ABSTRACT:
The in vivo metabolism and excretion of RWJ-333369 [1,2-ethanediol, 1-(2-chlorophenyl)-, 2-carbamate, (S)-], a novel neuromodulator, were investigated in mice, rats, rabbits, and dogs after oral administration of 14C-RWJ-333369. Plasma, urine, and feces samples were collected, assayed for radioactivity, and profiled for metabolites. In almost all species, the administered radioactive dose was predominantly excreted in urine (>85%) with less than 10% in feces. Excretion of radioactivity was rapid and nearly complete at 96 h after dosing in all species. Unchanged drug excreted in urine was minimal (<2.3% of the administered dose) in all species. The primary metabolic pathways were O-glucuronidation (rabbit > mouse > dog > rat) of RWJ-333369 and hydrolysis of the carbamate ester followed by oxidation to 2-chloromandelic acid. The latter metabolite was subsequently metabolized in parallel to 2-chlorophenylglycine and 2-chlorobenzoic acid (combined hydrolytic and oxidative pathways: rat > dog > mouse > rabbit). Other metabolic pathways present in all species included chiral inversion in combination with O-glucuronidation and sulfate conjugation (directly and/or following hydroxylation of RWJ-333369). Species-specific pathways, including N-acetylation of 2-chlorophenylglycine (mice, rats, and dogs) and arené oxidation followed by glutathione conjugation of RWJ-333369 (mice and rats), were more predominant in rodents than in other species. Consistent with human metabolism, multiple metabolic pathways and renal excretion were mainly involved in the elimination of RWJ-333369 and its metabolites in animal species. Unchanged drug was the major plasma circulating drug-related substance in the preclinical species and humans.

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Materials and Methods

Test Article. RWJ-333369, specifically labeled with 14C at the benzylic carbon (Fig. 1), was synthesized by Johnson & Johnson Pharmaceutical Research and Development (Beere, Belgium). The 14C label at this position has been proven to be metabolically stable, as evidenced by the lack of 14CO2 exhalation in the rat after oral dosing of 14C-RWJ-333369. The radiolabeled substance had a specific activity of 998 MBq/mmol (or 4.6 MBq/mg) and a radiochemical purity of 98.9%. The enantiomeric excess determined by the drug's radiochemical purity was 98.9%. The enantiomeric excess determined by the drug's radiochemical purity was 98.9%. The enantiomeric excess determined by the drug's radiochemical purity was 98.9%. The enantiomeric excess determined by the drug's radiochemical purity was 98.9%.
chiral-HPLC method was found to be 98.7%. Unlabeled RWJ-333369 was synthesized by Janssen-Cilag (Schaflhausen, Switzerland) and had a chemical purity of 100%. Appropriate amounts of radiolabeled and unlabeled RWJ-333369 were combined to adjust the specific activity for dosing. For the identification of metabolites, various synthetic reference compounds were used and the synthetic procedures are not detailed in this article (data on file).

**Animal Experiments.** All animal experiments were conducted according to protocols approved by the Johnson and Johnson animal care and use committee. All animals were treated with a single dose of 14C-RWJ-333369 followed by collection of plasma, urine and feces at predefined intervals (see individual studies below). At the end of sample collections, cage debris and cage washings were collected for determination of total radioactivity (TR) mass balance.

**Mouse study.** Twenty-four male and female specific pathogen-free albino (CD1) mice (approximately 6–8 weeks of age) were obtained from Charles River (Kem, UK). Animals were divided into groups A and B within each sex; the former group was used for generating plasma metabolite profiles and the latter for the metabolism-excretion balance study. Group A animals were placed and acclimatized in grid-bottomed polycarbonate cages (four per cage, of same sex) and group B were placed in glass metabolism cages (4 per cage, of same sex). A suspension of 14C-RWJ-333369 in 0.5% Methocel F4M Premium EP (Dow Corning, Midland, MI) was prepared with a specific activity of 70 kBq/mg, and the mice were dosed orally at a 50 mg/kg dose (3.5 MBq/kg). The dose of 50 mg/kg corresponded to the low dose in an oral mouse carcinogenicity study with RWJ-333369. Plasma samples were collected from group A animals at 1-, 3-, 8-, and 24-h postdose intervals (three per time point per sex), and urine and feces were collected from group B animals from 0 to 96 h postdose at defined time intervals (0–12 h, 24–48 h, 48–72 h, and 72–96 h for urine and every 24 h for 4 days for feces).

**Rat study.** Twenty male and 20 female young Sprague-Dawley rats (approximately 8–9 weeks of age) were obtained from Charles River (Sulzfeld, Germany). Animals were divided into two groups, A and B, within each sex; the former group was used for generating plasma metabolite profiles and the latter for the metabolism-excretion balance study. Per sex, animals from the pharmacokinetics group were placed and acclimatized in Macrolon cages (3 per cage, total 12 animals), and animals for mass balance and metabolite profiling were placed in stainless steel metabolic cages (1 per cage, total 5 animals per sex). The remaining animals were kept as reserve animals. A solution (in water) of 14C-RWJ-333369 was prepared with a specific activity of 94.3 kBq/kg, and the rats were dosed orally by gavage at 30 mg/kg dose (0.36 MBq for males and 0.31 MBq for females). The dose of 30 mg/kg corresponded to the low dose in a 3-month oral rat toxicity study. Plasma samples were collected from group A animals at 1-, 3-, 8-, and 24-h postdose intervals (three per time point per sex), and urine and feces were collected from metabolism group (group B) animals from 0 to 96 h postdose at defined time intervals.

**Rabbit study.** After the acclimatization period, two of the four female New Zealand White albino rabbits (approximately 16–30 weeks of age, procured from Centre Lago, Vonnas, France) were dosed orally by gavage at a 100 mg/kg dose (~5.54 MBq) and the third rabbit received a 200 mg/kg dose (~10.52 MBq) with a suspension of 14C-RWJ-333369 in 0.5% Methocel F4M Premium EP (Dow Corning, Midland, MI) (specific activity of 16.7 kBq/mg). The one remaining rabbit was kept as a reserve animal. The dose of 200 mg/kg corresponded to the highest dose used in an oral developmental toxicity study in the rabbit. Plasma samples were collected at 1, 2, 3, 4, 8, 24, 48, and 72 h after dosing. Urine was collected from 0 to 24 h, 24 to 48 h, and 48 to 72 h after dose administration. Feces were collected daily for up to 3 days (72 h) after dosing and weights were recorded.

**Dog study.** After the acclimatization period, four of the six dogs (Janssen Animal Breeding Centre, Beersel, Belgium) were dosed orally by gavage at a 20 mg/kg dose (~12.39 MBq) with a solution of 14C-RWJ-333369 (specific activity, 49.3 kBq/mg). The two remaining dogs were kept as reserve animals. The oral dose of 20 mg/kg corresponded to the low dose level in a 3-month oral toxicity study in the dog. Plasma samples were collected at 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 12, 24, 32, 48, 72, 96, and 168 h after dosing. Urine was collected once before dosing and at intervals of 0 to 4, 4 to 8, 8 to 24, 24 to 48, 72 to 96, 120 to 144, and 144 to 168 h after dose administration. Feces were collected daily for 7 days after dosing and weights were recorded.

**Sample Analysis.** Predose plasma, urine, and feces samples of all tested species were spiked with known quantities of 14C-RWJ-333369 and stored during the period of the studies to allow verification of the stability of the drug in these media.

**Mass balance-radioactivity.** The TR was measured in all urine and plasma samples that were collected at various intervals in all studies. Aliquots of urine (0.25 ml in the case of rats, rabbits, and dogs, and 0.20 ml in the case of mice) in duplicates were mixed with 10 ml of UltimaGold (PerkinElmer Life and Analytical Sciences, Boston, MA) as scintillation cocktail. The TR in urine samples was determined by liquid scintillation counting using a Packard Tri-Carb 2100 TR liquid scintillation spectrometer (PerkinElmer Life and Analytical Sciences). Similarly, the TR in plasma samples of animal species was measured in duplicate aliquots.

For the measurement of TR in the feces samples, each sample was extracted with three different aliquots of methanol using a homogenizer (Ultra-Turrax; Janke and Kunkel GmbH and Co., Staufen, Germany) and a Büchner funnel (Merck Eurolab Holding GmbH, Zaventem, Belgium). The radioactivity was measured in duplicate aliquots of the resultant pooled methanolic extracts by liquid scintillation spectrometer after mixing with water and 10 ml of UltimaGold scintillation cocktail (PerkinElmer Life and Analytical Sciences). The fecal residues after methanolic extraction were air-dried and ground to a fine powder in an Ultra Centrifugal Mill (Retsch GmbH, Haan, Germany). Four weighted aliquots of each homogenized sample of approximately 80 to 120 mg were weighed in Combust-Cones (PerkinElmer Life and Analytical Sciences) and combusted in a Packard Sample Oxidizer model 307. Carbosorb (8 ml; PerkinElmer Life and Analytical Sciences) was used to absorb the 14CO2 and Permafluor (PerkinElmer Life and Analytical Sciences) was used as scintillation cocktail. The radioactivity in the combusted fecal residues was measured by liquid scintillation counting, using a Packard Tri-Carb 1900TR or 2100TR scintillation spectrometer. The TR in feces was calculated as the sum of the radioactivity in the methanolic fecal extracts and the feces residues.

**Sample preparation for metabolite profiling.** Where appropriate, per gender, per time interval (e.g., 0–12 h, 12–24 h, 24–48 h, etc.), urine pools or methanolic extract pools were prepared by mixing constant fractions of the original sample volume collected during the same interval across the subjects. The overall urine or methanolic extract pools across a broader time interval (0–24 h or 0–48 h) were prepared by mixing the constant fractions of the different time interval pools. At selected time points, plasma pools per gender, per time point were prepared by mixing the equal volumes of plasma samples from across the subjects.

Plasma was deproteinized by addition of acetonitrile (1.5 ml of acetonitrile per ml of plasma). The precipitated proteins were removed by centrifugation (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 (750–850 μl) of redissolved samples were injected on the radio-HPLC system. Similarly, fortified predose samples were processed and analyzed along with the test samples. Urine samples were centrifuged and the resultant clear supernatant was injected onto the radio-HPLC system. Aliquots of the methanolic extracts of feces samples were evaporated under nitrogen, and the residues were reconstituted in dimethyl sulfoxide/water (1:1 v/v). Aliquots of these samples were injected onto the radio-HPLC system.

**Chromatography and Spectroscopic Studies.** HPLC analyses. The HPLC apparatus consisted of a Waters Alliance 2695 gradient pump (Waters, Milford, MA), equipped with automatic injector (Waters Alliance 2695) and a stainless steel Kromasil C18 column (30 cm × 4.6-mm i.d., 5 μm; Akzo Nobel, Arnhem, The Netherlands). A gradient system with linear steps was applied to the column. Elution started at 1.0 ml/min, with a gradient from 100% of an aqueous solution of 0.05 M ammonium acetate, pH 7.5 (solvent system A), to 45% solvent system A and 55% solvent system B over 40 min. Solvent B was
composed of an aqueous solution of 0.5 M ammonium acetate, adjusted to pH 7.5/methanol (20:80) (solvent system B). This mobile phase composition was held for 5 min. Subsequently, a linear gradient over 1 min to 100% solvent system B was applied, and this solvent composition was held for another 5 min before returning to the starting conditions. The column elutes were monitored by UV detection set at 218 nm using a Waters 996 diode array detector. On-line radioactivity detection of HPLC elutes was carried out with a Berthold Radioactivity Monitor LB-509 (Berthold Technologies, Bad Wildbad, Germany). The elutes were mixed with Ultimo Flo AP (PerkinElmer Life and Analytical Sciences) as a scintillation cocktail delivered by a Berthold LB-5035-3 pump at a flow rate of 4.0 ml/min. Detector output was connected to the Millennium (Waters) chromatography data system.

The concentrations of RWJ-333369 and its metabolites in plasma and urine samples and in fecal extracts were calculated based on the recovery of the radioactivity in the samples and on the areas of the radioactivity peaks obtained after reversed-phase radio-HPLC of appropriate aliquots of these samples. Areas of the radioactivity peaks were converted into disintegrations per minute (dpm) by the data system, after introduction of a calibration curve prepared by injection of known amounts of 14C-RWJ-333369 and a linear regression analysis of the corresponding areas of the radioactivity peaks. The quantitation limit was calculated from the quantitation limit of 14C-RWJ-333369 (200 dpm) and from the amount of radioactivity injected. Samples with known amounts of 14C-RWJ-333369 were injected regularly as controls of the validity of the calibration curve over the whole series of analyses.

**Metabolite identification.** The metabolites in plasma, urine, and methanolic fecal extracts were identified by HPLC chromatography with synthetic reference compounds, enzymatic hydrolysis, and/or LC-MS/MS analysis and/or LC-NMR spectroscopy. For the identification of metabolites by HPLC chromatography, a mixture of synthetic reference standards (see Fig. 2) was cochromatographed with the plasma samples, urine samples, and the methanolic fecal extracts. The synthetic reference standards were monitored by UV detection (218 nm) (see Fig. 3), whereas the radioactive metabolites were monitored by liquid scintillation spectrometry.

For the identification of glucuronide and sulfate conjugates of RWJ-333369 and/or its metabolites in plasma and urine, a comparison was made between the radio-HPLC chromatograms of samples before and after enzymatic hydrolysis with β-glucuronidase/arylsulfatase from Helix pomatia (Boehringer Ingelheim GmbH, Ingelheim, Germany; 10 μl/ml acetate-buffered sample, pH 5.0), β-glucuronidase from Escherichia coli (Boehringer Ingelheim GmbH, Ingelheim, Germany; 10 μl/ml phosphate-buffered sample, pH 7.0), or arylsulfatase from Aerobacter aerogenes (Sigma-Aldrich, St. Louis, MO; 10 μl/ml phosphate-buffered sample, pH 7.0). The incubations were performed at 37°C for 24 h.

Representative urine and plasma samples were analyzed by LC-MS/MS (LCQ; Thermo Electron Corporation, Waltham, MA; and/or Waters QTof-Ultima), using a Waters Alliance 2695, a Waters Alliance 2795, or a Surveyor (Thermo Electron) separation module with a Waters 996 photodiode array detector or a Surveyor (Thermo Electron) photodiode array detector to confirm the identity of the metabolites and a radioactive monitor (Berthold Technologies). A gradient system with linear steps was applied to the column and the chromatographic conditions were identical to those in radio-HPLC analyses. A post column splitter was used to introduce 20% of the HPLC effluent to the electrospray ionization (ESI) source and 80% to the waste. LCQ used with ESI and/or atmospheric pressure chemical ionization operated in the positive and negative ion modes. The settings (lens voltages, quadrupole and octapole voltage offsets, etc.) of the mass spectrometer were optimized for maximum intensity for RWJ-333369 by using the auto-tune function within the LCQ Tune program. Acquired data were processed using Xcalibur (version 1.2; Thermo Electron).

The hybrid quadrupole time-of-flight mass spectrometry (QTof) was used with a dual electrospray ionization probe and was operated in the positive and
negative ion mode at a resolution of 3000 [full-width half-maximum at m/z 409.9418 (lock reference)]. The source temperature was 100°C, desolvation temperature was 250°C, and the cone voltage was set at 40 V. The second LockSpray ESI probe, which provided an independent source of the lock mass calibrant H$_3$PO$_4$ (M $+$ H$^+$) ion at m/z 196.9616, was used as the calibrant. Data were acquired in the centroid mode with a scan time of 1 s and finally processed using MassLynx 4.0 software (Waters).

To facilitate the identification of metabolites, several aliquots of urine samples from rat, rabbit, and dog studies were concentrated and the metabolites (M8 and M9 from rat; M21, M23, and M24 from rabbit; M11 from dog) were isolated and purified by chromatographic technique. For the confirmation of stereochemistry and the position of conjugation, purified conjugated metabolites (M21 and M23) were subjected to enzymatic hydrolysis, followed by chiral analysis, and LC-MS/MS. Chromatographic separation of stereoisomers was achieved by isocratic elution of extracts using a mobile phase (0.02 M ammonium acetate in ethanol/hexane, 20:80) on a chiral column (Chiralpak AD, 5 cm × 4.6 mm i.d.; Daicel Chemical Industries Ltd., Tokyo, Japan) maintained at ambient temperature. Column elutes were monitored by an API 3000 triple quadruplet mass spectrometer (Applied Biosystems, Foster City, CA) operated with a turboionspray interface under positive ion mode.

Data Analysis. The TR excreted in urine and feces was expressed as a percentage of the administered radioactivity. The mass balance of RWJ-333369 and its metabolites was presented as the percentage of the dose of radioactivity accounted for by these radiolabeled compounds. Radioactivity levels in plasma were expressed as microgram-equivalents to RWJ-333369 per milliliter. In plasma, the levels of unchanged RWJ-333369 and metabolites was presented as the percentage of the dose of radioactivity accounted for by these radiolabeled compounds. Radioactivity eluates were monitored by an API 3000 triple quadruplet mass spectrometer (Applied Biosystems, Foster City, CA) operated with a turboionspray interface under positive ion mode.

**TABLE 2**

Pharmacokinetic parameters of total radioactivity in plasma samples after single oral administration of $^{14}$C-RWJ-333369 in mice, rats, rabbits, and dogs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mice (50 mg/kg)$^a$</th>
<th>Rats (30 mg/kg)$^a$</th>
<th>Female Rabbits$^b$</th>
<th>Male Dogs$^c$ (20 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (µg-Eq/ml)</td>
<td>25.3</td>
<td>20.2</td>
<td>15.6</td>
<td>15.6</td>
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<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>1.0</td>
<td>1.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>3.7</td>
<td>4.1</td>
<td>6.6</td>
<td>5.6</td>
</tr>
<tr>
<td>AUC$_{0-t}$ (µg-Eq · h/ml)</td>
<td>95.8</td>
<td>93.6</td>
<td>174.8</td>
<td>177.5</td>
</tr>
<tr>
<td>AUC$_{0-\infty}$ (µg-Eq · h/ml)</td>
<td>97.3</td>
<td>95.6</td>
<td>192.0</td>
<td>188.0</td>
</tr>
</tbody>
</table>

$^a$ Data were derived from pooled plasma from mice and in rats.

$^b$ Data from female rabbits were either the mean of two animals (100 mg/kg dose) or data from one animal (200 mg/kg dose).

$^c$ Data are the mean (±S.D.) from four animals.

FIG. 4. Representative urine sample radio-HPLC chromatographic profiles of mouse (a), rat (b), rabbit (c), and dog (d) after single oral administration of $^{14}$C-RWJ-333369 (metabolites are indicated with numbers and RWJ-333369 as UD [unchanged drug]).
Results

Excretion of Radioactivity. Mean percentages of recovery of TR in urine and feces for different animal species are summarized in Table 1. After oral administration of \(^{14}C\)-RWJ-333369 to male and female mice, male and female rats, female rabbits, and male dogs, radioactivity was predominantly (all species 86.6–97.8% of the administered dose, except female mice 60%) recovered in urine and to a very minor extent (4.0–9.8% of the administered dose) in feces. The rate of excretion was rapid in almost all preclinical species; within the first 24 h postdose, \(>90\%\) of the administered dose (approximately) was recovered. Overall, excretion was nearly complete at 96 h in all preclinical species.

Pharmacokinetics of Total Radioactivity. Mean pharmacokinetic parameters of TR in plasma for mice, rats, rabbits, and dogs are summarized in Table 2. Except in rats, peak plasma concentrations of TR were achieved within 1 to 2 h. The terminal \(t_{1/2}\) of radioactivity ranged from 3.7 h to 6.6 h in mice and rats and \(~15\) h in rabbits. In dogs, large variations were observed in AUC and \(t_{1/2}\) values, and the \(t_{1/2}\) was in the range of 9.3 to 84.1 h.

Identification of Metabolites. Representative pooled urine radiochromatographic profiles of mouse, rat, rabbit, and dog are shown in Fig. 4. Metabolites were given a numerical code based on the retention time of metabolites detected in all species.

The MS/MS product-ion ESI mass spectrum of RWJ-333369 shows characteristic fragment ions useful for metabolite identification (Fig. 5.). The fragmentation behavior was dominated by cleavages in the side chain. The MS/MS product-ion ESI spectra of the unchanged drug (RWJ-333369) (MH\(^+\) 216) and the retention time of the reference compound R264397 (= RWJ-333369) are identical (Table 3). The presence of other marker ions related to sodium (MH\(^+\) 232) and ammonium (MH\(^+\) 238) adducts of RWJ-333369 confirms the identity of RWJ-333369.

The metabolites of RWJ-333369 identified in various preclinical species along with the method of identification are listed in Table 3. Metabolites M1, M4, M5, M14, M21, M22, M23, and M25 were identified with the synthetic reference compounds as 2-chlorophenylglycinic acid (R342141), 2-chlorobenzoic acid (R300100), 2-chloromandelic acid (R288222; CMA), N-acetyl 2-chlorophenylglycine (R338228), O-glucuronide of RWJ-333369 (R289876), 4-hydroxy RWJ-333369 (R300592), O-glucuronide of RWJ-452399 (R382574), and 1-chloro-2-(1,2-dihydroxyethyl)benzene (R288223), respectively (Fig. 2.). HPLC retention time and MS-MS fragmentation patterns of the synthetic reference compounds were identical to the radioactive peaks (Figs. 3 and 4; Table 3.).

Identity of M2, M3, M7, M15, and M17. Identity of these metabolites was not known. M2 was present in rat and dog, M3/M17 were unique to dog, M7 was present in all preclinical species, and M15 was present in rat and mouse.

Identity of M8 and M9. Metabolites M8 and M9 were identified as premercapturic acid conjugates (pre-MACs) of RWJ-333369. The QTof exact masses of M8, M9, and fragment ions are very close to the calculated exact mass of protonated sodium adduct of pre-MACs of RWJ-333369 (Table 4.) and fragment ions. The protonated molecular ion sodium adduct (M + Na\(^+\) of M8 and M9 was observed at \(m/z\) 417.0501 and 417.0476, respectively, and the fragmentation patterns were the same for M8 and M9. The major fragment ions were \(m/z\) 359.0481 (due to loss of sodium and two water molecules), \(m/z\) 316.0452 (M8 – Na – 2H\(_2\)O – CONH\(_2\)), and \(m/z\) 274.0321 (M8 – Na – 2H\(_2\)O – CONH\(_2\) – COCH\(_3\)). Based on the fragmentation patterns and NMR analysis (not shown) of the corresponding dehy-
drated purified metabolites (MACs of RWJ-333369) from rat urine, M8 and M9 were proposed as pre-MACs of RWJ-333369.

To determine the position of conjugation and hydroxylation on the aromatic ring, attempts were made to synthesize a number of possible pre-MACs of RWJ-333369. None of the attempts were successful. However, attempts to synthesize corresponding MACs of RWJ-333369 were successful. Approximately 10 aromatic MACs were synthesized and used as reference compounds for the purpose of cochromatographic comparison. The procedure for the synthesis of the 10 MACs and the HPLC separation data are described in Mannens et al. (2007). pre-MACs of RWJ-333369 in rat urine were converted to MACs of RWJ-333369 and were cochromatographed with 10 different reference compounds of aromatic MACs of RWJ-333369. Based on these investigations and NMR analysis (data not shown), it was proposed that the N-acetyl cystine side chains for metabolites M8 and M9 were attached to the aromatic ring at position 5 (meta-position with respect to aliphatic side chain of RWJ-333369) for metabolite M8 and at position 4 (para-position with respect to aliphatic side chain of RWJ-333369) for metabolite M9. The exact position of the hydroxy function and sterechemistry of the metabolites were not determined. Based on the above analysis, the metabolites M8 and M9 were identified as pre-MACs of RWJ-333369. In the rat urine, LC-MS/MS analyses revealed that, additionally, at least three MACs were observed in trace amounts.

Identity of M10, M11, M13, M20, and M16. The ESI mass spectra for metabolites M10, M11, M13, and M20 display the same protonated molecular ion at m/z 312 (Table 3). A mass shift of 96 compared with RWJ-333369 (protonated molecular ion: 216) points to hydroxylation (16 units), with concomitant sulfate conjugation (80 units). The major fragment ion was observed at m/z 294, arising from the loss of a water molecule directly from the protonated molecular ion indicating the presence of free hydroxy group in the molecule (see Fig. 6 for protonated mass spectra of M11 in dog urine). Additional fragment ions at m/z 294, 329, and 346 resulting from mono- and di-ammonium adducts of the metabolite were observed in the spectra. In addition to mass spectral analysis, radio-HPLC profiles clearly demonstrated that upon enzymatic hydrolysis (with β-glucuronidase/arylsulfatase or arylsulfatase), peaks related to metabolites M11 and M13 disappeared, and there was a concomitant increase in peaks of aglycons (hydroxy RWJ-333369), M16, and M22 (R300592), respectively. These findings and the LC-MS/MS results demonstrate that metabolites M11 and M13 are hydroxy-sulfate conjugates of RWJ-333369, and M16 and M22 (R300592) are monohydroxylated metabolites of RWJ-333369. The position of hydroxylation and sulfate conjugation was determined for metabolite M11 using 1H NMR and 13C NMR spec-

### Table 3

<table>
<thead>
<tr>
<th>Metabolite No.</th>
<th>Species</th>
<th>Identification Method</th>
<th>Identity</th>
<th>MH or M* or MA</th>
<th>Typical MS or MS&lt;sup&gt;2&lt;/sup&gt; Fragments</th>
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<tr>
<td>UD</td>
<td>M, R, Rh, D</td>
<td>Cochromatography, LC-MS/MS</td>
<td>RWJ-333369, UD</td>
<td>216, 232, 238</td>
<td>119, 137, 155, 198</td>
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<tr>
<td>M1*</td>
<td>M, R, Rh, D</td>
<td>Cochromatography, LC-MS/MS</td>
<td>R342141, 2-chlorophenylglycine</td>
<td>186</td>
<td>140, 169</td>
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<tr>
<td>M4*</td>
<td>M, R, Rh</td>
<td>Cochromatography, LC-MS/MS</td>
<td>R300100, 2-chlorobenzoic acid</td>
<td>155</td>
<td>111</td>
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<tr>
<td>M5*</td>
<td>M, R, Rh, D</td>
<td>Cochromatography, LC-MS/MS</td>
<td>R288222, 2-chloromandelic acid (CMA)</td>
<td>185</td>
<td>141, 123, 111</td>
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<td>M8</td>
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<td>M, R</td>
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<td>Pre-MAC of UD</td>
<td>417, 395</td>
<td>274, 316, 359, 377</td>
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<td>M10</td>
<td>D</td>
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<td>Sulfate conjugate of hydroxy UD</td>
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<td>M11</td>
<td>M, R, D</td>
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<td>Sulfate conjugate of 3-hydroxy UD</td>
<td>312, 329, 346</td>
<td>294</td>
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<td>M12a,b*</td>
<td>M, R</td>
<td>Hydrolysis</td>
<td>A mixture of glucuronides of CMA and 2-chlorophenylglycine</td>
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<td>M, R, D</td>
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<td>Sulfate conjugate of hydroxy UD</td>
<td>312, 329, 346</td>
<td>294</td>
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<td>M14*</td>
<td>M, R, D</td>
<td>Cochromatography, LC-MS/MS</td>
<td>R338228, N-acetyl derivative of 2-chlorophenylglycine</td>
<td>228</td>
<td>140, 169, 182, 186, 210</td>
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<td>M16</td>
<td>D</td>
<td>Hydrolysis, LC-MS/MS of M11</td>
<td>3-hydroxy UD</td>
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<td>84, 85, 113, 157, 175, 210</td>
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<td>M18</td>
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<td>347</td>
<td>84, 85, 113, 157, 175, 210</td>
</tr>
<tr>
<td>M19</td>
<td>R</td>
<td>Hydrolysis, LC-MS/MS</td>
<td>Glucuronide of M25</td>
<td>347</td>
<td>84, 85, 113, 157, 175, 210</td>
</tr>
<tr>
<td>M20</td>
<td>M, D</td>
<td>LC-MS/MS</td>
<td>Sulfate conjugate of hydroxy UD</td>
<td>312, 329, 346</td>
<td>294</td>
</tr>
<tr>
<td>M21*</td>
<td>M, R, Rh, D</td>
<td>Cochromatography, Hydrolysis, LC-MS/MS</td>
<td>R289876, glucuronide of UD</td>
<td>409</td>
<td>198, 216, 374, 392</td>
</tr>
<tr>
<td>M22*</td>
<td>M, R</td>
<td>Cochromatography, LC-MS/MS</td>
<td>R300592, 4-hydroxy UD</td>
<td>230</td>
<td>211, 169, 230</td>
</tr>
<tr>
<td>M23*</td>
<td>M, R, D</td>
<td>LC-MS/MS, NMR, chiral analysis, cochromatography</td>
<td>Glucuronide of RWJ-452399 (the other enantiomer of UD)</td>
<td>409, 392</td>
<td>198, 216, 374, 392</td>
</tr>
<tr>
<td>M24</td>
<td>M, R</td>
<td>LC-MS/MS, NMR</td>
<td>Sulfate conjugate at the alcohol function of UD and/or of its enantiomer</td>
<td>330, 313, 295</td>
<td>119, 137, 155, 198, 238, 233, 216</td>
</tr>
<tr>
<td>M25*</td>
<td>R</td>
<td>Cochromatography</td>
<td>R288223, 1-chloro-2-(1,2-dihydroxyethyl)benzene</td>
<td>228</td>
<td></td>
</tr>
</tbody>
</table>

MA, molecular adduct of NH₃ or Na⁺.
* Identified with synthetic reference standard.
troscopy (chemical shifts and coupling constants are not shown), and identity was given as 3-hydroxy sulfate (ortho to chloro in the aromatic ring) conjugate of RWJ-333369. Disappearance of metabolites M10 and M20 in the enzyme-treated samples was not conspicuous; moreover, the percentage of their presence in the urine samples was very low. Based on the LC-MS/MS analysis alone, identity was given for metabolites M10 and M20 as hydroxy-sulfate conjugates of RWJ-333369.

Identity of metabolites M12a,b, M18, and M19. Comparison of radio-HPLC chromatograms of enzyme-treated (β-glucuronidase or arylsulfatase) versus untreated urine samples clearly demonstrated that metabolites M12a,b, M18, and M19 disappeared with a concomitant appearance of corresponding aglycon peaks. Disappearance of these peaks was observed only in samples with β-glucuronidase-treated but not arylsulfatase-treated samples; these findings imply that these metabolites are glucuronide conjugates. Careful examination of radio-HPLC profiles of treated samples indicated that with the disappearance of M12a,b there was a concomitant increase in the peak intensities of metabolites M5 and M4. Based on this finding, it was thought that M12a,b was a mixture of two glucuronides (M12a, M12b), and M12a,b was identified as a mixture of glucuronides of M4 and M5. Similarly, with the disappearance of M18 and M19 peaks there was a concomitant increase in M25. Hence, M18 and M19 are thought to be glucuronides of metabolite M25. Further ESI spectra of M18 and M19 in negative mode showed a deprotonated molecular ion at m/z 347. Marker fragment ions in MS² spectra at m/z 175 (glucuronide), 157 (glucuronide − H₂O), and 113 (glucuronide − H₂O − CO₂) were indicative of the presence of a glucuronide in the molecule. Based on molecular and marker fragment ions together with cochromatography of the aglycon (R288223), the identities of M18 and M19 were given as glucuronides of M25 [1-chloro-2-(1,2-dihydroxyethyl)benzene].

Identity of M24. In rabbit urine, M24 was extensively studied in both positive and negative modes of ESI iontrap and QToF instruments. The nominal mass of M24 was found to be 295 mass units, but the spectra were dominated by molecular adduct ions (prototated ammonium adduct at m/z 313, protonat 2× ammonium adduct ion at m/z 330) (see Fig. 7). A mass shift of 80 units from unchanged RWJ-333369 (nominal mass 215) points toward sulfate conjugation. Additional fragmentation patterns [at m/z 216, 233 (ammonium adduct of m/z 216), 238 (sodium adduct of m/z 216), 198, 155, 137, and 119] are the same as that obtained for unchanged RWJ-333369 (Fig. 5), indicating the direct sulfate conjugation of RWJ-333369. Exact mass data from QToF spectra (in negative mode) (Table 4) are very close to the calculated exact mass of a deprotonated sulfate conjugate of RWJ-333369; further, it points in the same direction as sulfate conjugate of RWJ-333369. From rabbit urine M24 was isolated, purified, and subjected to 1H NMR spectroscopic analysis. Results indicate (raw data of chemical shifts and coupling constants are not shown) that sulfate conjugation occurred on the aliphatic hydroxy of the side chain of RWJ-333369. Therefore, metabolite M24 is identified as the sulfate conjugate of RWJ-333369.

Metabolite Profile in Urine. Urinary metabolites expressed as percentage dose radioactivity in different animal species are listed in Table 5. In all preclinical species, radioactivity was predominantly excreted in urine. In rats, after oral administration of 14C-RWJ-333369, less than 2.3% of the dose was excreted unchanged in urine of male and female rats over 0 to 48 h. Urinary metabolites M1 (2-chlorophenylglycine), M12a,b (mixture of glucuronides of 2-chloro-romandelec acid and 2-chloro-benzoic acid), M13 (N-acetyl 2-chlorophenylglycine), and M21 (glucuronide of RWJ-333369) were the major metabolites in rats, each ranging from 7.7 to 23.2% of the administered dose. Other metabolites, M2 (unknown), M4 (2-chloro-benzoic acid), M5 (2-chloro-benzoic acid), M8 (pre-MACs of RWJ-333369), M9, and M13 (sulfate conjugates of hydroxy RWJ-333369), M18 and M19 (glucuronide conjugates of M25), M22 (4-hydroxy RWJ-333369), M24 (sulfate conjugate at the alcohol function of RWJ-333369), and M25 [1-chloro-2-(1,2-dihydroxyethyl)benzene], together contributed to ~29% of the administered dose within the same period. Metabolites M2, M19, and M25 were specific to rats and were not observed in other species. Metabolite M24 appeared to be specific to female rats and was not detected in male rats.

In mice, less than 2.3% of the dose was excreted unchanged in male and female mice over 0 to 24 h (Table 5). Metabolite M21 was the major metabolite contributing to ~44% of the administered dose in male mouse urine within 24 h postdose. M1 and M12a,b were the other predominant metabolites in mice. pre-MACs of RWJ-333369, M8 and M9, were specific to mice and rats, and were not observed in urinary radio-HPLC profiles of dogs and rabbits.

In rabbits, administered radioactivity was almost completely recovered in urine (Table 5). Glucuronide (M21, M23) and sulfate (M24)-conjugated metabolites were the major metabolites in rabbits and in terms of percentage of dose excreted in urine was 43.6, 15.8, and 31.7%, respectively. The remainder of the dose was recovered as metabolites M1, M4, M5 (2-chloro-romandelec acid), M7 (unknown), and M23 (O-glucuronide of R-isomer of RWJ-333369). Metabolites M21 and M23 have been isolated from rabbit urine for NMR spectroscopy and chiral analysis, which clearly showed that the aglycon of M21 was RWJ-333369, and the aglycon of M23 was the enantiomer of RWJ-333369 (RWJ-45239). In dogs, metabolites M5, M11, M21, and M23 were the predominant metabolites in urine and in total,
contributing to approximately 50% of the administered dose radioactivity within 24 h postdose. Metabolites M3 (unknown), M16 (hydroxy RWJ-333369), and M17 (unknown) were found to be specific to the dog and were not observed in other preclinical species.

**Metabolite Profile in Feces.** The percentage of administered radioactivity excreted in feces was very limited in all preclinical species. The extraction recovery of radioactivity from fortified 14C-RWJ-333369 predose fecal samples was in the range of 91.7% to 98.7% of the theoretical value. In all animals, the percentage of total radioactivity recovered in the feces was <10% of the administered dose and the majority of the sample radioactivity was contributed by unchanged drug (RWJ-333369). In rats, apart from the unchanged drug, low amounts of metabolites M4, M5, M14, M15, M22, and M25 contributed to sample radioactivity. In dogs, in addition to unchanged drug, low amounts of metabolites M16, M17, M22, and M23 were observed in dog methanolic feces extracts. In mice and rabbits, unchanged drug was by far the predominant peak in the methanolic feces extracts.

**Metabolite Profile in Plasma.** The percentages of the plasma sample radioactivity accounted for by the parent drug and various metabolites are listed in Table 6. In the blank plasma samples fortified with 14C-RWJ-333369, the recovery of radioactivity after deproteinization was in the range of 89 to 131% of the theoretical value. In all preclinical species, unchanged drug (RWJ-333369) was the predominant component present in plasma. In plasma samples, a degradation peak X was observed, resulting from the carbamoyl migration in RWJ-333369 during sample storage or the sample preparation phase and confirmed with synthetic reference compound (R307716 structure; see Fig. 2). A similar phenomenon was seen in the predose plasma sample spiked with 14C-RWJ-333369. Hence, parent RWJ-333369 was, in fact, the sum of the radioactive peaks of both RWJ-333369 and its degradation product X (R307716). Carbamoyl migration has been reported in human plasma samples (Mannens et al., 2007).

In the animal species studied, O-glucuronide of RWJ-333369 (M21) was the common circulating metabolite present in plasma. In humans, only trace amounts of M21 were detected and the levels were below the limit of quantitation of the radio-HPLC method (Mannens et al., 2007). In plasma samples of rats and mice, a mixture of glucuronide conjugates of M4 and M5 (M12a,b) was present, but this was not seen in the dog and rabbit plasma. In contrast to this, M23 (O-glucuronide of the R-enantiomer of RWJ-333369) was mainly present in dog and rabbit plasma and was absent in mouse and rat plasma. Among the metabolites in rabbit plasma, a sulfate conjugate of RWJ-333369 (M24) was present in significant percentages and was not seen in plasma samples of other animals. In mice, plasma-conjugating metabolites were not detected beyond the 3-h sample point; it appears that metabolites had shorter plasma half-lives in mice than in other species.

**Discussion**

The overall metabolism of RWJ-333369 in mice, rats, rabbits, and dogs after single oral administration was evaluated on the basis of the metabolic profiles in urine, feces, and plasma. Given the high recovery of the administered radioactive dose in urine (>85% in almost all species), the overall metabolism was characterized on the basis of urine data. The in vivo metabolic pathway of RWJ-333369 in various preclinical species is presented in Fig. 8.

Similar to its metabolism in humans (Mannens et al., 2007), RWJ-333369 was extensively metabolized in all preclinical species; only
minor amounts were excreted unchanged in urine and feces. Multiple metabolic pathways and renal excretion are involved in the elimination of RWJ-333369 and its metabolites. Some of the major metabolic reaction pathways were glucuronidation, hydrolysis, sulfation, and oxidation.

In mice and rabbits, O-glucuronidation was the predominant and most important pathway in the metabolic elimination of RWJ-333369 (Table 5; Fig. 8.). As a primary metabolic step, this pathway accounted for approximately 24.6 to 44.0% of this elimination in mice and 44% in rabbits. The O-glucuronidation of RWJ-333369 (M21) is a single-step reaction (primary metabolic step) due to the presence of the secondary alcohol on the chiral carbon (”S”-enantiomer). O-Glucuronidation also occurred in combination with chiral inversion at the alcohol function of RWJ-333369 (M23, O-glucuronide of the ”R”-enantiomer of RWJ-333369). The chiral inversion of RWJ-333369 is a multistep biotransformation. Although the mechanism of inversion or reversibility of inversion was not elucidated for RWJ-333369, different pathways can be hypothesized as shown for structurally similar chiral alcohols such as stiripentol (Zhang et al., 1994), 1-hydroxyethylpyrene (Landsiedel et al., 1998), and 2-butanol (Krikun and Cederbaum, 1984). Either redox sequence or sulfate conjugate-mediated inversion mechanisms have been demonstrated for the chiral inversion of alcohol functions. M23 was predominantly present in rabbits compared with other species studied. In rats, glucuronidation of secondary metabolites (M4, M5) was more significant than O-glucuronidation of UD. Glucuronides of secondary metabolites were not detected in rabbits.

Another distinction among the species was sulfate conjugation (M24), which is a significant metabolic pathway in rabbits but less predominant in other preclinical species (Table 5; Fig. 8). It appeared that sulfate conjugation, as a primary metabolic step, was specific to female animals and absent in male species. With the exception of M24, there were no major gender differences in the metabolism of RWJ-333369 in rodents. Except in rabbits, in all preclinical species examined, there were a number of sulfate conjugates resulting from a secondary metabolic reaction, i.e., aromatic hydroxylation followed by sulfate conjugation (M11, M13).

Metabolites resulting from the hydrolytic, oxidative, and transamination pathways were common to all species (Fig. 8). In rats and dogs, the combined hydrolytic and oxidative pathways were more predominant than in the other species. Carbamate ester hydrolysis was a key step, which proceeded via multistep biotransformation pathways to different metabolites. Ester hydrolysis is a common metabolic reaction mediated by carboxylesterase(s) and has been observed with many drugs such as felbamate (Yang et al., 1991), irinotecan (Humerickhouse et al., 2000), and delapril (Takai et al., 1997). It has been shown that human carboxylesterase isoform-2 mediated the hydrolysis of the carbamate ester in irinotecan. Hydrolysis of the carbamate ester group in RWJ-333369 resulted in the formation of 2-chlorophenylethyl glycol (M25), which was oxidized subsequently to 2-chloromandelic acid (M5), and probably mediated by alcohol and aldehyde dehydrogenases. The α-hydroxy carboxylic acid (2-chloromandelic acid) was converted to 2-chlorobenzoic acid (M4) via oxidative decarboxylation, a metabolic reaction probably common for α-hydroxy acids. A similar biotransformation has been demonstrated for α-hydroxy bile acid in rat hepatocytes (Merrill et al., 1996).
Parallel to the formation of 2-chlorobenzoic acid, 2-chloromandelic acid was biotransformed by transaminases to 2-chlorophenyglycine (M1), most probably via oxidation to 2-chlorophenylglyoxylic acid (an intermediate product).

A similar pathway has been reported for the formation of phenylglycine in the metabolism of styrene (Haufroid et al., 2002; Manini et al., 2002). Styrene was first metabolized to mandelic acid [reference to the formation of 2-chloromandelic acid (M5) in the present study] and subsequently to phenylglycine via phenylglyoxylic acid. Secondary to the above phase I biotransformations, glucuronide conjugates of 2-chlorophenylethyl glycol (M25), 2-chloromandelic acid (M5), and 2-chlorobenzoic acid (M4) were observed in rodents and dogs, but not in rabbits. In rodents and dogs, N-acetyltransferases further biotransformed the 2-chlorophenyglycine (M1) to N-acetyl 2-chlorophenylglycine (M14). This conjugative pathway was absent in rabbits. The aromatic hydroxylation of RWJ-333369 was a minor pathway, most probably cytochrome P450-mediated, and it was followed by sulfation of the phase I hydroxy metabolite. This pathway was more predominant in dogs than in any other species.

Two minor metabolites, pre-MACs (premercapturic acid conjugates) of RWJ-333369 (M8, M9), were observed in rodents, each accounting for <2% and <3% of the dose in urine of mice and rats, respectively. Identification of metabolites revealed that the N-acetyl cysteine side chains of M8 and M9 were attached to the aromatic ring. These metabolites were most likely formed via arene oxidation, followed by glutathione conjugation, and formation of pre-MACs. The presence of pre-MACs strongly suggests that these metabolites were derived from an arene oxide intermediate, which usually undergoes spontaneous rearrangement to restore aromaticity. However, M8 and M9 appear to be stable pre-MACs of RWJ-333369. Previously stable pre-MACs in urine have been described for bromobenzene (Lertratanakoon and Horning, 1987). Trace amounts of M8 and M9 were observed in dog urine, and were quantifiable only by LC-MS/MS and not by radio-HPLC analysis (below the limit of quantitation of the radio-HPLC method, <0.3% of the dose). Similar to the dog, in human urine only trace amounts of M8 and M9 were detected by LC-MS/MS analyses (Mannens et al., 2007). Among the four animal species studied, the rabbit was unique in apparently not forming, to a significant extent, metabolites resulting from the oxidative pathways.

Similarities in the metabolic fate of RWJ-333369 in animals and humans include O-glucuronidation, and hydrolysis of the carbamate ester followed by oxidation to 2-chloromandelic acid and subsequent metabolism to 2-chlorophenylglycine and 2-chlorobenzoic acid (Mannens et al., 2006). Notable differences were that the N-acetylation pathway and arene-oxidation/glutathione pathway were absent or negligible in humans and nonrodents but present in rodents to a minor extent. All metabolic pathways and metabolites observed in humans were also observed in the preclinical species. Although some glucuronide- and sulfate-conjugated metabolites were present in plasma, the parent drug was the major plasma circulating drug-related substance in all species studied, including humans.

In summary, the disposition of orally administered RWJ-333369 in animals is characterized by a near complete absorption and extensive metabolism. Consistent with human metabolism, multiple pathways and renal excretion were mainly involved in the elimination of RWJ-333369 and its metabolites in animals.

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References


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