THE DISPOSITION AND METABOLISM OF ROFECOXIB, A POTENT AND SELECTIVE CYCLOOXYGENASE-2 INHIBITOR, IN HUMAN SUBJECTS

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ABSTRACT:

The disposition and metabolism of rofecoxib, a selective cyclooxygenase-2 inhibitor, were examined in healthy human subjects and in cholecystectomy patients. After oral administration of [14C]rofecoxib (125 mg, 100 μCi) to healthy subjects, the mean concentrations of total radioactivity and rofecoxib in plasma as a function of time indicated that the t_max was achieved at 9 h postdose. After t_max levels of both radioactivity and rofecoxib decreased in a parallel, exponential fashion (effective t_1/2 ~ 17 h). A similar result was obtained after oral administration of [14C]rofecoxib (142 mg, 100 μCi) to cholecystectomy patients equipped with an L-tube. In healthy subjects, radioactivity was recovered predominantly from the urine (71.5% of dose), with a small amount excreted in feces (14.2%). In patients with an L-tube, half the radioactive dose was recovered in feces, with a lesser amount excreted in urine (28.8%) and a negligible fraction in bile (1.8%). Rofecoxib underwent extensive metabolism in humans, and very little parent drug was recovered unchanged in urine (<1%). Products resulting from both oxidative and reductive pathways were identified by a combination of 1H NMR and liquid chromatography-tandem mass spectrometry analyses, and included rofecoxib-3′α′-trans-dihydrodiol, 4′-hydroxyrofecoxib-O-β-D-glucuronide, diastereomeric 5-hydroxyrofecoxib-O-β-D-glucuronide conjugates, 5-hydroxyrofecoxib, rofecoxib-erythro-3,4-dihydroxy acid, and rofecoxib-threo-3,4-dihydroxy acid. Interconversion of rofecoxib and 5-hydroxyrofecoxib appeared not to be a quantitatively important pathway of rofecoxib disposition in human subjects, in contrast to previous findings in rats.

Rofecoxib (3-phenyl-4-[4-(methylsulfonyl)phenyl]-2-(5H)-furanone; VIOXX, a registered trademark of Merck & Co., Inc.) is a potent and highly selective inhibitor of cyclooxygenase-2 (COX-2) (Prasit et al., 1999), an inducible isoform of cyclooxygenase that plays a key role in inflammatory processes. Rofecoxib has been approved for the treatment of arthritis and pain, and was developed on the premise that selective inhibition of COX-2 would result in decreased inflammation without the adverse gastrointestinal effects associated with inhibition of COX-1 (Vane and Botting, 1996; Donnelly and Hawkey, 1997; Jouzeau et al., 1997). This hypothesis has been supported by the results of recent clinical trials, including the VIGOR study, in which rheumatoid arthritis patients treated with rofecoxib displayed significantly fewer clinically important gastrointestinal events than patients treated with naproxen, a nonselective COX inhibitor (Bombardier et al., 2000).

Disposition and metabolism of rofecoxib in rats and dogs were reported recently (Halpin et al., 2000); metabolites identified in both species included 5-hydroxyrofecoxib, 5-hydroxyrofecoxib-O-β-D-glucuronide, and trans-3,4-dihydro-rofecoxib, while additional metabolites identified in rat only were cis-3,4-dihydro-rofecoxib, rofecoxib-3′,4′-dihydrodiiol, and 4′-hydroxyrofecoxib sulfate. Of note, following oral administration of rofecoxib to intact rats, was the appearance of a second C_max which was not observed in bile duct-cannulated rats. This phenomenon was found to be due to a novel form of enterohepatic recycling, which involved reversible metabolism of rofecoxib to 5-hydroxyrofecoxib (Baillie et al., 2001).

The studies presented here describe the disposition and metabolism of rofecoxib in human subjects. The specific objectives of these studies were: 1) to examine the absorption and excretion of [14C]rofecoxib in healthy adult volunteers and to compare the results with those obtained from animals, 2) to identify the urinary metabolites of rofecoxib in humans, 3) to examine the extent of conversion of 5-hydroxyrofecoxib to rofecoxib following oral administration of 5-hydroxyrofecoxib to healthy subjects, and 4) to investigate the possible involvement of biliary elimination in the excretion of rofecoxib and its metabolites in humans.
Experimental Procedures

Materials and Dosing Solutions. Chemicals. [14C]Rofecoxib and internal standard (the 4'-methylphenyl) analog of rofecoxib) were synthesized by the Labeled Compound Synthesis Group (Merck Research Laboratories, Rahway, NJ) with the radiolabel located at the 4-position of the furanone ring (Halpin et al., 2000). The chemical purity of these materials was 99%. Unlabeled rofecoxib was synthesized by the Department of Process Research (Merck Research Laboratories, Rahway, NJ), and rofecoxib metabolites (5-hydroxy-rofecoxib, rofecoxib-erythro- and rofecoxib-threo-3,4-dihydrohydroxycacid, cis- and trans-dihydorofecoxib) were synthesized by the Department of Medicinal Chemistry (Merck Frosst Canada, Kirkland, QC, Canada). Due to the sensitivity of rofecoxib to light, the compound and all biological samples were handled under yellow light.

Indocyanine green (ICG) was obtained from BD Biosciences (Cockeysville, MD) as Cardio-Green. β-Glucuronidase (Helix pomatia, type H-5) was obtained from Sigma-Aldrich (St. Louis, MO), trifluoroacetic acid (TFA) was purchased from Aldrich Chemical Co. (Milwaukee, WI), and ammonium hydroxide (NH4OH), sodium acetate, mehtanol (MeOH), methylene chloride, and acetonitrile (CH3CN) were obtained from Fisher Scientific (Pittsburgh, PA).

Human Studies. All human study protocols were approved by the Ethics Review Boards of the respective study sites. All subjects understood the procedures and agreed to participate in the study by giving written informed consent. Except for the 14-day rofecoxib dosing study (see below), blood, procedures and agreed to participate in the study by giving written informed consent. Except for the 14-day rofecoxib dosing study (see below), blood, urine, and feces were collected at predose and through 120 h postdose. Blood samples were collected into tubes that contained 5 M phosphoric acid (10 μl/ml blood) and potassium EDTA (10 μl/ml blood of a 1 mg/ml aqueous solution) to stabilize the 5-hydroxyrofecoxib. The final pH of the mixture was approximately 4. Plasma was obtained promptly by centrifugation, and all biological samples were stored at −20°C until analyzed.

Single oral rofecoxib dose. In an open, four-way, oral single-dose crossover study, six healthy men received single doses of 125 mg oral rofecoxib as either a single tablet or a solution in 12.5 ml of polyethylene glycol-400 (each formulation was administered twice). One of these two solutions included [14C]rofecoxib (100 μCi). Polyethylene glycol was included in the solutions due to the poor solubility of rofecoxib in water. Two hours after each administration, the subjects were given apple juice (2 h postdose) and a standard lunch (4 h postdose), and were required to drink sufficient fluid (750 ml/8 h) to maintain an adequate urinary flow. Only the results from the administration of radiolabeled drug are discussed in this presentation.

Single oral [14C]rofecoxib dose to patients with cholecystectomy. Four adult women who had undergone elective gallbladder surgery and had an L-tube (Vishnesky’s cholecdochal drainage) inserted into the common bile duct were enrolled into the study. Two hours prior to oral administration of [14C]rofecoxib (142 mg, 100 μCi), patients were given single i.v. paces of ICG (0.5 mg/kg) to evaluate the efficiency of bile collection.

Fourteen-day oral dose of unlabeled rofecoxib. In a multiple-dose clinical study, subjects received single daily doses of rofecoxib, as tablets, for a 14-day period. Urine (0–8 h) was collected on day 14 from one of these subjects receiving daily doses of 250 mg. Single oral doses of 5-hydroxyrofecoxib at 5, 10, and 25 mg, and a single oral dose of rofecoxib at 50 mg. A placebo-controlled, double-blind, six-period study was conducted in which 12 healthy subjects between the ages of 18 and 50 years were administered single oral doses of 5-hydroxyrofecoxib or rofecoxib, and two healthy subjects were given placebo throughout the study. During periods 1 to 3, sequential oral doses of 5, 10, and 25 mg of 5-hydroxyrofecoxib were administered as dry-filled capsules. During periods 4 to 6, the same 12 subjects were randomized to receive single doses of 50 mg of rofecoxib orally as a 25-mg compressed tablet, and 1 mg of rofecoxib and 1 mg of 5-hydroxyrofecoxib each in 200 ml of normal saline by 30-min i.v. infusion in a crossover fashion. Actual weights of i.v. solution infused were recorded for each subject. A 12-day washout period was allowed between doses for periods 1 to 3 and 4 to 6, and a 4-week washout period was allowed between the end of period 3 and the beginning of period 4.

Measurement of Total Radioactivity. After centrifugation of plasma, aliquots (0.5–1.0 ml) were added to polyethylene vials containing scintillation cocktail (15 ml; Ready Safe, Beckman Coulter, Inc., Fullerton, CA). Urine was brought to 37°C and shaken vigorously to dissolve particles, and 1.0 ml was taken for liquid scintillation counting.

Fecal samples were homogenized in approximately 4 to 5 volumes of water using an Omni homogenizer (Omni International, Waterbury, CT). Two aliquots of bile and fecal homogenates (~1 g each) were weighed into separate, tared copper beakers and, after drying overnight, the samples were combusted in a Packard Tricarb Sampler Oxidizer (model B306; Packard Instrument Company, Inc., Downs Grove, IL) prior to scintillation counting. Total radioactivity in these samples was determined using a Beckman LS5000CE liquid scintillation spectrometer (Beckman Coulter).

Preparation of Plasma Samples for Rofecoxib Assay. After centrifugation, an aliquot of plasma (1 ml) and internal standard (25 μl of a 150 ng/ml solution in CH3CN) was added to 1 ml of buffer (0.1 M sodium acetate, pH 5). Liquid-liquid extraction (50:50 mixture of methylene chloride and hexane; 8 ml) of the sample was carried out on an Eberbach shaker. The recovery was 97% and 80% for rofecoxib and 5-hydroxyrofecoxib, respectively. After centrifugation (3000 rpm for 5 min), the aqueous layer was frozen in an acetone/dry ice bath, and the organic phase was decanted into a separate tube. The organic phase was evaporated to dryness under a flow of nitrogen in a Turbo VAP LV evaporator (50°C, 20 min; Zymark Corp., Hopkinton, MA). The residue was reconstituted in CH3CN (500 μl) and water (1.5 ml) immediately before analysis.

Preparation of Urine, Bile, and Fecal Samples for Metabolite Profile Analysis. Urine. Since urine specimens from radiolabeled studies in healthy subjects and cholecystectomy patients afforded virtually identical HPLC profiles, samples from selected time points were pooled for metabolite isolation. Aliquots from each time interval were combined in proportion to their respective volumes to yield a representative 0- to 24-h sample from each subject. The pH of the pooled samples (30 ml) was adjusted to ~4.5 by the addition of glacial acetic acid (~100 μl). Representative pooled urine samples from two subjects were similarly prepared for time periods of 24 to 36 and 36 to 48 h.

Before radiochromatography, an aliquot of pooled urine (2 ml) was treated with a 2:1 mixture of CH3CN/MeOH (12 ml). After centrifugation, the supernatant was isolated and evaporated to dryness (N2, 44°C). The residue was reconstituted in an 85:15 mixture of 0.1% aqueous TFA/CH3CN (300 μl), after which the pH was adjusted to 3.0 with NH4OH. The reconstituted samples were sonicated for 5 min, and 200-μl aliquots were injected into the HPLC system. The extraction recoveries were 75 to 90%.

Feces. Samples from the two radiolabeled studies were homogenized in 4 to 5 volumes of water and mixed thoroughly. Aliquots (1 ml) were mixed with 1.5% glacial acetic acid in CH3CN (4 ml). After centrifugation, aliquots of the supernatant (200 μl) were taken for scintillation counting (extraction efficiency >70%), while the remaining supernatants were evaporated to dryness. The residues were reconstituted in 15% CH3CN in 0.1% aqueous TFA/NH4OH, pH 3 (500 μl). After the samples were vortexed and sonicated, centrifugation was carried out and the supernatants (300 μl) were analyzed by HPLC.

Bile. Bile samples from the cholecystectomy patients, collected at time intervals of 0 to 2, 2 to 4, 4 to 6, 6 to 9, 9 to 12, 12 to 16, 16 to 20, and 20 to 24 h, were thawed and pooled in proportion to the sample volumes to produce representative 0- to 24-h samples for each subject. Aliquots of bile (10 ml) were combined with 40 ml of 1.5% glacial acetic acid in CH3CN. After centrifugation, the supernatant was isolated and evaporated to dryness (N2, 44°C). The residue was reconstituted in 500 μl of 15% CH3CN in 0.1% aqueous TFA/NH4OH (pH 3), sonicated, and analyzed by HPLC. Extraction recovery was ~100%.

Analytical Techniques. Rofecoxib and 5-hydroxyrofecoxib assay. Concentrations of rofecoxib and 5-hydroxyrofecoxib in human plasma samples were determined by a modified HPLC/fluorescence method using a postcolumn photochemical reactor to generate highly fluorescent products through a stilbene-phenanthrene type of photo-cyclization (Woolf et al., 1999). The modification involved the use of a mobile phase consisting of CH3CN/water (33:67, v/v) to resolve 5-hydroxyrofecoxib from endogenous components of plasma. The limit of quantitation for each analyte for 1-ml samples was 0.5 ng/ml. The intraday accuracy (determined as mean observed concentration/nominal concentration × 100) and precision (coefficient of variation) at the lower limit of quantitation of rofecoxib were 96.0% and 2.5%, respectively. The correspond-
ing values at the remaining standard rofecoxib concentrations (5, 10, 20, 50, 100, 200, 500, and 1000 ng/ml) were ≥99.4 and ≤1.8%, respectively.

**Indocyanine green.** With minimal exposure to light, bile was diluted with 0.05 M sodium phosphate buffer, pH 5 (1:1, v/v), and centrifuged to remove insoluble particles. The supernatant was transferred to an amber autosampler vial and subjected to analysis by reversed-phase HPLC. A PerkinElmer pump (200 series) was equipped with a μ-Bondapak C18 column (Waters Corp., Milford, MA), which was eluted with a mobile phase of 0.05 M sodium phosphate buffer (pH 6)/CH2CN/MeOH (50:47.3, v/v/v) delivered at a flow rate of 1 ml/min. The run time of each injection (50 μl) was 20 min. ICG was detected by an Applied Biosystems (Foster City, CA) programmable absorbance detector (785A) with a tungsten lamp set at 700 nm (Rappaport and Thiessen, 1982). The retention time of ICG was approximately 11.0 min. The limit of quantitation for a 1-ml sample was 1.0 ng/ml. The intraday accuracy (determined as mean observed concentration/nominal concentration × 100) and precision (coefficient of variation) at the lower limit of quantitation of ICG was 98.6%. The corresponding values at the remaining standard concentrations (2, 5, 10, 20, 50, and 100 ng/ml) were all ≥99.2%.

**Metabolite profile and purification of unlabeled rofecoxib metabolites.** The HPLC system consisted of a Waters 600E instrument, an analytical C18 column (4.6 × 150 mm, 25°C), and a solvent system comprising 0.1% aqueous TFA/NH4OH, pH 3 (Solvent A), and 0.1% TFA in CH3CN (Solvent B) at a flow rate of 1 ml/min. Gradient elution began at 10% Solvent B and increased to 60% Solvent B at a rate of 2%/min for a total run time of 30 min. A postcolumn photochemical reactor was installed between the column exit and a fluorescence detector to effect photochemical cyclization of the metabolites. The effluent was monitored by fluorescence detection (λex = 250 nm and λem = 375 nm), and six metabolites were detected at retention times between 16 and 28 min. The fluorescence detector then was disconnected, and the same HPLC conditions as above were used for purification of the metabolites, except that a semipreparative column (9.4 × 250 mm), UV detector (λ = 280 nm), and fraction collector were used, and the mobile phase was delivered at a flow rate of 3 ml/min. In addition, the volumes of urine injected were 1 ml.

**Metabolite profiles and purification of radiolabeled rofecoxib metabolites.** The HPLC system for radiocromatography was a Hewlett Packard (Palo Alto, CA) instrument (model 1050). Prepared urine and fecal samples were injected onto a Zorbax RX-C18 analytical column (4.6 × 250 mm, 40°C; Alpha Omega Technologies Inc., Brielle, NJ) and were eluted at 1 ml/min with a solvent system consisting of 0.1% aqueous TFA/NH4OH (pH 3) and CH3CN, starting at 15% CH3CN and increasing the fraction of organic phase linearly at 1%/min to 60% CH3CN, yielding a run time of 45 min. The effluent was monitored by UV detection (λ = 280 and 220 nm) and by an in-line radiochemical detector (Ramona LS-5; Raytest, Wilmington, NC) with a 3 ml/min flow rate of scintillation cocktail. Between injections, the starting solvent conditions were delivered for 5 min to re-equilibrate the column. For the purification of metabolites, an aliquot of pooled, filtered urine (0–24 h, 0.5 ml) was injected onto a Zorbax RX-C18 semipreparative column (9.4 × 250 mm). The solvent system, gradient elution, and detection systems were the same as those described above except that the flow rate was 3 ml/min. NH3OH was omitted from the aqueous mobile phase, and the mobile phase gradient was increased from 15 to 45% CH3CN to give a run time of 30 min. Fractions were collected at 1-min intervals.

Approximately 80% of the radioactivity eluted in three contiguous fractions, which were pooled and evaporated to dryness. The residues were reconstituted in 10% aqueous CH3CN and were rechromatographed on an analytical column as described above, except that the solvent system did not contain NH3OH, and the mobile phase gradient was 0.5%/min increased in CH3CN, from 20 to 40% in 40 min. Fractions collected at 0.5-min intervals, the solvent was evaporated, and the residues were stored at −20°C until analyzed by 1H NMR spectroscopy.

**1H NMR analysis.** Purified samples were dissolved in deuterated methanol (CD3OD), acetonitrile (CD3CN), or dimethyl sulfoxide (DMSO-d6), and spectra were obtained using a Varian (Palo Alto, CA) Unity spectrometer (500 MHz). Chemical shifts are reported in parts per million (δ) downfield from tetramethylsilane, and coupling constants (J) are reported in Hertz. Where applicable, the NMR spectra of isolated metabolites were compared with those of the corresponding reference compounds prepared by synthesis.

**LC-MS/MS analysis.** Aliquots (1 ml) of pooled urine (0–24 h) from six healthy volunteers who received 14C-rofecoxib were mixed with CH3CN (4 ml). After centrifugation, the supernatants were evaporated to dryness in a centrifuge evaporator (SpeedVac; Thermo Savant, Holbrook, NY), and the residues were reconstituted in 40% aqueous CH3CN (600 μl). Aliquots (60 μl) of the reconstituted urine specimens were injected into a Hewlett Packard (model 1090) HPLC instrument that was equipped with a Luna Kromasil C18 microbore column (2 × 250 mm, 100 Å; Phenomenex, Torrance, CA). The analytes were eluted with a solvent system consisting of 0.1% aqueous N-methylmorpholine and CH3CN at a flow rate of 0.2 ml/min. After an isocratic phase of 10% CH3CN for 3 min, a linear gradient commenced that increased delivery of CH3CN to 60% in 29 min. The column was washed with 90% CH3CN for 3 min and re-equilibrated at 10% CH3CN for 10 min before each injection.

The detection of 14C-labeled metabolites of rofecoxib in human urine was carried out using an IN/US β-RAM radiochromatographic HPLC detector (IN/US, Tampa, FL). For all analyses, the detector was operated in the homogeneous liquid scintillation counting mode, with a 100-μl flow cell and a flow rate of 0.8 ml/min scintillation cocktail (True-Count; IN/US).

The HPLC system then was interfaced to a Finnigan TSQ 7000 mass spectrometer (ThermoQuest, Piscataway, NJ), and mass spectral analyses were carried out using electrospray ionization in the negative ion mode. The capillary temperature was 230°C and the electrospray ionization ionizing voltage was maintained at −5.0 kV for all analyses. Tandem mass spectrometry (MS/MS) was based on collision-induced dissociation of ions entering the radiofrequency-only octapole region where argon was used as the collision gas at a pressure of 1.7 mtorr. A combination of collision offsets ranging from 20 eV to 40 eV was used for all MS/MS analyses.

**β-Glucuronidase Treatment of Urine.** From the radiolabeled rofecoxib study in healthy subjects, aliquots (2 ml) of urine (0–24 h) from each subject were placed in duplicate tubes. β-Glucuronidase (2 mg, 1000 units, Helix pomatia) in 0.2 M sodium acetate buffer (1 ml, pH 5.0) was added to one tube, while buffer alone was added to the second tube which served as a control. The samples were incubated for 22 h at 37°C in a shaking water bath, following which enzyme activity was terminated by the addition of 4 volumes of MeOH. After centrifugation, the supernatants were evaporated to dryness and the residues were reconstituted for radiochromatography as described above. para-Nitrophenol glucuronide served as a positive control for the β-glucuronidase reaction; under the conditions used, hydrolysis of this conjugate proceeded essentially to completion.

**Pharmacokinetic Methods.** Actual times of sample collection were used for all calculations. Maximum concentrations observed in plasma (Cmax) and the time of occurrence of Cmax (tmax) following oral administration were obtained by inspection. Since preclinical studies suggested that rofecoxib and 5-hydroxyrofecoxib undergo reversible biotransformation, it was anticipated that standard methods of pharmacokinetic analysis could not be used. Strictly speaking, plasma concentration-time profiles for both parent and metabolite from separate i.v. administration of each would be required under such conditions. In any event, only the administered compound was detectable in plasma samples after each i.v. administration. Thus, although concentrations of rofecoxib were measurable after i.v. administration of 5-hydroxyrofecoxib, this was the case only for a few subjects, and the data obtained were sparse enough that little useful information could be extracted. Additionally, examination of the plasma profiles following oral doses revealed that the extent of interconversion between rofecoxib and 5-hydroxyrofecoxib was modest, so that the correction to the pharmacokinetic results that would be expected from use of i.v. data on reversibility should be minor. Therefore, pharmacokinetic parameters were estimated by standard methods without correcting for interconversion between parent and metabolite.

Total areas under the plasma concentration versus time curve for oral doses were estimated by the trapezoidal method until a sample with a concentration less than the limit of quantitation was encountered. This sample was assigned a concentration of zero for calculation purposes. Since the presence of secondary peaks in the terminal phase of the plasma-concentration curve precludes estimation of the half-life of rofecoxib by the standard technique of fitting an exponential curve to this phase, the method of Kwan et al. (1984) was used to estimate an effective plasma t1/2 for rofecoxib.
activity and rofecoxib levels decreased in a parallel, exponential fashion (Fig. 1B; Table 1). These profiles, however, did not exhibit a secondary $C_{\text{max}}$, and absolute concentrations of both total radioactivity and rofecoxib were approximately 30 and 20%, respectively, of those observed in the plasma of healthy subjects. The bioavailability of rofecoxib in the cholecystectomized patients was estimated at $\sim$25% relative to that in healthy subjects. In addition, in cholecystectomy patients, only about one-third of the total radioactivity in plasma was accounted for by parent compound, whereas in healthy subjects, approximately two-thirds of the total radioactivity in plasma represented unchanged rofecoxib.

Total recovery of radioactivity in excreta of healthy subjects was 85.7% of the administered dose, of which an average of 71.5% was excreted in urine and 14.2% in feces (Table 2). In cholecystectomy patients, the total recovery of radioactivity varied widely between subjects and averaged 80.6% of dose (Table 2). In these individuals, radioactivity was recovered mostly in feces (50%), although approximately one-third was excreted in urine. Only a minute fraction of the dose was recovered in bile ($\sim$1.8%, Table 2). Recovery of ICG in bile averaged $\sim$86%, indicating efficient bile collection from these patients.

**Rofecoxib Metabolite Profiles by HPLC Analysis.** As part of a 14-day multiple-dose study in which rofecoxib was dosed at 250 mg/day to healthy subjects, the urine of one subject obtained on day 14 (0–8 h) was analyzed by HPLC. The components detected by fluorescence included the parent compound and four products believed to be drug-related, with retention times of 17.5, 18.5, 22.5, and 25.5 min (Fig. 2). These components were isolated and analyzed by $^1$H-NMR spectroscopy.

For both [14C]rofecoxib studies (i.e., those in healthy subjects and cholecystectomy patients), radiochromatographic profiles of urine and fecal samples were qualitatively similar for all subjects; representative chromatograms are shown in Fig. 3. In feces, only one component, corresponding to unchanged rofecoxib, was present, whereas in the biliary excretion study, analysis of bile samples revealed the presence of three very polar components which collectively accounted for an insignificant fraction of the dose ($<0.3%$, data not shown). Urine specimens were characterized by the absence of parent compound but revealed numerous minor components and two major, unresolved peaks.

To determine whether glucuronide conjugate(s) were present in urine, an aliquot was treated with $\beta$-glucuronidase (Fig. 4), which led to a 6 to 11% increase in the area of the peak at 24 min, with a proportional decrease in area of the major, unresolved peaks that eluted between 15 and 17 min (Fig. 3A). The unresolved peaks were collected and rechromatographed with a slower gradient in an effort to separate and purify the individual components. It was concluded that these metabolites were somewhat unstable under the conditions used.
in these studies, in that they appeared to give rise to two new components that eluted just prior to the parent compound (Fig. 5). All four of these components were collected and submitted for structural characterization by $^1$H NMR analysis.

Identification of Rofecoxib Metabolites by $^1$H NMR Spectroscopy. unlabeled Rofecoxib Study. $^1$H NMR analysis was carried out on rofecoxib metabolites purified from human urine. Salient details of their spectra are presented in Table 3. In the NMR spectrum of the HPLC component eluting at 17.5 min (Fig. 2), the resonances associated with the unsubstituted aromatic ring were absent, and three new signals (at 6.20, 5.85, and 5.54 ppm) appeared that were consistent with a conjugated diene system. In addition, a signal at 4.39 ppm, integrating for two hydrogens with a vicinal coupling constant of 11.3 Hz, pointed to the presence of a CH-(OH) system in a trans conformation. Taken together, these data indicated that the component eluting at 17.5 min was trans-rofecoxib-$3^4$-dihydrodiol.

The HPLC component eluting at 18.5 min (Fig. 2) yielded an NMR spectrum indicating that the metabolite was rofecoxib-$4^\prime$-$O$-$\beta$-$D$-glucuronide. The presence of two aromatic systems with an AA$XX$ pattern demonstrated that metabolism had occurred at the $4^\prime$ position of rofecoxib. The anomic proton resonated as a doublet at 4.85 ppm with a coupling constant of 7.3 Hz, indicating a $\beta$-configuration. In addition, signals were observed between 3.36 and 3.09 ppm, corresponding to protons of the glucuronosyl CH-(OH) moiety ($H_g$ and $H_h$).

The NMR spectrum derived from the split peak at 22.5 min (Fig. 2) indicated a mixture of glucuronide conjugates. Since the resonance for the C-H moiety adjacent to the furanone ring oxygen ($H_o$) integrated for only one proton and was shifted downfield relative to the corresponding signal in the parent compound, this metabolite was identified as 5-hydroxyrofecoxib-$O$-$D$-glucuronide. The NMR spectra of the early-eluting, minor component and the later-eluting, major component of the split peak showed that these metabolites were diastereomeric 5-hydroxyrofecoxib-$O$-$D$-glucuronide conjugates.

The HPLC component that eluted at 25.5 min (Fig. 2) yielded an NMR spectrum very similar to that of the earlier-eluting conjugate, although no signals corresponding to the glucuronide moiety were observed; thus, this metabolite was identified as free 5-hydroxyrofecoxib.

![Fig. 2. HPLC chromatogram of urine (0–8 h) collected from a healthy human subject on day 14 after oral administration of rofecoxib (250 mg/day) for 14 days.](image)

The column effluent was monitored by fluorescence detection with excitation at 250 nm and emission at 385 nm.

![Fig. 3. Radiochromatograms of: (upper panel) pooled urine (0–24 h, n = 6), and (lower panel) fecal homogenate from healthy subjects following oral administration of $^{14}$C-rofecoxib (125 mg, 100 μCi).](image)

### Table 2

<table>
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<th>Healthy Subjects</th>
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<tr>
<td>Subject</td>
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*a* Dose was 125 mg, 100 μCi.

*b* Dose was 142 mg, 100 μCi.
Thus, was identified as the furanone double bond had been reduced in this metabolite, which yielded an NMR spectrum that was similar to that of the parent compound, except that two new signals were present at 4.57 and 4.26 ppm, each integrating for one proton, which were part of a CH-CH-CH2 spin system. The aromatic (H_a, H_b, and H_c) and furanone (H_d) signals all were shifted upfield, indicating that the highly conjugated π-electron system of rofecoxib had been lost. It was concluded that the furanone double bond had been reduced in this metabolite, which thus was identified as cis-3,4-dihydrorofecoxxib. The structure and cis-stereochemistry were confirmed by direct comparison with a synthetic standard prepared by catalytic hydrogenation of rofecoxib (Nicoll-Griffith et al., 2000). A coupling constant of 8 Hz between the two vicinal protons (H_i and H_j) was consistent with the cis-configuration.

The component eluting at 26 min (Fig. 5) yielded a NMR spectrum very similar to the metabolite eluting at 24 min, except that the coupling constant between H_i and H_j was 12.4 Hz; thus, this product was identified as the corresponding trans isomer of 3,4-dihydrorofecoxxib. The structure was confirmed by comparison with a standard formed by spontaneous isomerization of the synthetic cis isomer.

NMR analysis of the metabolite eluting at 15 min (Fig. 5), which was unstable during isolation and purification, indicated the presence of the ring-opened, 3,4-dihydroxydiacid (DHHA) metabolite, together with the corresponding cis-3,4-dihydrorofecoxxib lactone, in a ratio of 2:1. 3H NMR analysis of a synthetic standard of rofecoxib-erythro-3,4-dihydroxydiacid verified the former assignment, while control experiments demonstrated that this DHHA species cyclized readily to the lactone species under mildly acidic conditions.

Finally, the NMR spectrum of the component eluting at 16 min (Fig. 5) was essentially identical to that of 5-hydroxyrofecoxxib-O-β-D-glucuronide, a major metabolite identified in the study with unlabeled rofecoxib.

LC-MS/MS Analysis of Metabolites of Rofecoxib. LC-β-RAM analysis of pooled urine from six healthy subjects who received [14C]rofecoxib showed the presence of five major peaks in the radiochromatogram (Fig. 6A). LC-MS/MS analysis of the peaks with retention times of 17.8, 27.2, 32.2, and 33.1 min yielded data that supported their identification by NMR analysis (described above) as 5-hydroxyrofecoxxib-O-β-D-glucuronide ([M – H]^− = m/z 505; minor metabolite), 5-hydroxyrofecoxxib ([M – H]^− = m/z 329; minor metabolite), cis-3,4-dihydrorofecoxxib ([M – H]^− = m/z 315; minor metabolite) and trans-3,4-dihydrorofecoxxib ([M – H]^− = m/z 315; major metabolite), respectively. The most polar peak, with a retention time of 8.1 min, which had been shown by NMR to be unstable under acidic isolation conditions, yielded an [M – H]^− ion of m/z 333, 18 Da higher than the cis- and trans-3,4-dihydrorofecoxxib lactones. An extracted ion chromatogram of m/z 333 yielded two peaks around 8 min (Fig. 6B), which were of approximately equal intensity. A product ion spectrum obtained by collision-induced dissociation of m/z 333 (Fig. 6C) suggested that addition of water to the cis- and trans-3,4-dihydrorofecoxxib lactones had taken place, yielding diastereomeric DHHA metabolites. Product ions at m/z 259 (loss of CO_2 and formaldehyde from m/z 333) and at m/z 244 (loss of a methyl group from the methylsulfonfonyl moiety) further indicated that the lactone ring had undergone hydrolysis to form DHHA. Taken together, these two peaks were identified as rofecoxxib-erythro-3,4-dihydroxydiacid and rofecoxxib-threo-3,4-dihydroxydiacid. The absolute stereochemistry of these diastereomers was not determined.

Biotransformation of 5-Hydroxyrofecoxxib to Rofecoxib. Following single oral doses (5, 10, and 25 mg) of 5-hydroxyrofecoxxib to 12 healthy subjects, the concentrations in plasma of both rofecoxxib and 5-hydroxyrofecoxxib increased as a function of time (Fig. 7), with 5-hydroxyrofecoxxib reaching an earlier t_{max} than rofecoxxib (~1 h versus ~10 h) and then declining more rapidly. The mean profiles depicted in Fig. 7 are representative of the shapes of the individual profiles. The pharmacokinetic parameters for 5-hydroxyrofecoxxib estimated following administration of 1 mg i.v. and single oral doses of 5, 10, and 25 mg are listed in Table 4. The behavior of orally administered 5-hydroxyrofecoxxib was found to be linear with dose in the range of 5 to 25 mg. Approximately 15% of the dose appeared in plasma as 5-hydroxyrofecoxxib, whereas some 20% was accounted for...
TABLE 3

\(^1\)H NMR data for rofecoxib and metabolites isolated from human urine (500 MHz, CD\(_2\)OD)

<table>
<thead>
<tr>
<th>Protons</th>
<th>Rofecoxib</th>
<th>Rofecoxib-3,4'-dihydrodiol(^b)</th>
<th>4'-Hydroxyrofecoxib-O-(\beta)-D-glucuronide(^c)</th>
<th>5-Hydroxyrofecoxib-O-(\beta)-D-glucuronide(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Major Isomer</td>
<td>Minor Isomer</td>
<td>Major Isomer</td>
<td>Minor Isomer</td>
</tr>
<tr>
<td>a</td>
<td>7.93 (AA'XX', 2H, J = 8.6)</td>
<td>7.96 (AA'XX', 2H, J = 8.5)</td>
<td>7.96 (AA'XX', 2H, J = 8.5)</td>
<td>7.87 (AA'XX', 2H, J = 8.5)</td>
</tr>
<tr>
<td>b</td>
<td>7.62 (AA'XX', 2H, J = 8.6)</td>
<td>7.66 (AA'XX', 2H, J = 8.5)</td>
<td>7.64 (AA'XX', 2H, J = 8.5)</td>
<td>7.69 (AA'XX', 2H, J = 8.5)</td>
</tr>
<tr>
<td>c</td>
<td>7.39 (m, 5H)</td>
<td>6.20 (t, 1H, J = 2.0)</td>
<td>7.27 (AA'XX', 2H, J = 8.7)</td>
<td>7.45, 7.33 (m, 5H)</td>
</tr>
<tr>
<td>c'</td>
<td>5.85 (dd, 1H, J = 9.9, 2.4)</td>
<td>5.54 (dt, 1H, J = 9.9, 2.0)</td>
<td>7.04 (AA'XX', 2H, J = 8.7)</td>
<td>7.43, 7.36 (m, 5H)</td>
</tr>
<tr>
<td>c''</td>
<td>4.39 (m, 2H, J = 11.3)</td>
<td>4.39 (m, 2H, J = 11.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d</td>
<td>5.34 (s, 2H)</td>
<td>5.17, 5.06 (AB, 2H, J = 17.3)</td>
<td>5.36 (AB, 2H, J = 17.8)</td>
<td>6.88 (s, 1H)</td>
</tr>
<tr>
<td>d'</td>
<td>3.12 (s, 3H)</td>
<td>3.08 (s, 3H)</td>
<td>3.24 (s, 3H)</td>
<td>3.24 (s, 3H)</td>
</tr>
<tr>
<td>e</td>
<td>3.12 (s, 3H)</td>
<td>3.08 (s, 3H)</td>
<td>3.24 (s, 3H)</td>
<td>3.22 (s, 3H)</td>
</tr>
<tr>
<td>f</td>
<td>3.12 (s, 3H)</td>
<td>3.08 (s, 3H)</td>
<td>3.24 (s, 3H)</td>
<td>3.22 (s, 3H)</td>
</tr>
<tr>
<td>g</td>
<td>3.12 (s, 3H)</td>
<td>3.08 (s, 3H)</td>
<td>3.24 (s, 3H)</td>
<td>3.22 (s, 3H)</td>
</tr>
<tr>
<td>h</td>
<td>3.12 (s, 3H)</td>
<td>3.08 (s, 3H)</td>
<td>3.24 (s, 3H)</td>
<td>3.22 (s, 3H)</td>
</tr>
<tr>
<td>i</td>
<td>3.12 (s, 3H)</td>
<td>3.08 (s, 3H)</td>
<td>3.24 (s, 3H)</td>
<td>3.22 (s, 3H)</td>
</tr>
<tr>
<td>j</td>
<td>3.12 (s, 3H)</td>
<td>3.08 (s, 3H)</td>
<td>3.24 (s, 3H)</td>
<td>3.22 (s, 3H)</td>
</tr>
</tbody>
</table>

\(^a\) Splitting patterns: s = singlet, d = doublet, t = triplet, dd = doublet of doublets, dt = doublet of triplet, m = multiplet, AA'XX' = multiplet, characteristic of para-substituted benzene ring. Coupling constants (J) are expressed in Hz.

\(^b\) Spectrum recorded in CD\(_2\)OD.

\(^c\) Metabolite dissolved in DMSO-d\(_6\).

\(^d\) Data reported for protons H\(_a\), H\(_b\), and H\(_j\) were obtained from analysis of synthetic standard; these signals in the human urine sample were obscured by impurities.
as rofecoxib (based on oral versus i.v. administration). Although the extent of conversion of 5-hydroxyrofecoxib to rofecoxib was determined to be only ~20%, concentrations of 5-hydroxyrofecoxib in plasma were observed to be lower than those of rofecoxib, since the plasma clearance of 5-hydroxyrofecoxib is about 8 times greater than that of rofecoxib (estimated from separate i.v. administration). Interestingly, following a single oral administration of rofecoxib (50 mg), only a minute amount of 5-hydroxyrofecoxib (~4% of the dose) was detected in plasma throughout the sampling period (Fig. 8).

**Discussion**

The results of the present study indicate that rofecoxib is extensively absorbed following oral administration to human subjects and that the bulk of the dose undergoes metabolism to products that are eliminated via the kidneys into the urine. Although a small fraction (~14%) of the administered radioactivity was recovered in feces, the (eliminated via the kidneys into the urine. Although a small fraction that the bulk of the dose undergoes metabolism to products that are sively absorbed following oral administration to human subjects and detected in plasma throughout the sampling period (Fig. 8).

For samples that were below the limit of quantitation (5 ng/ml), a value of zero was used in determining the mean. The mean profiles presented here are representative of individual profiles.

The structures of the metabolites of rofecoxib identified in human urine are depicted in Fig. 9, and reflect the operation of both oxidative and reductive pathways of biotransformation, together with hydrolysis and conjugation. Oxidation of the phenyl ring gave rise to small amounts of the 4'-phenol (excreted as its glucuronide conjugate) and the 3'4'-dihydriodiol. However, based upon radiochromatographic profiles of urine specimens, the products of reductive metabolism of the 2-furanone ring, which were excreted as the ring-opened hydroxy acid derivatives erythro- and threo-DHHA, represented the major metabolites of rofecoxib in humans, while the corresponding cis- and trans-lactones were observed upon exposure of urine extracts to mineral acid. Reduction of the 3,4-carbon-carbon double bond of rofecoxib, which theoretically could occur either before or after hydrolytic opening of the 2-furanone moiety, probably reflects the presence of an α, β-unsaturated carbonyl functionality in the parent structure, since examples of similar C=C reductions have been reported for other conjugated carbonyl groups (Cook et al., 1974; Jauch et al., 1975). With the aid of oxygen-18 labeled variants of rofecoxib, recent studies in rats demonstrated that the 2-furanone ring of the drug is subject to rapid, reversible ring-opening in vivo (Baillie et al., 2001). Thus, if a similar process operates in humans, it seems likely that hydrolysis of the 2-furanone precedes reduction to DHHA, the erythro isomer of which epimerizes spontaneously to the thermodynamically more stable threo form.

In contrast to the rat, where 5-hydroxylation is the major pathway of rofecoxib metabolism (Halpin et al., 2000), relatively small amounts of 5-hydroxyrofecoxib and its glucuronide conjugate were
excreted in the urine or feces of humans. This observation is sig-
ificant in light of the fact that 5-hydroxyrofecoxib can be reduced back
in vivo (Baillie et al., 2001). Indeed, the glucuro-
nide conjugate of 5-hydroxyrofecoxib has been implicated in the
enterohepatic recycling of rofecoxib in rats (Baillie et al., 2001), and
it was speculated that a similar enterohepatic cycling in humans might
contribute to the secondary peaks, which are evident in the plasma
concentration versus time profiles for rofecoxib in humans. However,
this appears not to be the case, based on the very small fraction (<2%) of
a radiolabeled dose of the drug which was recovered from the bile of
cholecystectomy patients examined in this study. Moreover, follow-
oral administration of 5-hydroxyrofecoxib itself to healthy
subjects, a relatively small fraction of the dose, estimated at no more
than 20%, underwent conversion to rofecoxib. Thus, although clear-
ance of rofecoxib in humans depends largely upon metabolism, it is
evident that hydrolysis and reduction of the 2-furanone moiety, rather
than oxidative events, dominate the overall process. It may be antic-
ipated, therefore, that rofecoxib will exhibit a relatively low propen-
sity to interact with coadministered therapeutic agents that undergo
metabolism by cytochrome P-450-mediated pathways, and clinical
experience to date with rofecoxib is consistent with this prediction
(Schwartz et al., 1998).

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tance in preparing the manuscript.

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TABLE 4
Pharmacokinetic parameters of 5-hydroxyrofecoxib determined following administration of a 1-mg i.v. dose and single oral doses of 5, 10, and 25 mg

<table>
<thead>
<tr>
<th>Route of Administration</th>
<th>Dose (mg)</th>
<th>AUC (ng h/ml)</th>
<th>Cmax (ng/ml)</th>
<th>tmax (h)</th>
<th>t1/2 (h)</th>
<th>Cl (ml/min)</th>
<th>Bioavailability</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.v.</td>
<td>1</td>
<td>18.7 (4.5)</td>
<td>1.3</td>
<td>843 (213)</td>
<td>843 (213)</td>
<td>0.149</td>
<td></td>
</tr>
<tr>
<td>Oral</td>
<td>5</td>
<td>5.9 (3.1)</td>
<td>3.6 (1.3)</td>
<td>0.9</td>
<td>4.0</td>
<td>0.149</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>29.9 (9.4)</td>
<td>4.5 (1.5)</td>
<td>1.6</td>
<td>3.3</td>
<td>0.149</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>68.2 (12.1)</td>
<td>14.4 (6.3)</td>
<td>1.0</td>
<td>3.3</td>
<td>0.149</td>
<td></td>
</tr>
</tbody>
</table>

*Median.

†Harmonic mean.

For samples that were below the limit of quantitation (5 ng/ml), a value of zero
was used in determining the mean.

Absolute stereochemistry was not determined for the dihydrohydroxy acid (DHHA) metabolites or for their corresponding cis- and trans-3,4-dihydrorofecoxib lactones.


