drug as the only measurable plasma metabolites. With the use of very sensitive liquid chromatography-tandem mass spectrometry techniques, only traces of aromatic (pre)mercapturic acid conjugates were detected in urine (each <0.3% of the dose), suggesting a low potential for reactive metabolite formation. In conclusion, the disposition of RWJ-33369 in humans is characterized by virtually complete absorption, extensive metabolism, and unchanged drug as the only significant circulating species.
Materials and Methods

Test Article and Formulation. RWJ-333369 was specifically labeled with $^{14}$C at the benzylic carbon (Fig. 1). The $^{14}$C label at this position has been proven to be metabolically stable, as evidenced by the lack of $^{14}$CO$_2$ exhalation in the rat after oral dosing of $^{14}$C-RWJ-333369.

$^{14}$C-RWJ-333369 with a specific radioactivity of 998 MBq/mmol was diluted with unlabeled RWJ-333369 to the desired specific activity of approximately 3.7 kBq/mg (0.1 μCi/mg). Therefore, unlabeled RWJ-333369 (10.0 g) was dissolved in 12.0 ml of methanol (refluxed at 70°C, until all material is dissolved). The mixture was allowed to cool and 37 MBq of $^{14}$C-labeled RWJ-333369, dissolved in 1.90 ml of methanol, was added at a temperature before crystallization occurred (approximately 50°C). The mixture was stirred overnight at room temperature and 6.0 ml of water (pH 3.73) was added. This mixture was cooled to 5 ± 5°C, stirred for 2 h at this temperature, and filtered. The filter cake was washed three times with 3 ml of water and the material was dried at 42°C (±5°C) under vacuum (Büchi TO-51; Q-Lab, Belgium) to yield the desired polymorph A as colorless needles. The specific radioactivity of the dried material resulting from the filtration was 998 MBq/mmol.

The formulation was prepared as follows: $^{14}$C-RWJ-333369 (specific activity of 3.7 kBq/mg), 250.0 mg per capsule; microcrystalline cellulose (Avicel PH-101; FMC International, Cork, Ireland), 130.0 mg per capsule; sodium starch glycolate (Primojel; Avebe, Veendam, The Netherlands), 25.5 mg per capsule; magnesium stearate (Univar Benelux NV, Brussels, Belgium), 2.5 mg per capsule; total capsule fill weight, 408 mg per capsule; and Swedish Orange Size 00 gelatin capsules (Capsugel, Bornem, Belgium), 1 capsule. The formulation was stored at 4 ± 2°C in amber vials. The radioactivity concentration was 2.268 kBq/mg formulation (100.0%), the total RWJ-333369 concentration was 0.604 mg/mg formulation (98.5%), the radiochemical purity was 99.9%, and the enantiomeric excess was 99.2%.

Subjects. The protocol of the clinical trial was approved by an internal protocol review committee and an independent Ethics Committee. The trial was performed in accordance with the Declaration of Helsinki (1964) and its subsequent revisions. The radioactivity dose was approved by an external Radiation Protection expert. Eight healthy subjects were recruited according to the following inclusion criteria: male gender; age 40 to 60 years; nonsmoking; no history of serious idiosyncratic reactions to drugs; or abnormal liver, kidney, or heart function. All subjects gave their full informed consent before the start of the study.

Before dose administration, subjects had fasted overnight for at least 8 h. Intake of water was allowed until 2 h before dose administration. Eight subjects were enrolled to ensure that at least six subjects would complete the study. Each subject was administered two 250-mg capsules of $^{14}$C-RWJ-333369 with 240 ml (8 oz.) of water. Subjects refrained from drinking water until 2 h after dosing and fasted until 4 h after dosing. Water was allowed ad libitum beyond 2 h after dosing. Subjects were allowed to resume a normal diet, as provided by the clinical unit, after measurement of vital signs and collection of biological samples 4 h after drug administration.

Sample Collection. Venous blood samples (10 ml, unless otherwise indicated) were collected in heparinized tubes before dosing (0 h; 20 ml), and at 0.5, 1 (20 ml), 1.5, 2 (20 ml), 3, 4 (20 ml), 8 (20 ml), 12, 24 (20 ml), 36, 48, 72, 96, 120, 144, and 168 h after dosing. An aliquot of 2 ml of whole blood from samples taken at 0, 1, 2, 4, 8, and 24 h after dosing was transferred to separate tubes and stored at −18°C for the measurement of total radioactivity in whole blood. The remaining blood samples were processed for plasma and kept at −18°C until analysis.

Urine collections were obtained within 2 h before and during the intervals 0 to 4, 4 to 8, 8 to 12, and 12 to 16 h; 16 to 24 h; 24 to 36 h; 36 to 48 h; 48 to 72 h; 72 to 96 h; 96 to 120 h; 120 to 144 h; and 144 to 168 h. Feces (per stool) were collected starting before dosing on day −1 and continuing until 7 days after dosing.

Sample Analysis. Blank samples of plasma, urine, and feces were fortified with known quantities of radiolabeled RWJ-333369 and stored during the period of the studies to allow verification of the stability of the drug in these media.

Radioactivity balance in urine. Levels of total radioactivity in urine samples were determined by liquid scintillation counting of duplicate 0.25-ml aliquots of the urine samples, using 10 ml of UltimaGold (Packard) as scintillation cocktail and a Packard Tri-Carb 1900 TR or 2100 TR liquid scintillation spectrometer.

Radioactivity balance in feces. The first five to eight feces stools were homogenized in methanol using an Ultra-Turrax homogenizer. After centrifugation of the suspension, the residues were extracted twice more with methanol. After the last extraction, the methanol extracts were separated from the residues by filtration of the suspensions through a Büchner funnel. The methanol extracts of each fecal stool were combined and the volume of the combined extracts was measured, using a volumetric cylinder. Levels of TR in the methanol extracts were determined by liquid scintillation counting. Therefore, duplicate 0.25-ml aliquots were diluted with distilled water up to 1 ml and mixed with 10 ml of UltimaGold (Packard) as a scintillation cocktail. The fecal residues were air-dried and the weight of the residues was measured. The dried residues were ground to a fine powder in an Ultra Centrifugal Mill ZM100 (Retsch GmbH, Haan, Germany). Four weighed samples of 80 to 120 mg of each residue sample were combusted in a Packard Sample Oxidizer model 307. Carbosorb (8 ml; Packard) was used to absorb the $^{14}$CO$_2$ and Permafluor (10 ml; Packard) was used as a scintillation cocktail. TR in the combusted residues was measured by liquid scintillation counting, using a Packard Tri-Carb 1900TR or 2100 TR liquid scintillation spectrometer. The methanol fecal extracts were stored in brown glass containers and the fecal residues were stored in plastic bags, both at 4 ± 2°C.

No methanol extracts were prepared for the fecal samples collected at the end of the first week’s collection. The latter fecal samples were lyophilized and homogenized, and the dried fecal residues were ground in an Ultra Centrifugal Mill ZM100 (Retsch GmbH). Four weighed aliquots of 80 to 120 mg of each residue sample were combusted in a Packard Sample Oxidizer and the radioactivity of the liberated $^{14}$CO$_2$ was counted as described above.

Radioactivity in blood and plasma. Blood radioactivity levels were measured in the samples (from predose and at 1, 2, 4, 8, and 24 h after dosing) after combining and quadruplicate dried 0.25-ml aliquots in a Packard Sample Oxidizer model 307. Carbosorb (9 ml; Packard) was used to absorb the $^{14}$CO$_2$ and Permafluor (11 ml; Packard) was used as a scintillation cocktail. TR in the combusted blood samples was measured in a Packard Tri-Carb 1900TR liquid scintillation spectrometer. Plasma levels of total radioactivity were determined by liquid scintillation counting of duplicate 0.25-ml aliquots of the plasma samples using UltimaGold as a scintillation cocktail.

Bioanalysis of unchanged RWJ-33369 in plasma and urine. Plasma and urine concentrations of RWJ-33369 were determined using a validated LC-MS/MS method. To 100-μl aliquots of plasma or urine, 200 ng of a stable isotope-labeled internal standard (R293792; racemic mixture of $S$-isomer and $R$- isomer) were added. After buffering each sample with 1 ml of 0.1 M phosphate buffer (pH 7), samples were extracted with 3 ml of methyl tert-butyl ether. The organic layer was evaporated under nitrogen at 65°C and the residue dissolved in 500 μl of injection mixture (0.01 M ammonium acetate/methanol; 70:30). Forty microliters of injection mixture were injected on a 5 × 4.6 mm i.d. column, packed with 5 μm of Hypersil BDS C18 (Alttech Associates, Deerfield, IL). The mobile phase was a 0.01 M ammonium acetate/methanol mixture (55:45 v/v) with a flow rate of 1.5 ml/min, yielding a retention time for both compounds of 1 min. LC-MS/MS analysis was performed using a Sciex API 3000 (Applied Biosystems, Foster City, CA) equipped with a Turboion-spray source operated in the positive ion mode. The analyte and its internal standard were monitored in the multiple reaction monitoring (MRM) mode at 254.0 > 198.0 and 363.0 > 208.0 m/z. The concentration was 0.604 mg/mg formulation (98.5%), the radiochemical purity was 99.9%, and the enantiomeric excess was 99.2%.

FIG. 1. Chemical structure of $^{14}$C-RWJ-333369 with the position of the $^{14}$C label indicated with an asterisk.

Cl

O

H

NH2

O

OH

Fig. 1. Chemical structure of $^{14}$C-RWJ-333369 with the position of the $^{14}$C label indicated with an asterisk.
standard were monitored at transitions m/z 216.0 → 155.0 and m/z 220.1 → 159.0, respectively. The validated range for both plasma and urine was 100 to 50,000 ng/ml.

The plasma concentrations of the separate S- and R-enantiomers (RWJ-333369 and RWJ-452399, respectively) were determined in the 0.5-, 1-, 2-, 3-, 4-, 8-, 12-, and 24-h samples of all subjects. The samples were analyzed by a chiral LC-MS/MS method as follows. To 100 μl of plasma, 200 ng of the stable isotope-labeled internal standard (R293792; racemic mixture of R- and S-enantiomer) was added. After adding 1 ml of 0.1 M phosphate buffer (pH 7), the samples were extracted with 2.5 ml of methyl tert-butyl ether. The organic layer was evaporated under nitrogen at 65°C and the residue was dissolved in 500 μl of a hexane/ethanol (50:50) mixture. The extracts were analyzed by LC-MS/MS. The injection volume was 40 μl. Chromatographic separation was done on a 50 × 4.6 mm i.d. Chiralpak AD HPLC column (Daicel Chemical Industries Ltd., Tokyo, Japan). The mobile phase was composed of 0.02 M ammonium acetate in ethanol/hexane (20:80), pumped at 1 ml/min. Separation was performed at ambient temperature. A Scieix API 3000 triple quadrupole mass spectrometer, equipped with a TurboionSpray interface, was operated in the positive ion mode. Ionspray voltage was 4500 V. The turbo gas temperature was 50°C. RWJ-333369 and RWJ-452399 were monitored at transitions m/z 216.0 to m/z 155.0. The internal standards (both enantiomers) were monitored at m/z 222.1 to m/z 161.0. Total chromatographic run time was approximately 5 min. The limit of quantitation was 30.0 ng/ml for either enantiomer.

Radio-HPLC analysis. Where appropriate, individual or overall pools of the urine samples or the methanol extracts of the fecal samples were prepared by mixing constant fractions of the individual samples or the individual pools, respectively. Overall pools of the plasma samples were prepared by mixing equal volumes of the individual samples.

Plasma was deproteinized by addition of acetonitrile (1.5 ml/ml plasma). The precipitated proteins were removed by centrifugation (approximately 10 min at 3000g), and the supernatants were collected and evaporated to dryness. The evaporation residues were redissolved in 1 ml of dimethyl sulfoxide/water mixture (20:80 v/v). Aliquots of 750 μl of the redissolved samples were injected on the radio-HPLC system.

Urine samples were injected on the radio-HPLC system after centrifugation. For feces, 10-ml aliquots of the methanol extracts of some fecal samples and of the spiked fecal sample were evaporated under nitrogen, and the residues were reconstituted in 600-μl aliquots of a dimethyl sulfoxide/water mixture, 20:80 (v/v). Aliquots of 300 μl of these samples were injected on the radio-HPLC system.

The HPLC apparatus consisted of a Waters (Milford, MA) Alliance 2695 system, equipped with a Waters automatic injector. The samples were chromatographed on a stainless steel column (300 × 4.6 mm i.d.) packed with Kromasil C-18 (5 μm, EKA Chemicals, Bohus, Sweden). The columns were packed by a balanced density slurry procedure (Haskel DSTV 122-C pump, Haskel Inc., Burbank, CA). UV detection was performed at 218 nm using a Waters 996 diode array detector. On-line radioactivity detection of HPLC eluates was carried out with a Berthold Radioactivity Monitor LB 507 A system (Berthold Technologies, Bad Wildbad, Germany) equipped with a flow-through cell of 1000 l/min. The Q-Tof Ultima mass spectrometer was equipped with a dual electrospray ionization probe and was operated in the positive ion and negative mode at a resolution of 10,000 (full-width half-maximum). The source temperature was 100°C, the desolvation temperature was 250°C, and the cone voltage was set at 40 V. The second LockSpray ESI probe provided an independent source of the lock mass calibrant H3PO4 ([M + H]+) ion at m/z 196.9166 was used as the calibrant). Data were acquired in the centroid mode with a scan time of 1 s and processed using MassLynx v. 4.0 software (Waters).

Chemical Synthesis of Mercapturic Acid Conjugates. To facilitate the identification of mercapturic acid conjugates, in total, 10 aromatic mercapturic acid conjugates were chemically synthesized on the four unsubstituted positions of the aromatic ring including the chlorine shift, and for the R- as well as for the S-enantiomer. An overview of the authentic mercapturic acid conjugates and a representative synthetic sequence (for compounds 7a–e, S and R) is depicted in Fig. 3. The experimental details are given below for compound N-acetyl-S,S′-[1-[(methylamino)carbonyloxy]-l-hydroxyethyl]-2-chlorophenyl-l-cysteine (compound 7a, S-enantiomer, JN-26641836-AAA).

Experimental details. 1-Chloro-3-nitro-2-vinylbenzene (9d) was synthesized according to the literature (Mundla, 2000). All chemicals were commercially available. Specifically, compounds 1a and 1c were obtained from Sigma-Aldrich NV (Bornem, Belgium), and compounds 1b and 1e were purchased from Acrros Organics (Geel, Belgium).

(3-Amino-2-chlorophenyl)ethanone (2a). A solution of 2-chloro-3-nitrobenzoic acid (1a; 44.6 mmol, 9.0 g) in dry tetrahydrofuran (100 ml) was cooled on ice and lithium aluminium hydride (49 mmol, 1.86 g) was added portion-wise to the stirred mixture. After 1 h, the cooled mixture was decomposed with water (2.5 ml), 5 N aqueous sodium hydroxide solution (1.9 ml), and water (8.2 ml). The precipitate was filtered and rinsed with tetrahydrofuran/methanol (1:1 v/v); the filtrate was concentrated and chromatographed over silica with dichloromethane/methanol (95:5 v/v) to give 4.5 g of (2-chloro-3-nitrophenyl)methanol (53%). This material (19 mmol, 3.6 g) was hydrolyzed for 18 h in a mixture of platinum on carbon (5%, 1 g), a 4% solution of thiophene in diisopropyl ether (0.5 ml), triethylamine (1 ml), and tetrahydrofuran (50 ml). The mixture was filtered over dicalite and concen-
trated at aspirator pressure to yield 2.9 g of 2a (98%), which was used as such in the next reaction step.

2-Chloro-1-iodo-3-vinylbenzene (3a). (3-Amino-2-chlorophenyl)methanol (2a; 18.4 mmol, 2.9 g) was suspended in a cold solution of water (10 ml) and concentrated hydrochloric acid (10 ml). A solution of sodium nitrite (20.4 mmol, 1.41 g) was dropped into the suspension and the reaction mixture was stirred for 2 h on ice. The solution of the diazonium salt was then slowly transferred to a solution of potassium iodide (55.4 mmol, 9.2 g) in water (20 ml) and then stirred at room temperature for 48 h. The precipitate was filtered, dissolved in dichloromethane, and was made alkaline with 1 N aqueous sodium hydroxide solution. The organic layer was separated, dried, and concentrated to yield 4.3 g of (2-chloro-3-iodophenyl)methanol (86%). The material was stirred for 48 h in a suspension of dry tetrahydrofuran (40 ml) and dry diethyl ether (40 ml) containing activated manganese(IV) oxide (6.4 g, 74 mmol). The reaction mixture was filtered over dicalite, rinsed with tetrahydrofuran, and concentrated to give 3.0 g of 2-chloro-3-iodobenzaldehyde (70%). A suspension of methyltriphenylphosphonium iodide (8.4 mmol, 3.4 g) and dry tetrahydrofuran (20 ml) was stirred under argon atmosphere. n-Butyllithium (2.1 M in hexane, 8.4 mmol, 4.0 ml) was dropped into the cooled suspension and the reaction mixture was allowed to stir for 1 h at room temperature. It was then cooled again on ice, and a solution of 2-chloro-3-iodobenzaldehyde (7.6 mmol, 2.0 g) in dry tetrahydrofuran (8 ml) was dropped into it and the reaction mixture was stirred for 18 h at room temperature. The mixture was filtered over dicalite, which was then rinsed with ether and

![Chemical structures of the authentic compounds cochromatographed with urine, feces, and plasma samples from male subjects dosed with 14C-RWJ-333369.](image-url)
tetrahydrofuran. The filtrate was concentrated at aspirator pressure and the residue was stirred several times each with hexane. The combined hexane layers were chromatographed over silica (eluate pure hexane) to afford 2-chloro-1-iodo-3-vinylbenzene 1.27 g (3a; 64%, 38.9% three-step yield).

A mixture of AD-mix-H9251 (5.7 mmol, 8.0 g), water (25 ml), and tert-butanol (25 ml) was stirred under argon. At 0°C, 3a (1.27 g, 4.8 mmol) was slowly added; the mixture was allowed to come to room temperature and was stirred for 18 h. The reaction mixture was again cooled on ice and sodium sulfite (12 g) was spooned into it. Water (50 ml) was added and the whole was thoroughly extracted with ethyl acetate. The organic layers were combined, dried on magnesium sulfate, and concentrated. The residue was stirred at room temperature for 2 h with a mixture of hexane/ethyl acetate (95:5 v/v, 8 ml) and the solid was filtered off to afford 4a, S-enantiomer (1.34 g, 93%) with an enantiomeric excess >99%.

(4S)-4-(2-Chloro-3-iodophenyl)-1,3-dioxolan-2-one (5a, S-enantiomer). A reaction flask fitted with a Dean-Stark trap was charged with diethylcarbonate (1.17 ml), sodium methanolate powder (0.37 mmol, 20 mg), and 4a (4.1 mmol, 1.22 g). The mixture was heated under nitrogen atmosphere for 15 min to 140°C and was then allowed to cool to room temperature. The solvent was removed at 60°C (aspirator pressure) and the residue was taken up in water/dichloromethane (1:1 v/v, 20 ml). The layers were separated and the water layer was extracted three times with dichloromethane. The organic layers were combined, dried, and concentrated and the residue was chromatographed over silica [eluate: dichloromethane/methanol (98:2 v/v)] to yield 5a, S-enantiomer (900 mg, 67%).
Overall yields obtained for 1-(2-chloro-6-nitrophenyl)ethane-1,2-diol enantiomers as described for 9d 1-chloro-3-nitro-2-vinylbenzene (order from that described for the other compounds. Commercially available pounds

The nitro group was then reduced to the aniline function, which was trans-

A suspension of 6a (2.6 mmol, 850 mg) in 2-propanol (6 ml) was slowly

N-Acetyl-S-(3-{(1S)-2-[(aminocarbonyl)oxy]-1-hydroxyethyl}-2-chlorophe-

(2S)-2-(2-Chloro-3-iodophenyl)-2-hydroxyethyl carbamate (7a, S-enanti-

8c, 58.3 mmol, 10.0 g) in dry tetrahydrofuran (350 ml) was

(5-Amino-2-chloro-benzoic acid (8c, 58.3 mmol, 10.0 g) in dry tetrahydrofuran (350 ml) was treated with lithium aluminum hydride (116 mmol, 4.5 g) as described for 2a, to yield 3.1 g of 2c (34%).

Evolution of Radioactivity in Urine and Feces. By far, the major part of the 14C-RWJ-33369-related radioactivity was excreted in urine (Table 1). At 24 h after a single oral dose of 500 mg of 14C-RWJ-33369 in healthy male subjects, 56.6 to 70.5% (mean 63.9%) of the dose was excreted in urine. At 1 week after dosing, 80.4 to 101.3% of the administered radioactivity (mean 96.3% of the dose) had been excreted. The cumulative excretion of the radioactivity in urine after 1 week accounted for 79.8 to 99.8% (mean 93.8%) of the dose. The cumulative excretion of radioactivity in feces accounted for 0.6 to 5.1% (mean 2.5%) of the dose. The TR in fecal samples was calculated as the sum of the TR in the methanol extracts and the fecal residues prepared from these samples.

Plasma Kinetics of TR and Unchanged RWJ-33369. The pharmacokinetic parameters calculated from the plasma levels of TR and unchanged RWJ-33369 are presented in Table 2. Plasma concentration-time profiles for TR (expressed as nanogram-equivalents to RWJ-33369 per milliliter of plasma) and for RWJ-33369 are shown in Fig. 4. The Cmax of TR in plasma was attained between 0.5 and 1 h after dosing. Highest UD

were analyzed by standard noncompartmental analysis (WinNonlin v.4.0.1; Pharsight, Mountain View, CA). Based on the individual plasma concentration-time data, the following pharmacokinetic parameters were calculated, as follows: AU(t0→t), area under the plasma concentration-time curve from 0 to the time of the last quantifiable concentration, by linear trapezoidal summation; AU(t→∞), area under the plasma concentration-time curve extrapolated to infinity using A2; Cmax, peak plasma concentration, by visual inspection of the data; A2, linear terminal slope determined by linear regression of the terminal points of the plasma concentration-time curve; t1/2, terminal half-life, defined as 0.693/A2; and tmax, time to reach the peak plasma concentration, by visual inspection of the data.

Results

Dose Received. The nominal dose was 500 mg, the actual dose ingested was 493.1 mg (divided over two capsules), and the radioactive dose was 1852 kBq (or 50.0 μCi) per subject. This radioactive dose was calculated to result in a radiation exposure of less than 1 mSv. An effective dose between 100 and 1000 μSv is categorized as a category IIa project (a minor level of risk, covering doses to the public from controlled sources) (ICRP, 1993).

Excretion of Radioactivity in Urine and Feces. Excretion of radioactivity in urine and feces after oral administration of 500 mg of 14C-RWJ-33369 was measured as the percentage of the administered dose. This excretion is presented in Table 1. The excretion of radioactivity in urine and feces was expressed as a percentage of the administered radioactive dose. The mass balance of RWJ-33369 and its major metabolites was presented as the percentage of the sample or dose radioactivity accounted for by these radiolabeled compounds.

In plasma, the levels of unchanged RWJ-33369 and its major metabolites were presented as a percentage of the administered dose. The mass balance of RWJ-33369 and its major metabolites was presented as the percentage of the sample or dose radioactivity accounted for by these radiolabeled compounds.

Table 1: Excretion of radioactivity in urine and feces of eight healthy male adult subjects after single oral administration of 500 mg of 14C-RWJ-33369.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Subject 101</th>
<th>Subject 102</th>
<th>Subject 103</th>
<th>Subject 104</th>
<th>Subject 105</th>
<th>Subject 106</th>
<th>Subject 107</th>
<th>Subject 108</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>0–4 h</td>
<td>12.58</td>
<td>11.98</td>
<td>14.50</td>
<td>11.34</td>
<td>13.35</td>
<td>11.53</td>
<td>11.31</td>
<td>10.52</td>
<td>12.14 ± 1.29</td>
</tr>
<tr>
<td>4–8 h</td>
<td>13.11</td>
<td>13.46</td>
<td>22.53</td>
<td>12.89</td>
<td>15.35</td>
<td>7.13</td>
<td>12.94</td>
<td>12.70</td>
<td>13.76 ± 4.25</td>
</tr>
<tr>
<td>8–12 h</td>
<td>12.14</td>
<td>13.53</td>
<td>5.74</td>
<td>12.97</td>
<td>13.43</td>
<td>13.16</td>
<td>14.61</td>
<td>14.40</td>
<td>12.50 ± 2.84</td>
</tr>
<tr>
<td>12–16 h</td>
<td>4.21</td>
<td>12.80</td>
<td>11.92</td>
<td>12.42</td>
<td>13.40</td>
<td>12.09</td>
<td>12.15</td>
<td>5.87</td>
<td>10.61 ± 3.50</td>
</tr>
<tr>
<td>24–36 h</td>
<td>18.13</td>
<td>16.74</td>
<td>16.76</td>
<td>15.74</td>
<td>15.60</td>
<td>12.14</td>
<td>15.35</td>
<td>11.77</td>
<td>15.28 ± 2.23</td>
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<tr>
<td>36–48 h</td>
<td>7.69</td>
<td>7.21</td>
<td>6.59</td>
<td>7.93</td>
<td>6.99</td>
<td>9.19</td>
<td>7.60</td>
<td>6.28</td>
<td>7.44 ± 0.90</td>
</tr>
<tr>
<td>48–72 h</td>
<td>6.38</td>
<td>5.84</td>
<td>5.05</td>
<td>5.65</td>
<td>4.98</td>
<td>6.71</td>
<td>5.73</td>
<td>3.55</td>
<td>5.49 ± 0.98</td>
</tr>
<tr>
<td>72–96 h</td>
<td>1.68</td>
<td>1.27</td>
<td>0.99</td>
<td>1.75</td>
<td>1.23</td>
<td>1.79</td>
<td>1.72</td>
<td>1.30</td>
<td>1.47 ± 0.30</td>
</tr>
<tr>
<td>96–120 h</td>
<td>0.28</td>
<td>0.21</td>
<td>0.00</td>
<td>0.31</td>
<td>N.D.</td>
<td>0.47</td>
<td>0.37</td>
<td>0.32</td>
<td>0.33 ± 0.09</td>
</tr>
<tr>
<td>120–144 h</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.13</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.C.</td>
<td>N.C.</td>
</tr>
<tr>
<td>144–168 h</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Sum (0–168 h)</td>
<td>96.94</td>
<td>97.96</td>
<td>99.84</td>
<td>96.59</td>
<td>95.53</td>
<td>88.11</td>
<td>96.08</td>
<td>79.82</td>
<td>93.78 ± 6.58</td>
</tr>
<tr>
<td>Feces (0–168 h)</td>
<td>1.75</td>
<td>2.26</td>
<td>1.50</td>
<td>4.57</td>
<td>1.64</td>
<td>2.43</td>
<td>5.13</td>
<td>0.57</td>
<td>2.48 ± 1.57</td>
</tr>
<tr>
<td>Total (0–168 h)</td>
<td>98.69</td>
<td>99.62</td>
<td>101.34</td>
<td>101.16</td>
<td>97.17</td>
<td>100.54</td>
<td>100.21</td>
<td>80.39</td>
<td>96.27 ± 7.34</td>
</tr>
</tbody>
</table>

N.D., concentration of the total radioactivity in the urine sample was below the limit of quantitation; N.C., not calculated.
plasma concentrations were 9.0 to 14.6 μg/ml (on average 11.3 μg/ml). Based on the \(C_{\text{max}}\) values for TR and unchanged RWJ-333369, unchanged RWJ-333369 accounted for 94.0 ± 4.6% of plasma TR. Based on the AUC0-\(t\) values for TR and unchanged RWJ-333369, unchanged RWJ-333369 accounted for 76.5 ± 2.3% of total plasma radioactivity. At any time point during the first 24 h after dosing, the amount of any individual metabolite was very small relative to RWJ-333369 (less than 4% of the 1- to 8-h plasma radioactivity and less than 10% in 24-h plasma, as estimated from radio-HPLC analysis).

The average blood to plasma concentration ratio of TR ranged from 0.89 ± 0.04 to 0.99 ± 0.06 during the first 24 h after dosing. This means that RWJ-333369 and its metabolites are not bound to blood cells to a significant extent. The overall R/S-enantiomer exposure ratios (AUC0-24h) ranged from 3.64% to 6.11%, indicating that the R-enantiomer is a minor circulating metabolite.

**Metabolite Profile of RWJ-333369 after Oral Administration.**

The major metabolites of RWJ-333369 identified in this human AME trial are listed in Table 3. The structures of the identified metabolites are shown in the metabolic scheme (Fig. 5). The metabolites were given a numerical code based on the retention time of all metabolites detected so far in the animal studies.

The MS/MS product-ion electrospray ionization (ESI) mass spectrum of RWJ-333369 shows characteristic fragment ions, useful for metabolic identification (Fig. 6; Table 3).

The retention time and the MS/MS product-ion ESI spectra of the radioactive peak UD (MH + 216) and the reference compound RWJ-333369 were identical. Therefore, UD is identified as the parent drug RWJ-333369.

Metabolite 1 (MH + 186) is coeluting with reference compound R342141. The fragmentation patterns of the reference compound and metabolite 1 were identical. Therefore, metabolite 1 is identified as 2-chlorophenyl glycine (R342141).

Metabolite 4 (MH + 155) is coeluting with reference compound R300100. The protonated molecular ion of the reference compound and metabolite 4 were identical. Therefore, metabolite 4 is identified as 2-chlorobenzoic acid (R300100).

Metabolite 5 (MH + 185) is coeluting with reference compound R288222. The protonated molecular ion of the reference compound and metabolite 5 were identical. Therefore, metabolite 5 is identified as 2-chloromandelic acid (R288222).

The mass difference of 96 units of the protonated molecular ion (m/z 312) of metabolite 11 compared with RWJ-333369 suggests sulfation and oxidation. A loss of \(H_2O\) gives the fragment ion m/z 294. The electrospray spectra in the negative mode confirm the proposed structure (m/z 310). The exact mass Q-Tof spectrum in negative and positive mode of metabolite 11 fell within 5 ppm of the calculated exact mass of a sulfate conjugate of hydroxylated RWJ-333369. Metabolite 11 was further identified by co-injection with urine collected from beagle dogs, after a single oral dose of \(^{14}C\)-RWJ-333369, as a sulfate conjugate of an aromatically hydroxylated metabolite of RWJ-333369. Enzymatic hydrolysis of metabolite 11 with arylsulfatase released the aglycon. NMR identification showed that metabolite 11 was a sulfate conjugate at the 3-position of the phenyl ring of RWJ-333369.

The ESI mass spectrum of metabolite 21 reveals the protonated molecular ion at m/z 392. A mass shift of 176 units as compared with RWJ-333369 points to glucuronidation of the molecule. The retention time and the fragmentation behavior of the reference compound R289876 and metabolite 21 were identical. Therefore, metabolite 21 was identified as the O-glucuronide of RWJ-333369.

The fragmentation behavior and the retention time of reference compound R382574 and metabolite 23 were identical. The structure of the metabolites 21 and 23 was further confirmed by enzymatic hydrolysis with \(\beta\)-glucuronidase from \(H.\) pomatia and from \(E.\) coli, showing that metabolites 21 and 23 were hydrolyzed to RWJ-333369 and its R-enantiomer, respectively. Metabolite 23 was therefore identified as a glucuronide of the enantiomer of RWJ-333369.

**Metabolite Profile in Urine.**

The separation of the authentic substances used for HPLC cochromatography is shown in Fig. 7, together with the metabolite patterns obtained from the analysis of a urine sample. Parent RWJ-333369 was stable in urine, as indicated by the results of the radio-HPLC analysis of a blank urine sample that was fortified with the formulation. Unchanged RWJ-333369 represented 1.1 to 2.4% (1.7% on average) of the dose in 0- to 72-h urine of the eight subjects, indicative of extensive metabolism. Besides parent RWJ-333369, the identity of metabolites 1, 4, 5, 21, and 23 could be confirmed by cochromatography with known authentic substances (Fig. 7).

The by far major metabolite 21 is derived from glucuronidation of RWJ-333369. In urine treated with \(\beta\)-glucuronidase, the major metabolite 21 was clearly hydrolyzed to parent RWJ-333369. In human urine, this glucuronide (O-glucuronide of RWJ-333369) and the glucuronide of the enantiomer RWJ-452399 (metabolite 23) could be hydrolyzed by \(\beta\)-glucuronidase from \(E.\) coli. Metabolites 21 and 23 have been isolated from rabbit urine for NMR spectroscopy and chiral analysis, which clearly showed that the aglycon of metabolite 21 was RWJ-333369, and the aglycon of metabolite 23 was the enantiomer of RWJ-333369 (RWJ-452399). The O-glucuronide of RWJ-333369 (metabolite 21) accounted for 37.3 to 52.4% of the dose (44.4% on average).

Metabolite 4, which coeluted with 2-chlorobenzoic acid (R300100), and metabolite 1 (2-chlorophenyl glycine, R342141) were the second
TABLE 3
Identification of metabolites of RWJ-333369 in humans after a single oral dose (500 mg) of [14C]-RWJ-333369

<table>
<thead>
<tr>
<th>Metabolite Code</th>
<th>Identification Method</th>
<th>Identity</th>
<th>MH⁺</th>
<th>Typical MS Fragments (see Fig. 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UD</td>
<td>Cochromatography</td>
<td>Unchanged RWJ-333369</td>
<td>216</td>
<td>198, 155, 119</td>
</tr>
<tr>
<td>2</td>
<td>Cochromatography</td>
<td>2-Chlorophenyl glycine (R342141)</td>
<td>186</td>
<td>169, 140</td>
</tr>
<tr>
<td>4</td>
<td>Cochromatography</td>
<td>2-Chlorobenzoic acid (R300100)</td>
<td>155</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Cochromatography</td>
<td>2-Chloromandelic acid (R288222)</td>
<td>185</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>LC-MS/MS, co-elution with dog metabolite, NMR</td>
<td>Sulfate conjugate at the 3-position of the phenyl ring of RWJ-333369</td>
<td>312</td>
<td>294</td>
</tr>
<tr>
<td>21</td>
<td>Cochromatography</td>
<td>O-Glucuronide of RWJ-333369 (R289876)</td>
<td>392</td>
<td>374, 216, 198</td>
</tr>
<tr>
<td>23</td>
<td>Cochromatography</td>
<td>O-Glucuronide of RWJ-452399 (enantiomer of RWJ-333369) (R382574)</td>
<td>392</td>
<td>374, 216, 198</td>
</tr>
</tbody>
</table>

Fig. 5. Metabolic pathways of RWJ-333369 after a single oral dose of 500 mg in healthy male subjects. The structures shown between brackets have not been detected as such but are presumed intermediates.
shown in Figs. 2 and 5, respectively.

The identities of the authentic substances (indicated with their laboratory codes in the lower chromatogram) and of the metabolites are shown in Figs. 2 and 5, respectively.

FIG. 7. Radio-HPLC chromatogram for the 0- to 24-h urine pooled sample from eight subjects after a single oral dose of 500 mg of 14C-RWJ-333369 (upper panel), and separation of the authentic substances used for cochromatography (UV detection at 218 nm; lower panel). The identities of the authentic substances (indicated with their laboratory codes in the lower chromatogram) and of the metabolites are shown in Figs. 2 and 5, respectively.

major metabolites in urine. Metabolite 4 accounted for 9.1 to 21.0% of the dose (14.5% on average). Metabolite 1 accounted for 11.7 to 19.5% of the dose (16.2% on average). Metabolite 5, coeluting with 2-chloromandelic acid (R288222), accounted for 3.0 to 6.5% of the dose (5.0% on average). Overall, the metabolites resulting from carbamate hydrolysis (i.e., the metabolites 1, 4, and 5) made up 35.7% of the dose on average. The formation of 2-chlorophenyl glycin (metabolite 1) involves the hydrolysis of the carbamate and further oxidation to 2-chloromandelic acid (metabolite 5), which then was followed by transamination.

Another major metabolite in human urine was the glucuronide of the enantiomer of RWJ-333369 (metabolite 23), which accounted for 8.9 to 12.1% of the dose (10.6% on average). Metabolite 11, a sulfate conjugate at the 3-position of the phenyl ring of RWJ-333369, accounted for, on average, 4.8% of the dose (range 2.3–5.8%).

Premercapturic acid and mercapturic acid conjugates of RWJ-333369 were detected in measurable amounts in rat urine after a single oral dose of RWJ-333369 at 30 mg/kg (2–3% of the dose each; data on file). Because these metabolites were not detectable by radio-HPLC in human urine, more sensitive LC-MS/MS analysis (QToF) was used to examine for the presence of these types of metabolites in humans.

The premercapturic and mercapturic acids have a corresponding most abundant fragmentation ion of m/z 359.0468 (Fig. 8). Mass chromatograms with this ion (molecular ion of the mercapturic acid conjugates — H2O), 377.0574 (molecular ion of the mercapturic acid conjugates) or 399.0399 (sodium adduct of the mercapturic acid conjugates) and a mass window of 0.04 Da gave an indication of the relative presence of the premercapturic and mercapturic acids in the different samples (Fig. 9). For semiquantitative analysis, mass chromatograms were plotted to estimate the relative presence of the premercapturic and mercapturic acids in the different samples. The premercapturic acid conjugates could be differentiated from the mercapturic acid conjugates based on their shorter HPLC retention time and on the presence of the (sodium) adducts of the respective molecular ions.

Three peaks associated with premercapturic acid conjugates of RWJ-333369 were detected in human urine (indicated with an asterisk in Fig. 9). Three mercapturic acid conjugates were present and could be identified by coelution with the available authentic substances as JNJ-26641836-AAA (mercapturic acid conjugate ortho with respect to the chlorine), JNJ-26954213-AAA (mercapturic acid conjugate meta with respect to the chlorine and para to the side chain), and JNJ-26642083-AAA (mercapturic acid conjugate para with respect to
the mercapturic acid conjugates; twice the chlorination pattern).

mercapturic acid conjugates of RWJ-333369 in the 8- to 24-h urine of a male subject (acid) from the parent ion and the ion at 377.0574 (molecular ion of the mercapturic acid conjugates; gates), 377.0574 (molecular ion of the mercapturic acid conjugates; the chlorine). In all instances, radio-HPLC quantitation fell below the gates), 377.0574 (molecular ion of the mercapturic acid conjugates; the chlorine).

Radio-HPLC analysis of the methanol fecal extracts (61% on average). Only two extracts were analyzed for metabolite profiling. There was one major radioactivity peak in feces eluting at about 23 min. This metabolite represented 95% and 99%, respectively, of the quantifiable sample radioactivity in the extracts of the two analyzed samples. The metabolite did not coelute with any of the metabolites seen in urine or in enzymatically hydrolyzed urine (data not shown). Parent RWJ-333369 was detected in minor amounts in the fecal extract (5% of the sample radioactivity).

Metabolite Profile in Plasma. A typical plasma metabolite profile is shown in Fig. 10. The recovery of TR following deproteinization up to and including the 24-h plasma samples ranged from 78% to 107%.

In the blank plasma sample fortified with 14C-RWJ-333369, the recovery of TR after deproteinization was 102% of the theoretical value.

The deproteinization process of plasma introduced some conversion of RWJ-333369 to the positional isomer via carbamate migration (R307716 structure; see Fig. 2). The same phenomenon was seen in the radio-HPLC analysis of the blank plasma sample fortified with 14C-RWJ-333369. Carbamate migration has also been observed in the preparation of plasma samples from animal species.

Radio-HPLC analysis of the plasma samples collected up to 24 h showed that unchanged RWJ-333369 accounted for the bulk of the sample radioactivity and did not reveal important circulating metabolites (Fig. 10). The remainder of the plasma radioactivity was accounted for by the minor metabolite 21. The concentration of this glucuronide (metabolite 21) was below the limit of quantitation of the radio-HPLC method. As evidenced from the chiral analysis, the R-enantiomer of RWJ-333369 was also a minor circulating metabolite.

Discussion

The objective of this single-dose clinical study was to investigate the mass balance and metabolic profile after oral administration of a pharmacologic dose (500 mg) of 14C-RWJ-333369 in healthy human subjects. Blood, urine, and feces were collected for 7 days after dosing. The overall metabolism of RWJ-333369 in humans after single oral administration was evaluated on the basis of the metabolite profiles in urine, feces, and plasma. Metabolite profiles in the methanol fecal extracts could not be determined because of the very low levels and were not taken into account. However, given the high recovery of the administered RWJ-333369 dose in urine, the overall metabolism could be adequately characterized on the basis of urine and plasma metabolites only.

After a single oral dose of 500 mg of 14C-RWJ-333369 to eight healthy volunteers, the mean ± S.D. total recovery in the urine was 93.8 ± 2.3% and 2.5 ± 1.6% in the feces. Unchanged RWJ-333369 accounted for, on average, 1.7% of the dose in urine, and trace
amounts were excreted in feces. The very high recovery of TR in urine points to an almost complete absorption, whereas the low levels of parent drug excreted indicate that extensive metabolism of RWJ-333369 occurred in humans.

The mass balance of RWJ-333369 and its metabolites in urine after single oral administration of 500 mg of $^{14}$C-RWJ-333369 is shown in Fig. 11, and a metabolic scheme is presented in Fig. 5. The majority of the drug was eliminated by two metabolic pathways: O-glucuronidation (mean percentage of dose excreted in urine is 44%) and carbamate hydrolysis followed by oxidation to 2-chloromandelic acid, which is subsequently metabolized in parallel to 2-chlorophenyl glyoxylate. A similar biotransformation has been described for felbamate because of the low levels observed.

The O-glucuronidation of RWJ-333369 is a one-step pathway due to the presence of the secondary alcohol on the chiral carbon. O-Glucuronidation also occurred in combination with chiral inversion at the alcohol function (mean percentage is 11%). In plasma, the R-enantiomer accounted for approximately 5% of the S-enantiomer (RWJ-333369 itself) exposure. The chiral inversion of RWJ-333369 is a multistep biotransformation and probably occurs via oxidation reduction of the secondary alcohol on the chiral center followed by glucuronidation. The glucuronide of RWJ-333369 and the R-enantiomer itself were the only metabolites that could be detected in plasma. These two metabolites are unlikely to contribute to the pharmacologic action of RWJ-333369 in humans because of the low levels observed. Moreover, the glucuronide of RWJ-333369 was inactive in a rodent acute seizure model (data on file).

2-Chloromandelic acid is formed by an esterase-mediated hydrolysis of the carbamate to (as a possible intermediate) 2-chlorophenyl-ethyl glycol, which is subsequently oxidized by alcohol and aldehyde dehydrogenase to 2-chloromandelic acid. 2-Chloromandelic acid is further metabolized by decarboxylation to 2-chlorobenzyl alcohol (a possible intermediate), which is further oxidized to 2-chlorobenzoic acid. Parallel to the formation of 2-chlorobenzoic acid by oxidative decarboxylation, 2-chloromandelic acid is biotransformed by transamination to 2-chlorophenylglycine, most probably via oxidation to 2-chlorophenyl glyoxylic acid. A similar biotransformation has been described for metabolites of styrene (Haufroid et al., 2002; Manini et al., 2002). Styrene was shown to metabolize to mandelic acid in humans and further to phenylglycine via phenylglyoxylic acid. Accordingly, for RWJ-333369, the same pathways lead to the formation of chloromandelic acid (metabolite 5) and 2-chlorophenyl glyoxylate (metabolite 1).

Aromatic hydroxylation at the 3-position of the phenyl ring of RWJ-333369 followed by sulfate conjugation at the phenol accounted for, on average, 5% of the dose. The hydroxylation of RWJ-333369 is most probably P450-mediated and it is followed by sulfation of the phase-1 hydroxy metabolite.

Apart from the identification of major radiolabeled metabolites, additional investigations were performed to identify potential aromatic (pre)mercapturic acid conjugates of RWJ-333369 in urine, since these metabolites had been detected in rat urine. Ten aromatic mercapturic acid conjugates were chemically synthesized, and LC-MS/MS analysis was used to trace the (pre)mercapturic acid conjugates of RWJ-333369. (Pre)mercapturic acid conjugates could not be detected and quantified with the radio-HPLC method, indicating that each conjugate accounted for less than 0.3% of the dose. However, using a more sensitive exact mass LC-MS/MS method, (pre)mercapturic acid conjugates of RWJ-333369 could be detected in human urine. In all eight subjects, three premercapturic acid conjugates and at least two mercapturic acid conjugates were detected. The mercapturic acid conjugates were identified by coelution with the available authentic substances. All (pre)mercapturic acid conjugates were conjugated on the aromatic ring. The presence of premercapturic acid conjugates is strongly suggestive of an arene oxide pathway for the formation of these metabolites. Premercapturic acid conjugates derived from an arene oxide intermediate usually undergo spontaneous rearrangement to restore aromaticity. However, stable premercapturic acid conjugates in urine have been described before for bromobenzene (Lertratanan Kong and Horning 1987).

The dicarbamate antiepileptic drug felbamate has been associated with cases of idiosyncratic hepatotoxicity and aplastic anemia (Dieckhaus et al., 2002). The mechanism of felbamate idiosyncratic toxicity is believed to involve the metabolism of the drug to the αβ-unsaturated aldehyde metabolite, atropaldehyde (ATPAL) (Thompson et al., 1996; Kapetanovic et al., 1998; Pellock et al., 2002). ATPAL may react with cellular glutathione, a well established detoxification pathway, and be excreted as mercapturic acid conjugates in the urine. Alternatively, ATPAL may bind covalently to cellular proteins and, in conjunction with additional risk factors that are not well understood, ultimately lead to cases of idiosyncratic toxicity. RWJ-333369 cannot form an analogous αβ-unsaturated aldehyde metabolite because of the absence of one carbon on the side chain of the molecule. The observed trace amounts of RWJ-333369 (pre)mercapturic acid conjugates matched the available synthesized compounds and were most likely derived from aren oxide intermediate(s), which generally exhibit less reactivity than αβ-unsaturated aldehydes and are more commonly observed in drug metabolic pathways. In addition, the amount of mercapturic acid metabolites formed with RWJ-333369 is very low (each <0.3% of the dose). In humans, an average of 6.3% of the felbamate dose is excreted as ATPAL-derived mercapturic acid conjugates (Dieckhaus et al., 2000); this value is within the range of glutathione-derived conjugates observed in human urine with acetaminophen under therapeutic conditions (Chen et al., 1996; Esteban et al., 1996; Critchley et al., 2005).

In conclusion, the disposition of RWJ-333369 in humans is characterized by a virtually complete absorption, extensive glucuronidation, and carbamate hydrolysis. Unchanged drug is the only significant circulating drug-related species, and excretion of unchanged drug and metabolites is almost exclusively via the urine.

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References


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