Peripheral neuropathic pain consists of diverse syndromes such as postherpetic neuralgia, trigeminal neuralgia, painful diabetic neuropathy, and the complex regional pain syndromes (Scadding, 1989, 1992; Price et al., 1994; Kingery, 1997). Characteristic symptoms of peripheral neuropathic pain include persistent burning sensations associated with dull or throbbing pain, paroxysmal lancinating pains, tactile or cold allodynia, and hyperpathia in response to repeated stimulation. Its chronic nature and resistance to treatment by current pharmacological therapies (Bowsher, 1991; MacFarlane et al., 1997; Mackin, 1997) has a serious negative impact on patients with respect to emotional state, social relationships, and ability to function.

Voltage-gated sodium channels are essential for the initiation and propagation of neuronal impulses. Under conditions of abnormal neuronal firing, such as ectopic discharge from an injured sensory nerve, voltage-gated sodium channels determine the threshold for neuronal activation and modulate the frequency and duration of repetitive neuronal firing (Catterall, 1993; Kirsch, 1994; Urenjak and Obrenovitch, 1996). Thus Na⁺ channels are believed to play a critical role in the initiation and maintenance of neuropathic pain.

4-[4-Fluorophenoxy]benzaldehyde semicarbazone (Co 102862) is a potent blocker of neuronal voltage-gated sodium channels that interacts selectively with inactivated states as opposed to resting states of the channel. It has negligible action at other neuroeffector sites. Co 102862 demonstrated an increase in the threshold for mechanical allodynia at oral doses of 2.5 mg/kg (plasma levels of 480 ng/ml) in a 10% Tween 80 formulation. In the mouse Formalin test, Co 102862 blocks both early and late phase nociceptive responses with oral ED₅₀ values of 10.6 and 5.2 mg/kg, respectively. Co 102862 is being developed for the treatment of neuropathic pain based on its antiallodynic activity in the rat Chung model for pain (Kim and Chung, 1992; Carter et al., 1999).

In experimental seizure models, Co 102862 exhibits potent oral anticonvulsant activity against tonic seizures induced by maximal electroshock in both mice and rats and moderate activity against clonic seizures induced by pentylenetetrazol in mice. Moreover, Co 102862 exhibits efficacy in rats against fully kindled stage 5 seizures induced by cornel kindling (Carter et al., 1997). In contrast to carbamazepine (Burchiel, 1988) and lamotrigine (Catterall, 1987), which are proconvulsant at high doses in humans, Co 102862 does not reduce seizure threshold in mice. Thus, Co 102862 would not be expected to be proconvulsant in man and may demonstrate a broader margin of utility in therapeutic use than other clinically employed sodium channel blockers.

The purpose of this study was to characterize the absorption, distribution, metabolism, and excretion of [¹⁴C]Co 102862 in rats and to identify the major metabolites observed in rat plasma, urine, and bile after oral dosing using an HPLC method with radiometric and MS/MS detection methods (Ramu et al., 1999b). The study was conducted in albino male rats of the Sprague-Dawley strain, and a target dose of 10 mg of [¹⁴C]Co 102862/kg of body weight was used...
in this study. The strain and dose are consistent with the pharmacokinetic and toxicokinetic studies completed.

Materials and Methods

Chemicals. [14C]Co 102862 (1) with specific activity of 171.9 mCi/mmol, radiochemical purity of >97%, and chemical purity of >99% was synthesized by BioDynamics Radiochemicals (Cardiff, Wales, UK) and was stored refrigerated at ≤−20°C. Nonradiolabeled Co 102862 (1) was synthesized by CoCensys, Inc. (Irvine, CA) and stored at room temperature (Ramu et al., 2000). The reference standards Co 103084 (2) (carboxyl derivative) and Co 200653 (3) (carboxylic acid derivative) were also synthesized by CoCensys, Inc. and stored at room temperature (see Table 2).

Hydroxypropyl-β-cyclodextrin was supplied by Cerestar, Inc. (Hammond, IN) and was stored at ambient temperature before use. Halothane (Fluothane) was purchased from Zeneca (Macclesfield, Cheshire, UK), buprenorphine hydrochloride (Tengesic) from Reckitt and Colman (PL) Pharmaceuticals (Hull, Yorkshire, UK), providone-iodine USP (2.5% w/v) (Betadine) from Seton Healthcare Group (Oldham, UK), and Nobecucaine from Astraech (Stroud, Gloucestershire, UK). Sterile water for injection was obtained from Fresenius Healthcare Group (Basingstoke, Hampshire, UK) and sterile saline (0.9%, w/v) from Macopharma (London, UK).

Analytical HPLC grade ethanol, methanol, formic acid, methyl tertiary butyl ether, hexane, ethyl acetate, tetrahydrofuran and t-glucose were purchased from Fisher Scientific (Loughborough, UK) and HPLC grade water and acetonitrile from Rathburn Chemicals (Walkerburn, Scotland, UK). Sodium hydroxide, sodium taurocholate, sodium taurochenodeoxycholate, β-glucuronidase, and t-saccharic acid 1,4-lactone were supplied by Sigma-Aldrich Company (Poole Dorset, UK). Ultima Gold XR, Permafluor E, and Hionic-Fluor scintillants and the 14C CO2 absorbent Carbosorb were obtained from Canberra Packard (Pangbourne, Berkshire, UK). All products were subjected to appropriate conditions before use.

One-dimensional 1H NMR spectra of the synthetic standards were recorded on a Varian 300 MHz spectrometer (Varian, Palo Alto, CA) in CDCl3, with tetramethylsilane as reference. Melting points were determined on a Thomas-Hoover capillary melting point apparatus (Thomas-Hoover, Swedenboro, NJ) and are uncorrected. Elemental analyses were performed by Galbraith Laboratories (Knoxville, TN). Flash chromatography was carried out on silica gel (230–400 mesh; Mallinnckrodt, Hayward, CA). Acrodic nitrocellulose membrane filters were supplied by Gelman Sciences (Northampton, UK). Polyethylene tubing was purchased from Portex (Hythe, Kent, UK). All other chemicals and reagents were of the highest analytical grade available and were supplied and were supplied by Sigma-Aldrich Chemical Company (St. Louis, MO, or Poole, Dorset, UK) or from Fisher Scientific.

In Vivo Studies in Rats. Animals. Male albino rats of the Sprague-Dawley strain, approximately 2 months old, were obtained from Charles River UK Ltd. (Margate, Kent, UK) and quarantined for 1 week before dosing. The animals were housed in sawdust-lined polypropylene holding cages for the pharmacokinetic and tissue distribution studies and in glass metabolism cages for the mass balance/excretion and biliary excretion studies. The cages were in a room of constant temperature (22.5 ± 0.5°C) and humidity range of 55 to 74%, with a 12-h light/dark cycle. Rats were maintained on a commercial diet that was withdrawn 24 h before dosing and remained available ad libitum throughout the study.

Bile-duct cannulated rats. Bile ducts were cannulated under anesthesia (diethyl ether) and the cannulas exteriorized at the nape of the neck. Each rat was fitted with a saccle, which allowed collection of bile without restricting movement within the metabolism cage.

Dosing Formulation. Appropriate amounts of [14C]Co 102862 and nonradiolabeled Co 102862 were dispersed into a glass vial and hot ethanol was added, followed by sonication in a water bath (50°C), to dissolve the solids. The ethanol was then evaporated, under a stream of nitrogen at ambient temperature, and the residue suspended in an appropriate amount of hydroxypropyl-β-cyclodextrin [50% (w/w) in sterile water for injection], to achieve a target concentration of 2 mg of Co 102862g of vehicle and ≤27 μg/C of the mixture. The solution was sonicated overnight to ensure solubilization of the [14C]Co 102862 in the hydroxypropyl-β-cyclodextrin. The dosing solutions were stored at 4°C for up to 24 h before use and found to be stable. Pre- and postdose samples of the [14C]Co 102862 dosing solutions diluted in methanol (approximately 0.25 g each) were stored frozen (approximately ≤−20°C) until they were analyzed for total radioactivity and radiochemical purity by liquid scintillation counting and HPLC, respectively.

Dose Administration. Rats were anesthetized with halothane, and then a single dose of [14C]Co 102862 was administered orally (p.o.) by gavage to rats (mean values of 1.2 g, −10.0 mg/kg, −127.8 μCi/kg, 5 ml/kg).

Sample Collection. Pharmacokinetic study. Blood samples (−0.2 ml) were collected from rats (tail vein) in heparinized tubes (Mircovette CB300; Sarstedt, Nurmbrecht, Germany) after p.o. dosing at predose and 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 48, 72, 96, and 120 h postdose. At 120 h, the rats were anesthetized with halothane and exsanguinated via the dorsal aorta and the blood collected into a heparinized container. The blood samples were stored at -80°C until they were centrifuged (approximately 5000g for 5 min at room temperature). The plasma was harvested and retained frozen (approximately ≤−20°C) until analyzed for radioactivity.

Mass balance study. Based on the plasma radioactivity profiles obtained in the pharmacokinetic study above, urine was collected at predose, 0 to 12, 12 to 24, 24 to 48, 48 to 72, 72 to 96, and 96 to 120 h postdose; feces at predose, 0 to 24, 24 to 48, 48 to 72, 72 to 96, and 96 to 120 h postdose; and expired air at predose and 0 to 12 and 12 to 24 h postdose. The urine and feces up to 48 h were collected over solid CO2 and after this time at ambient temperature. The traps containing 5 M sodium hydroxide for collecting the expired air were divided at the point of collection into three aliquots, the first aliquot (approximately 1 ml) for radioactivity measurements and the remaining as two equal aliquots, one for HPLC and the other for LC-MS and subsequently LC-MS/MS analysis. Where there were insufficient fecal pellets to produce a homogenate, the samples were retained whole for total radioactivity determinations only.

At 120 h postdose, rats were sacrificed by an overdose of halothane followed by cervical dislocation, and the carcasses were retained for total radioactivity measurements. The metabolism cages were thoroughly washed with water/methanol (50:50, v/v), and the washings and debris were collected into a preweighed container and retained overnight. The supernatant was decanted and the liquid and solid debris retained separately for total 14C measurements. All samples (except the final cages washings and the sodium hydroxide solutions, which were stored at room temperature) were transferred to a −80°C freezer as soon as possible after collection and stored therein until analyzed.

Bile-duct cannulation study. In the case of bile-duct cannulated rats, bile was collected at predose, 0 to 1, 1 to 2, 2 to 3, 4 to 6, 6 to 12, 12 to 24, and 24 to 48 h postdose; urine at predose, 0 to 12, 12 to 24, and 24 to 48 h postdose; and feces at predose, 0 to 24 and 24 to 48 h postdose. All urine, feces, and bile samples were aliquoted as above and stored frozen before analysis.

Tissue distribution study. Based on the plasma radioactivity profiles obtained in the pharmacokinetic study above, the rats were anesthetized and exsanguinated as described above for the tissue distribution study (n = 3 rats per time point) at 8 h (Tmax) and 1, 2, 4, 7, 14, and 28 days postdose. Blood, plasma, and, following exsanguination, the tissues (adrenal, bone marrow, brain (including pituitary), brown fat, eyes, gastrointestinal tract (including contents), heart, kidney, liver, lung, lymph node, pancreas, salivary gland, skeletal muscle, skin, spleen, stomach (including contents), testes, and white fat) were collected and stored at approximately ≤−20°C until analyzed for total radioactivity. The carcasses were also retained frozen.

Chemical Synthesis of Proposed Metabolites of Co 102862. Co 200653: 4-(4-Fluorophenyl)benzoic acid (3). To a solution of 4-(4-fluorophenoxymethyl) benzaldehyde (Ramu et al., 2000) (published elsewhere; 1.55 g, 7.17 mmol) in 25 ml of tetrahydrofuran was added dropwise a solution of K2CO3 (2.32 g) in water (40 ml), followed by 2 N HCl (2 ml). The mixture was stirred at room temperature for 17 h. The solid was filtered out. To the filtrate, 20 ml of 2 N NaOH was added. The liquid was washed with 40 ml of 3:2 hexane/ethyl acetate. The aqueous solution was then acidified with 2 N HCl to pH 5. The resulting white solid was collected by filtration, washed with water, and dried in vacuo to yield a white solid product (1.10 g, 4.74 mmol, 66% yield). 1H NMR (CDCl3) δ: 6.98 (d, 2H, J = 8.6 Hz), 8.08 (d, 2H, J = 8.6 Hz), 7.14–7.05 (m, 4H).

Co 103084: 4-(4-Fluorophenyl)benzoylesemicarbazide (2). To a solution of
4-(4-fluorophenoxo)benzoic acid (2; 117 mg, 0.504 mmol) in 5 ml of CH₂Cl₂ was added carbonyl diimidazole (88 mg, 0.54 mmol). The mixture was stirred at room temperature for 30 min. The solvent was removed in vacuo. To the residue was added semicarbodiimide hydrochloride (541 mg, 4.84 mmol), followed by pyridine (5 ml). The mixture was stirred at room temperature for 20 h. The solution was carefully taken with a pipette and added to water (50 ml). The resulting white solid was collected by filtration, washed with water, acidified with 2 N HCl in water, and dried in vacuo to yield a solid (73 mg, 0.25 mmol, 50% yield), m.p. 218–219°C. 1H NMR (DMSO-d₆): δ 10.06 (s, 1H), 7.91 (d, J = 8.7 Hz, 2H), 7.85 (s, 1H), 7.32–7.03 (m, 4H), 7.01 (d, J = 8.7 Hz, 2H), 6.01 (s, 2H). Analysis calculated for C₅₂H₃₂FN₄O₂: C 58.13; H, 4.18; N, 14.53. Found: C, 58.23; H, 4.21; N, 14.45.

Quantitative Radiochemical Analysis. Dosing solutions, plasma, bile, urine, NaOH solutions, and washings. Known weights (0.01 to 1.0 g) of each of the dosing solutions, bile, plasma, and urine samples, urine container washings, syringe and cage washings, and NaOH solutions were mixed with 2 ml of Ultima Gold XR scintillator and counted. The scintillant for the NaOH solutions was Hionic-Fluor (2 ml). The radioactivity was determined by liquid scintillation counting techniques using a Canberra Packard 1900TR scintillation counter (Canberra Packard) with appropriate quench curves. The instrument was autocalibrated daily or before counting each batch of samples. Samples were routinely counted for 5 min or 2% sigma, but where levels of radioactivity were low, samples were counted for either 20 min or until 10,000 disintegrations were recorded.

Blood, feces, and cage debris. Known weights (approximately 0.2 g in triplicate) of each of blood, feces homogenates, and cage debris were combusted (Canberra Packard Sample Oxidizer 307) and the ¹⁴CO₂ produced trapped with the carbon dioxide absorbent Carbosorb. This was mixed with the scintillator Permafluor E° before counting. The efficiency of burning (98.21 ± 1.54%) was determined by spiking known amounts of radioactivity into control matrices and combusting before counting as described above.

Tissues and organs. Known weights of the organs/tissues or whole organs were combusted and counted as indicated above.

Carcases. Carcasses were individually dissolved, over several days, in approximately 1 liter of 2 M potassium hydroxide in methanol and the total weight of the digest recorded. Aliquots (×5) of known weight of the digests (approximately 0.5 g) were mixed with 2 ml of Hionic-Fluor scintillator and counted.

Pharmacokinetic Data Analysis. The plasma radioactivity profiles from rats in the pharmacokinetic study were analyzed using WinNonLin version 1.5 (Scientific Consulting, Inc., NC) and the pharmacokinetic parameters [Tmax, t₁/₂, Cmax, Con, AUC(t→∞)] were determined (Gibaldi and Perrier, 1982; Lam et al., 1985).

Preparation of Dosing Solution for HPLC Analysis. Diluted samples of each dosing solution were injected directly onto the HPLC system. Radioactive purity and dose administered were confirmed by data analysis.

Extraction of Plasma Samples for HPLC or LC/MS/MS Analysis. Weighed aliquots of pooled plasma (samples at a certain time point from pharmacokinetic or tissue distribution studies) were added dropwise to 3.0 ml of acetonitrile containing 0.05% formic acid and mixed vigorously by vortexing for 15 min. The samples were then centrifuged at approximately 1400g for 15 min and the supernatant retained in a preweighed glass test tube. The resulting pellet was resuspended in 2.0 ml of acetonitrile containing 0.05% formic acid, mixed, and centrifuged as before. The supernatant was removed and pooled with the initial supernatant. The extract was weighed and analyzed by liquid scintillation counting to determine the radioactivity recovery. The pooled extracts were dried under a stream of nitrogen at 40°C and reconstituted in 0.2 ml of methanol/water (70%, v/v) with thorough mixing. The reconstituted extracts were then centrifuged at approximately 13,000g for 5 min, transferred to HPLC vials, and placed on a cooled autosampler.

Filtration of Urine and Bile Samples for HPLC or LC/MS/MS Analysis. Pooled urine and bile were diluted 1:4 in methanol/water (70:30, v/v) and filtered through a 0.2-μm filter (Gelman nylon 13-mm Acrodisc) at 10,000 revolutions/min. The resulting solution was then transferred to HPLC vials and analyzed.

Solid-Phase Extraction of Urine and Bile Samples for LC/MS/MS Analysis. Pooled urine and bile samples were extracted using a simple one step solid-phase extraction on an Oasis cartridge (Waters Corporation, Milford, MA) using a 96-well extraction plate placed on a vacuum manifold. Each well on the plate contained 30 mg of Oasis HLB sorbent. The cartridge was conditioned with 1 ml of methanol/water (70%, v/v) with thorough mixing. The reconstituted extracts were then centrifuged at approximately 13,000g for 5 min, transferred to HPLC vials, and analyzed.

ENZYMATIC HYDROLYSIS OF POOLED URINE AND BILE. Samples of pooled urine (48 h) and bile (4 h) were lyophilized overnight and the dried material reconstituted in 0.01 M sodium acetate buffer pH 5, respectively. The recon-
stituted samples were incubated with β-glucuronidase/sulfatase enzyme in the presence and absence of inhibitor (o-saccharic acid 1,4-lactone). After incubation, the mixtures were stored frozen at approximately −80°C before HPLC and LC-MS/MS analysis.

**HPLC-UV, Radiometric, and LC-MS/MS Analysis of Samples.** Dosing solutions, plasma, urine, and bile samples (200-µl injections) were analyzed by HPLC and LC/MS/MS with radio and UV detection. Chromatography was performed on a Spherisorb ODS-2 5-µm HPLC column with 150 x 4.6-mm column attached to a guard column (ODS, 4 µm, 1 cm) maintained at 25°C with a column heater (model 7990; Jones Chromatography, Hengoed, Mid-Glamorgan, Wales). The samples were eluted with a step gradient mobile phase system: 0 min, 100% A to 100% B over 15 min; 20 min, 100% B to 100% A over 1 min; 30 min, 100% A (where A = water with 0.05% formic acid and B = acetonitrile with 0.05% formic acid) at a flow rate of 1 ml/min using a PU-980 HPLC pump with an LG-980-02S gradient mixing unit, an AS-950-10 autosampler maintained at 4°C, and a UV-975 UV detector (Jasco UK Ltd, Great Dunmow, UK). A Radiomatic 150TR scintillation analyzer (Canberra Packard) with a scintillator flow rate of 1.5 ml/min (Quickzint Flow 302; Zinsser Analytic, Maidenhead, Berkshire, UK) was used for radiometric detection. Data acquisition and processing for HPLC analysis was done using P.E. Nelson Turbochrom v 4.1 (Perkin-Elmer Limited, Beaconsfield, Bucks, UK), whereas MassLynx (version 3.0; Micromass, Manchester, UK) was used for LC/MS/MS analysis. Direct infusion of the reference standards into the mass spectrometer was carried out using a syringe pump (Model 11; Harvard Apparatus, Edenbridge, Kent, UK) at a flow rate of 0.6 ml/h.

The LC-MS interface used was electrospray ionization, operated in either positive ion or negative ion detection mode. The eluent from the liquid chromatography system at 1 ml/min was split 1 to 9 to give ca. 100 µl/min into the mass spectrometer and ca. 900 µl/min into the radiochemical detector. The mass spectrometric conditions were optimized for sensitivity and for providing structural information for the reference standards and unknown metabolites with capillary discharge at 3.5 kV, cone voltage at 20 V, extractor lens voltage at 3 V, source block temperature of 100°C, desolvation temperature of 250°C, nebulizer gas flow at its maximum, and desolvation gas (nitrogen) flow rate of 600 l/h. Single MS spectra were obtained on infusion by scanning the mass range 100 to 1500 with a scan speed of 3.95 s per scan. Single MS scans were performed during chromatography over the mass ranges 200 to 1200 or 900 to 1200 with a scan speed of 2.96 s per scan.

The mean recovery of radioactivity from the column was 103%. For quantification of [14C]Co 102862 and metabolites in plasma, urine, and bile, the percentage area associated with each component (limits of quantification >2% of the total area) was multiplied by the total microgram equivalents of the sample. The radiodetector was calibrated over a range of 0 to 6 × 10⁶ for total peak area.

**Results**

**Pharmacokinetic Study.** The mean plasma concentration-time profiles in rats after p.o. administration of [14C]Co 102862 are shown in Fig. 1. The typical radioactivity profile in plasma increased progressively peaking (Tmax) at 8 h with a Cmax of 30 µg equivalents/g and then declined in an exponential manner resulting in an AUC of 881 µg equivalents · h/g. In comparison, unchanged drug peaked at 1 h with a Cmax of 821 ng/ml and AUC of 7167 ng · h/ml. The terminal elimination half-lives for [14C]Co 102862 and unchanged drug were 37 and 2.1 h, respectively. The profile when compared with that of unchanged drug (Ramu et al., 1999a) was indicative of extensive metabolism of Co 102862.

**Mass Balance Study.** Over the 120-h collection period, a mean of >91% of the radioactive dose was recovered (Table 1). Of this, the bulk (~74%) was eliminated in the urine, and the majority of this was within the first 48 h. Excretion in the feces was low at 7%, and there was no evidence for elimination of radioactivity by the expired air. After 120 h, significant amounts (~10%) of radioactive dose was still associated with the carcass.

**Bile Duct-Cannulation Study.** After oral dosing of [14C]Co 102862 to bile-duct cannulated rats, there was extensive biliary secretion of radioactivity material, with approximately ~39% of the dose eliminated by this route and ~37% excreted in the urine (Table 1). The amount of radioactivity excreted in the feces of bile-duct cannulated rats over 48 h was similar to that in intact rats.

**Tissue Distribution Study.** After p.o. administration of [14C]Co 102862, the radioactivity was measured in selected tissues (expressed as microgram equivalents per gram). The radioactivity in most tissues and in plasma was highest during the study at the first sampling point of 8 h. It declined immediately as a function of time. The radioactivity in the fatty tissues (white fat and brown fat) and secretory gland...
tissues (lymph nodes, adrenals, salivary glands, testes, and pancreas), however, did not peak until as late as 96 h.

Although significant amount of radioactivity (10% of the total radioactivity in the tissues on day 5) was sequestered in the fatty and secretory gland tissue, the radioactivity did decline with time (Fig. 2a) with a terminal half-life of 14 days. On day 28, adipose tissue, skin, gastrointestinal tissue, muscle, and testes contained 3% of the administered dose. Radioactivity levels in all other tissues including brain were low, decreasing like those of plasma, and were not quantifiable after day 5. It should be noted that the major excretory organs, such as the liver and kidneys, did not accumulate radioactivity in excess of the blood concentrations. Presumably, this was due to the relatively rapid elimination in bile and urine.

### HPLC Analysis of Samples

**Plasma samples from mass balance and tissue distribution study.** Plasma samples were analyzed by HPLC radiodetection (Fig. 1). The extraction recovery of total radioactivity was 98%. The profiles of the plasma extracts from 4 to 96 h showed a single major radioactive peak (rt \( \approx 25.5 \text{ min} \), \( \approx 25.0 \text{ min} \) on UV), which coeluted with the carboxylic acid metabolite (Co 200653). Unchanged drug was observed only in the 4-h samples and eluted at approximately 23.5 min and accounted for 2.2% of the plasma radioactivity.

**Urine samples from mass balance/excretion study.** The HPLC profiles with radiodetection for the pooled samples of rat urine indicated that 0.1% of the dose was excreted as unchanged \([^{14}C]\)Co 102862 (at \( \approx 23.5 \text{ min} \)). The 12-h urines were comprised of 10 separable radioactive

### Table 2: Major metabolites of \([^{14}C]\)Co 102862 in rat urine and bile identified by LC-MS/MS

<table>
<thead>
<tr>
<th>Peak ID</th>
<th>HPLC Radiodetection rt min</th>
<th>LC-MS Radiodetection rt min</th>
<th>Structure</th>
<th>M + H Daughter Ion</th>
<th>M - H Daughter Ion</th>
<th>Mean % Dose 0–48 h (Urine)</th>
<th>Mean % Dose 0–48 h (Bile)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 10</td>
<td>25.3</td>
<td>21.3</td>
<td>![Structure](Co 200653 (3))</td>
<td>233/215, 111</td>
<td></td>
<td>16.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Parent</td>
<td>23.5</td>
<td>20.7</td>
<td>![Structure](Co 102862 (1))</td>
<td>274</td>
<td></td>
<td>-0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Peak 9</td>
<td>21.7</td>
<td>18.0</td>
<td>![Structure](Co 103084 (2))</td>
<td>290/215, 288/244, 187</td>
<td></td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Peak 8</td>
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<td>17.6</td>
<td>![Structure](Co 200653 (3))</td>
<td>290/273, 230</td>
<td></td>
<td>1.0</td>
<td>-0.1</td>
</tr>
<tr>
<td>Peak 7</td>
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<td>16.7</td>
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<td>409 (431)/255, 199</td>
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<td>20.4</td>
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<tr>
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<td>16.3</td>
<td>![Structure](Co 200653 (3))</td>
<td>290/247, 215</td>
<td>288/245</td>
<td>1.5</td>
<td>0.5</td>
</tr>
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</tr>
<tr>
<td>Peak 4</td>
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<td>13.7</td>
<td>![Structure](Co 200653 (3))</td>
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<tr>
<td>Peak 3</td>
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<td></td>
<td></td>
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<td>0.8</td>
</tr>
<tr>
<td>Peak 2</td>
<td>10.0</td>
<td>9.4</td>
<td>Mixture of polar metabolites (sulfate conjugates)</td>
<td></td>
<td></td>
<td>6.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Peak 1</td>
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<td>3.5</td>
<td>Mixture of polar metabolites (sulfate conjugates)</td>
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<td></td>
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<td>0.2</td>
</tr>
<tr>
<td>Others</td>
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</tr>
<tr>
<td>Total</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>-53.0</td>
<td>-36.0</td>
</tr>
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ID, identification.
peaks, excluding parent drug, although some of these were broad and clearly contained more than one metabolite (Fig. 3a).

The amount of each radioactive peak excreted in the urine over 48 h, expressed as a percentage of dose, is shown in Table 2. Quantitatively, the profiles are dominated by two major metabolites with retention times of ~19.7 and ~25.3 min. These correspond to the O-glucuronide of the carboxylic acid metabolite and the carboxylic acid metabolite, respectively. HPLC profiles of the urine samples at 72, 96, and 120 h were dominated by one major metabolite, namely the carboxylic acid with only trace amounts of other components.

When the 12-h pooled urines were incubated at pH 5.0, 37°C for 17 h (in the absence of any enzyme), the major change was the loss of the large peak at rt ~19.7 (peak 7, the glucuronide conjugate of the carboxylic acid) with a corresponding increase in the free carboxylic acid itself at rt ~25.5 min (Fig. 3b). This change also occurs after incubation with the β-glucuronidase/sulfatase mixture (Fig. 3c) and in the presence of the β-glucuronidase inhibitor saccharolactone (Fig. 3d). The loss of peak 7 made the two peaks at the leading and trailing edges of peak 7 much more distinguishable. The two polar peaks at approximately 4 and 10 min were lost after incubation with the β-glucuronidase/sulfatase mixture (even in the presence of the glucuronidase inhibitor) with a corresponding increase in the peaks at ~13.9 and ~22.0 min. This suggested that these may be sulfate conjugates or double conjugates of which one is a sulfate.

Bile samples from bile duct-cannulated rats. A representative chromatogram with radiodetection for the intervals (0–48 h postdose) of bile samples is shown in Fig. 4a. The metabolic profile of rat bile was similar (metabolite peaks 1–10) to that of rat urine from intact rats but with a few additional minor peaks. The amounts of each of the 10 metabolite peaks over 48 h, expressed as percentage of dose, are shown in Table 2. Except for peaks 4 and 7, all the other peaks were less than ~2% of the dose. The most striking feature of the time course of biliary metabolites is the dominance in the early bile samples (up to 6 h) of the glucuronide of the ring hydroxylated metabolite (rt ~16.6 min). At later sampling times, this metabolite was replaced as the dominant component by the glucuronide of the carboxylic acid (rt ~19.7 min).

On incubation of the 4-h bile samples at pH 5.0, 37°C for 17 h with the β-glucuronidase/sulfatase mixture, the two major peaks eluting at rt ~16.6 (peak 4, glucuronide of the ring hydroxylated metabolite) and at rt ~19.7 (peak 7, the glucuronide of the carboxylic acid metabolite) disappeared (Fig. 4b). They were replaced by two new major peaks eluting at rt ~20.5 min (peak 8, ring hydroxylated metabolite, which is the aglycone of peak 4) and at rt 25.5 min (peak 10, carboxylic acid metabolite, which is the aglycone of peak 7). Here again, after hydrolysis, the two peaks at the leading and trailing edges of rt 19.7 min were much more distinguishable. As in the case of the urine samples, peak 2 at approximately 10 min disappeared on incubation even in the presence of the β-glucuronidase inhibitor saccharolactone.

Urine samples from bile duct-cannulated rats. The HPLC profiles with radiodetection of urine samples from bile-duct cannulated rats were identical with the profiles of urine from intact rats.

LC-MS/MS Analysis of Plasma, Urine, and Bile Samples. Plasma, urine, and bile samples were analyzed by LC-MS/MS. The peaks associated with the mass ions and their daughter ions in positive and/or negative modes are summarized in Table 2. The metabolite profiles of the pooled urine sample using radiochemical, UV, and mass spectrometric detection (at m/z 233, m/z 274, m/z 290, m/z 466, and m/z [409 + 431]) along with the reference standards Co 103084, Co 102862, and Co 200653 are shown in Fig. 5. The metabolite peaks are labeled from 1–10.

Peak 10: Carboxylic acid metabolite (Co 200653). The metabolite peak at ~21.3 min (Fig. 5) showed a [M + H]+ ion at m/z 233 and a [M + Na]+ ion at 255, suggesting that the metabolite had a molecular weight of 232 (even number of nitrogens). It had the same chromatographic retention time as that of the standard Co 200653.

Peak 6: Carboxyl metabolite (Co 103084). Based on the radiochemical tracing (Fig. 5), four metabolite peaks (6, 7, 8, and 9) appeared to elute together at rt ~16.5 to 18.0 min. The metabolite peak at a retention time of 16.3 min showed a [M + H]+ ion at m/z 290. It had the same chromatographic retention time as that of the standard Co 103084 (Fig. 4). Comparing the mass spectrum and daughter ion mass spectrum of m/z 290 to those of the reference standard Co 200653, confirming the identity of peak 10 to be the carboxylic acid metabolite. The major peak (~21.6 min) seen in pooled rat plasma corresponded to that of the carboxylic acid metabolite.

Peak 7: Glucuronide of the carboxylic acid metabolite. The central eluting component at a retention time of ~16.7 min (Fig. 5), which was the major component of the radiochemical peak, showed a weak [M + H]+ ion at m/z 409 and a strong [M + Na]+ ion at m/z 431. The mass spectrum at 16.7 min and daughter ion spectrum of m/z 431 showed the neutral loss of 176 from m/z 431 to m/z 255, [M + Na] of the carboxylic acid (carboxylic acid), indicating that the metabolite was the glucuronide of the acid. A peak at m/z 233 was indicative of a neutral loss of 176 ion from m/z 409. The negative ion spectrum of this metabolite (Fig. 7a) showed [M − H]− at m/z 407 and daughter ion spectrum of m/z 407 (Fig. 7b) showed a fragment of m/z 231 that was a neutral loss of 176 from m/z 407 ion. These results suggested that the metabolite has a molecular weight of 408, and this would fit a glucuronide of the carboxylic acid metabolite (176 + 232 = 408).
Peak 8: Ring hydroxylated metabolite. The third component of the coeluting peak at retention time of ~17.6 min (Fig. 5) showed [M + H]^+ ions at m/z 290. The mass spectrum of peak 8 showed a single peak for m/z 290, and the daughter ion spectrum of m/z 290 showed a fragment at m/z 273. The daughter ion spectrum of peak 8 was different from that of the carbonyl metabolite Co 103084 (peak 6). The molecular weight of 289 suggested an addition of oxygen to the parent compound. The neutral loss of NH3 from m/z 290 to m/z 273 was characteristic of the semicarbazide moiety being intact. The ion fragmentation was consistent with the structure of a hydroxylated metabolite. The ion at m/z 127 suggested that the hydroxylation took place on the fluorinated ring. One would expect a fragment at m/z 111 if hydroxylation were to take place elsewhere (Fig. 6b).

Peak 9: Glycine conjugate of the carboxylic acid metabolite. The fourth component of the coeluting peak at retention time of ~18.0 min (Fig. 5) showed [M + H]^+ ions at m/z 290. The mass spectrum of peak 9 showed a major peak at m/z 290 and a minor peak at m/z 215, whereas the daughter ion spectrum of m/z 290 showed a fragment at m/z 215. The mass spectrum and daughter ion spectrum of peak 9 in the positive mode were similar to that of the carbonyl metabolite Co 103084. The intense ion at m/z 215 suggested that the fluoro-diphenylether carbonyl moiety was present. In the positive ion daughter ion spectrum, there was very little structural information between m/z 215 and m/z 290. The negative mode mass spectra of peak 9 and 6 were similar in that they showed a single peak at m/z 288. However, in the negative ion daughter ion spectrum of m/z 288 for peak 9, there was a characteristic loss of 44 (CO2) from the [M − H]− ion at m/z 288 to m/z 244 that was indicative of a carboxylic acid function in the conjugate. This was in contrast to a neutral loss of 43 (CONH) from the [M − H]− ion at m/z 288 to m/z 245 for peak 6 that was indicative of the semicarbazide moiety as in the case of Co 103084. One possible and most probable structure for this metabolite peak 9 was the glycine conjugate of the carboxylic acid metabolite.
Peak 4: Glucuronide of the ring hydroxylated metabolite. The metabolite peak at ~13.7 min (Fig. 5) showed a [M + H]+ ion at m/z 466 in the mass spectrum (Fig. 8a). In the daughter ion spectrum of m/z 466 (Fig. 8b), the major neutral fragment loss observed was 176 (giving m/z 290), suggesting that the unknown metabolite was a glucuronic acid conjugate. The fragment ions at m/z 290 and 273 suggested that this was most probably the glucuronide of the hydroxylated metabolite (peak 8). Enzymatic hydrolysis with the β-glucuronidase/sulfatase confirmed the presence of a glucuronide.

Peaks 1, 2, 3, and 5. The radiochemical trace shown in Fig. 5 also had peaks at ~3.5 (peak 1) min, ~9.4 (peak 2) min, and minor peaks at ~11.8 (peak 3) and ~14.7 (peak 7) min. The LC-MS spectrum of these peaks showed some prominent ions, but no identification was made from the limited mass spectral data of these peaks.

The LC-MS profiles of urine at 24 and 48 h showed primarily the presence of the carboxylic acid metabolite (peak 10) and its glucuronide (peak 7). The LC-MS analysis of the 12-h pooled urine samples after enzymatic hydrolysis confirmed the identification of the glucuronide conjugates of the carboxylic acid (peak 7) and the ring hydroxylated metabolites (peak 4) as seen after HPLC analysis. Additionally, the metabolite peaks 1 and 2 were proposed to be sulfate conjugates or double conjugates of which one is a sulfate. Using techniques and approaches similar to those used for the identification of metabolites in the pooled urine from intact rats, the same six metabolites were identified in the pooled urine and bile samples of bile-duct cannulated rats. Unlike urine, in which the carboxylic acid and its glucuronide were the major metabolites, the glucuronides of the carboxylic acid and the ring hydroxylated metabolites seemed to be the major metabolites in the bile. The glucuronide of the ring hydroxylated form was more dominant at early times (~4 h), whereas the glucuronide of the acid was more prevalent at later times (~6 h).

Discussion

[14C]Co 102862 showed multienzyme pharmacokinetics in rat plasma with a $T_{\text{max}}$ of 8 h. [14C]Co 102862 is extensively distributed into tissues of rats after an oral dose (~10 mg/kg). The fatty tissues and secretory gland tissues contain the highest radioactivity among the tissues. Elimination of radioactivity from the tissues was pro-
and has an estimated half-life of 14 days. A total of 91% of the administered radioactivity was recovered in both intact and bile-duct cannulated rats over 120 h and 48 h, respectively. Despite extensive biliary excretion, the majority of the radioactivity was slowly excreted in urine (74% in intact rats). Bile-duct cannulated experiments show the enterohepatic circulation is an important route of elimination and reabsorption. Approximately 10% of the total radioactivity remained in the tissues on day 5 and decreased with time to ~3% on day 28. Six metabolites, the carboxylic acid, glucuronide conjugate of the carboxylic acid, glycine conjugate of the carboxylic acid, the carbonyl, the ring hydroxylated and glucuronide conjugate of the ring hydroxylated metabolite, were identified (Fig. 9).

Ten metabolite peaks were seen in urine and bile. Carboxylic acid is the major urinary metabolite and the only metabolite seen in plasma. Bile had predominant levels of the glucuronides of both the carboxylic acid and the hydroxylated metabolites. The glucuronides undergo enterohepatic circulation where after reabsorption they could be further metabolized. The acyl glucuronide was fairly stable in urine at temperatures of 2 to 20°C and completely hydrolyzed to the acid metabolite at 37°C. No rearranged isomeric products of the glucuronide were observed. The acyl glucuronide and carbonyl metabolite were not observed in plasma even 15 min postdose. Co 102862 is synthesized via the aldehyde intermediate, and formation of aldehyde was seen as a degradation product during stability studies of Co 102862 in water. The aldehyde was not detected in the analysis of plasma, urine, and bile samples. The formation of the acid metabolite can be presumed to go through an aldehyde intermediate, which in turn can be formed as a result of hydrolysis across the C==N bond. The aldehyde intermediate can then be easily converted by enzyme systems to the acid. Hydrolysis of the carbonyl metabolite is a possible mechanism for formation of the acid, but only minor levels of the carbonyl metabolite were observed in urine. In vitro (S9, liver microsomes, hepatocytes, and liver slices) conversion of Co 102862 to its metabolites in rats was not observed. The carboxylic acid metabolite is probably responsible for the radioactivity in the tissues.

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References


Fig. 9. Proposed metabolic pathway for 14C-Co 102862 in rat urine and bile showing carboxylic acid (10), glucuronide conjugate of the carboxylic acid (7), glycine conjugate of the carboxylic acid (9), ring hydroxylated (8), glucuronide conjugate of the ring hydroxylated (4), and carbonyl metabolites (6) (metabolites labeled using peak numbers: *, 14C-label; #, only metabolite in rat plasma).