Metabolic Disposition of ^{14}C-Bromfenac in Healthy Male Volunteers

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The metabolic disposition of ^{14}C-bromfenac, an orally active, potent, nonsteroidal, nonnarcotic, analgesic agent was investigated in six healthy male subjects after a single oral 50-mg dose. The absorption of radioactivity was rapid, producing a mean maximum plasma concentration (C_{max}) of 4.9 ± 1.8 μg·equiv/mL, which was reached 1.0 ± 0.5 hours after administration. Unchanged drug was the major component found in plasma, and no major metabolites were detected in the plasma. Total radioactivity recovered over a 4-day period from four of the six subjects averaged 82.5% and 13.2% of the dose in the urine and feces, respectively. Excretion into urine was rapid; most of the radioactivity was excreted during the first 8 hours. Five radioactive chromatographic peaks, a cyclic amide and four polar metabolites, were detected in 0- to 24-hour urine samples. Similarity of metabolite profiles between humans and cynomolgus monkeys permitted use of this animal model to generate samples after a high dose for structure elucidation. Liquid chromatography/mass spectrometry (LC/MS) analysis of monkey urine samples indicated that the four polar metabolites were two pairs of diastereoisomeric glucuronides whose molecular weight differed by two daltons. Enzyme hydrolysis, cochromatography, and LC/MS experiments resulted in the identification of a hydroxylated cyclic amide as one of the aglycones, which formed a pair of diastereoisomeric glucuronides after conjugation. Data also suggested that a dihydroxy-cyclic amide formed by the reduction of the ketone group that joins the phenyl rings formed the second pair of diastereoisomeric glucuronides. Further, incubation of various reference standards in control (blank) urine and buffer with and without creatinine indicated that the hydroxy cyclic amide released from enzyme hydrolysis can undergo ex vivo *transformations to a condensation product between creatinine and an α-keto acid derivative of the hydroxy cyclic amide that is formed by oxidation and ring opening. Further experiments with a dihydroxylated cyclic amide after reduction of the keto function indicated that it too can form a creatinine conjugate.*

Journal of Clinical Pharmacology, 1998;38:744–752

Bromfenac is a phenylacetic acid derivative (Figure 1). It is an orally active, nonsteroidal, nonnarcotic, analgesic agent possessing antiinflammatory and antipyretic properties.[1–7] In animal models of pain, bromfenac was more potent as an analgesic agent than indomethacin, piroxicam, zomepirac, and suprofen.[1] Bromfenac demonstrated a rapid onset of analgesic activity (10 minutes) and a duration of action longer than that obtained with piroxicam, a once-a-day nonsteroidal antiinflammatory drug (NSAID), or suprofen.[1–7] The mechanism of action of this compound appears to be primarily related to the inhibition of prostaglandin synthesis.

Animal studies indicated that bromfenac was rapidly and widely distributed into tissues, with no appreciable accumulation in any tissue other than the organs of elimination and excretion.[1] Studies in rats and mice have shown a relationship between the pharmacologic activity of bromfenac and its concentration in plasma after oral administration. Drug levels in plasma of approximately 0.20 ± 0.50 μg/mL or higher have been associated with significant analgesic activity regardless of the route of administration.[1]

The pharmacokinetics of bromfenac in humans have been reported previously.[8–13] The drug is absorbed rapidly after oral administration in the fasted state. Maximum plasma concentrations (C_{max}) of unchanged drug after single oral doses of 25, 50, and 75 mg occurred less than 1 hour after administration. The apparent elimination half-life in plasma ($t_{½}$) was reported to be <1.5 hours. This agent exhibits a long duration of analgesic activity despite a short $t_{½}$. In this paper, we describe excretion and metabolite patterns of ^{14}C-bromfenac after oral administration to healthy human volunteers.

From Wyeth-Ayerst Research, Princeton, New Jersey. Submitted for publication November 11, 1997; accepted in revised form April 2, 1998.
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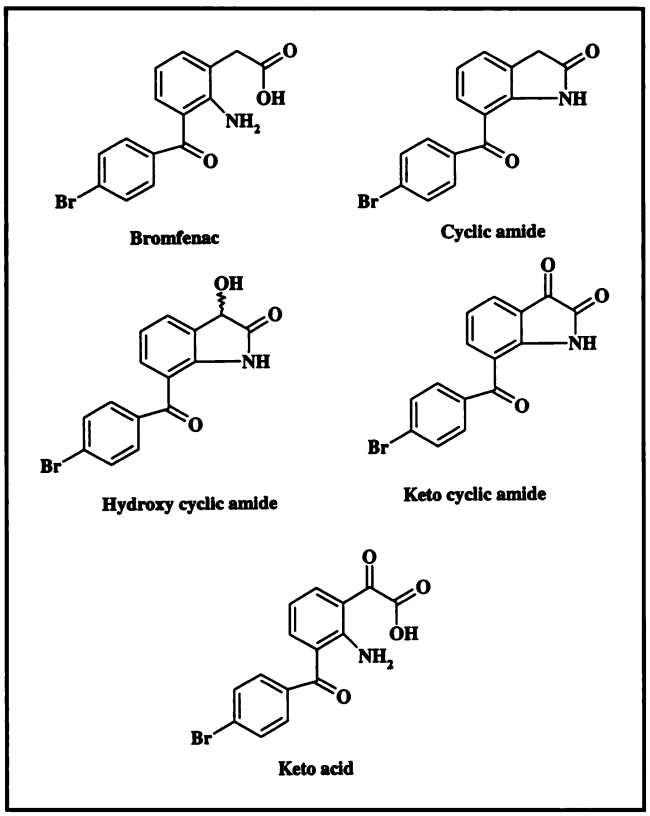

Figure 1. Chemical structures of bromfenac and other reference standards used in this study.

SUBJECTS AND METHODS

Chemicals and Reagents

^{14}C-Bromfenac [2-amino-3-(4-bromobenzoyl)-benzene acetic acid], as the sodium salt (lot #CFQ6438), was purchased from Amersham International, Buckinghamshire, England. The compound was labeled at the carbon bridging the two phenyl groups (Figure 1). Unlabeled bromfenac sodium (batch #142966A) was obtained from Wyeth-Ayerst Research (Rouses Point, NY). The reference metabolite standards 7-(4-(bromobenzoyl)-1,3-dihydro-2H-indole-2-one (cyclic amide), α-hydroxy-7-(4-(bromobenzoyl)-1,3-dihydro-2H-indole-2-one (α-hydroxy cyclic amide), α-keto-7-(4-bromobenzoyl)-1,3-dihydro-2H-indole-2-one (α-keto cyclic amide), and 2-amino-4-bromobenzoyl-benzene α-keto acetic acid (α-keto acid) were synthesized in-house (Figure 1).

^{14}C-Labeled cyclic amide was prepared by treating ^{14}C-bromfenac with acid. Briefly, ^{14}C-bromfenac (5.3 mg) and unlabeled bromfenac (118.1 mg) were dissolved in 150 mL of high-performance liquid chromatography (HPLC) grade water, to which 150 mL of 1*N* HCl solution was added. This solution was then heated for 1 hour at 45°C while stirring. Sufficient sodium carbonate was then added to raise the pH to 8. The cyclic amide was extracted from the solution with methylene chloride (3 × 50 mL), dried over anhydrous magnesium sulfate (10 g), and evaporated under nitrogen. The radiochemical purity of ^{14}C-bromfenac and ^{14}C-cyclic amide was determined to be >97% by thin layer chromatography (TLC) and HPLC.

Glusulase (*Helix pomatia*) with β-glucuronidase (416,800 units/g) and sulfatase activities (15,400 units/g) was obtained from Sigma Chemical Company (St. Louis, MO). Creatinine was obtained from Aldrich Chemical Company. Uridine-5′-diphospho (UDP)-glucuronic acid, saccharolactone, and uridine-5′-diphosphoglucuronyl transferase were purchased from Sigma Chemical Company. All other chemicals were HPLC grade from commercial sources.

Clinical Study

The clinical portion of the study was conducted by Dr. P. Leese at Quincy Research Center, Kansas City, Missouri, and was approved by the Quincy Research Center Review Board. Six men (18 to 42 years old, 60 to 85 kg) participated in this study. The general health of the volunteers was assessed before the study by medical history and physical examination, and found to be good. The subjects provided written informed consent before participating in the study.

After an overnight fast, the six volunteers were given orally 50 mg of bromfenac sodium containing 50 μCi of ^{14}C-bromfenac in capsules. No concomitant medications were given during the study. Participants drank 240 mL of water and were allowed to eat 4 hours after dose administration.

Venous blood samples (10 mL) were collected before (0 hours) and 0.25, 0.50, 0.75, 1, 2, 3, 4, 6, 8, 12, 24, 48, 72, and 96 hours after dose administration. An aliquot (3 mL) of each blood sample was removed for radioactivity analysis. The remaining blood was centrifuged and the plasma transferred to screw capped plastic tubes and frozen. Urine samples were collected for the intervals from 0 to 2, 2 to 4, 4 to 8, 8 to 12, 12 to 24, 24 to 48, 48 to 72, and 72 to 96 hours after dose administration. The urine samples were divided into two equal volumes for each time point. One volume was frozen as collected; to the other volume, 1 mol/L citric acid was added at 1.5 mL of acid solution per 100 mL of urine to stabilize, if any, acyl glucuronide conjugates. Fecal samples were collected for 96 hours after administration.

All specimens were stored frozen at −20°C and packed in dry ice before shipment to the Wyeth-Ayerst Research facility in Princeton, New Jersey.

All samples were kept frozen at −20°C until analysis.

Animal Studies

To assist in the determination of metabolic pathways of bromfenac, three animal studies were conducted. Three male cynomolgus monkeys (weight range 4 to 7 kg) from the in-house colony at Wyeth-Ayerst were used in the first study. All animals were fasted overnight and for 4 hours after drug administration. On the day of drug administration, appropriate amounts of labeled and unlabeled ^{14}C-bromfenac sodium were combined and dissolved in HPLC grade water. The monkeys were each given 3 mg/kg (50 μCi/kg) of dosing solution by nasogastric intubation. Dose volumes were based on the 24-hour predose body weight of the monkeys and a desired dose volume of 1 mL/kg. The animals were housed individually in stainless steel metabolism cages and maintained under a normal day/night cycle. Water was available ad libitum and food was returned 4 hours after drug administration. Blood was obtained before (0 hours) and 0.5, 2, 4, and 8 hours after administration. Urine was collected on citric acid (25 mL, 1 mol/L) and kept on wet ice for the periods from −12 and 0 hours before and 0 to 12 and 12 to 24 hours after administration. Daily urine collections were made on days 2 through 7 at ambient temperature.

In the second study, three female cynomolgus monkeys (weight range 3 to 5 kg) were each given 3 mg/kg of the ^{14}C-labeled cyclic amide intragastrically. Drug administration, animal housing, and other procedures used in this study were similar to ones used in the first study. The dosing compound was dissolved in a vehicle consisting of 32% polyethylene glycol-400, 36% dimethylacetamide, and 32% HPLC grade water. The dosing solution was made to a final concentration of 3 mg/mL and 12.5 μCi/mL. After an overnight fast, each animal was given 1 mL/kg and approximately 50 μCi of the radioactive dose. Blood was collected before (0 hours) and 0.5, 2.0, and 12.0 hours after administration. Urine was collected on 25 mL of 1 mol/L citric acid for the periods from 0 to 4, 4 to 8, 8 to 12, 12 to 24, and 24 to 48 hours after administration on wet ice.

In the third study, three female cynomolgus monkeys (weight range 3 to 5 kg) were given 30 mg/kg of bromfenac sodium orally after an overnight fast. On the day of drug administration, bromfenac sodium was weighed and dissolved in HPLC grade water to attain a concentration of 30 mg/mL. Each animal received 1 mL/kg of the dose. Urine for the period from 0 to 24 hours after administration was collected on citric acid (1 mol/L, 10 mL in each collection container). These samples were used for structure elucidation of the urinary metabolites by liquid chromatography/mass spectrometry (LC/MS).

Measurement of Radioactivity

Whole blood and plasma. Duplicate aliquots of whole blood were solubilized in 1 mL of a 1:1 mixture of Soluene 350 (Packard, Downeys Grove, IL) and isopropyl alcohol (EM Science, Gibbstown, NJ). The solution was allowed to stand at ambient temperature overnight to complete digestion. Samples were then decolorized using 0.5 mL of a 30% solution of hydrogen peroxide (EM Science) and allowing the samples to set for 1 hour at ambient temperature. Hionic Fluor (Packard) was added to each sample (10 mL) and the concentration of radioactivity was determined with a Packard 4640 liquid scintillation counter. Plasma samples were radioassayed in triplicate. Plasma (1 mL) was added to 15 mL of Aquasol-2 (New England Nuclear, Boston, MA).

Urine. The volume of the urine samples was measured using graduated cylinders. Triplicate urine samples (1 mL) were diluted in 15 mL of Aquasol 2 (New England Nuclear) and radioassayed.

Fecal samples. Feces were placed in a volume of water equal to approximately three times the sample weight. The samples were allowed to set in the water 24 hours and then homogenized using a Stomacher Lab Blender Model 3500 (Tekmar, Cincinnati, OH). After homogenization, aliquots (30 to 324 mg) were placed in scintillation vials (all samples were aliquoted in triplicate) and digested in 1 mL Soluene 350 (Packard Instrument Company, Meridan, CT). After standing at room temperature for 24 hours, 0.5 mL of isopropyl alcohol (EM Science) was added to each sample. The samples were then decolorized using 0.2 mL of 30% hydrogen peroxide solution (EM Science) and allowing the samples to stand overnight at ambient temperature to complete the decolorizing process. The samples were then diluted in 15 mL of Hionic Fluor (Packard Instrument Company) and radioassayed in a Tri-Carb 4640 liquid scintillation counter (Packard).

Creatinine Analysis in the Urine

Creatinine was measured in the urine samples from subjects 1 and 5 with an A-GENT diagnostic kit (Abbott Laboratories, Abbott Park, IL). The concentration of creatinine was compared in the samples treated with citric acid versus those not treated with citric acid. These samples were analyzed for creatinine concentration due to the unusually high or low overall recovery of radioactivity for these two subjects.

Metabolite Profiles in Plasma

Plasma profiles were determined by fraction collecting the HPLC effluent. The plasma proteins were first precipitated by adding acetonitrile (1 mL) to 1 mL of plasma. The samples were then filtered through 0.2-μm centrifuge filters (Rainin, Woburn, MA) by centrifuging for 15 minutes at 800 × *g*. Total radioactivity concentrations were measured after protein precipitation; all of the radioactivity was recovered after filtration. The filtrate was then chromatographed using an HPLC method. Sample injection volume was 500 μL. The mobile phase was a linear gradient from 10% to 45% acetonitrile in 0.1 mol/L ammonium acetate (pH 4.9) over 50 minutes, then held at 45% organic for an additional 10 minutes, giving a total run time of 60 minutes. The column (HiChrom, 15 cm × 4.6 mm, 5 μ; Regis, Morton Grove, IL) was reequilibrated for at least 15 minutes between injections. The flow rate for the system was 1 mL/min. The HPLC system comprised two Rabbit HPX pumps (Rainin), a Spectra-physics autosampler, and a Perkin-Elmer (model 235) diode array detector (Norwalk, CT). The wavelength used for detection was 270 nm.

Urinary Metabolite Patterns

Urinary metabolite profiles were determined by direct injection of urine samples onto an HPLC column. Hydrolysis of the conjugates was accomplished by incubating the urine samples (1 mL) at 37°C for 16 hours after addition of 1 mL of 0.1 mol/L ammonium acetate buffer (pH 5) and 100 μL of Glusulase (40,000 units). The samples were then centrifuged at 800 × *g* for 15 minutes and the supernatant was analyzed by HPLC as described for plasma.

Liquid Chromatography/Mass Spectrometry

Representative samples of nonhydrolyzed and hydrolyzed urine, and reference standards were analyzed by LC/MS to confirm the identity of metabolites. The HPLC conditions used were similar to those described above, with the exception of narrow-bore column (2 mm ID) for LC/MS analysis. A Hewlett Packard 1090 liquid chromatograph (Wilmington, DE) was used as the solvent delivery system. Separation was achieved by a 2 mm × 250 mm C18 DB column (Supelco, Bellefonte, PA) with gradient elution as described above. The flow rate was 200 μL/min. The LC eluant was transferred to a Finnigan-MAT TSQ 700 mass spectrometer (San Jose, CA) via fused silica capillary ending. The eluent was ionized by electrospray ionization (ESI) operated in the negative ion mode. The electrospray needle was set to −4.5 kV, the heated capillary was set to 200°C. For MS/MS experiments, the resolution of the precursor and product ions were 4 m/z units and 1 m/z units, respectively. The collision gas was 2 mtorr of argon, the collision energy was 25 eV.

In Vitro Synthesis of Glucuronides of Hydroxy Cyclic Amide

In vitro synthesis of the glucuronides of hydroxy cyclic amide was accomplished by modifying a procedure from Sigma Chemical Company. Briefly, to 7 mL of sodium phosphate buffer (pH 8; 0.5 mol/L) was added 10 mg of UDP-glucuronic acid, 0.5 g UDP-glucuronyl transferase, and 500 μg of cyclic amide. The mixture was incubated at 37°C in a shaking water bath for 15 minutes. After incubation, the reaction mixture was centrifuged at 9,000 × *g* for 15 minutes and an aliquot (200 μL) of the supernatant was analyzed by HPLC using diode array detection.

HPLC Separation of Enantiomers of Hydroxy Cyclic Amide

Separation of the enantiomers of racemic hydroxy cyclic amide reference standard was accomplished using a Chiral Pak-AS (10 μm, 4.6 × 250 mm) HPLC column. The mobile phase was isocratic and consisted of ethanol and hexane in a 50:50 ratio. The flow rate was 0.8 mL/min, and the injection volume was 15 μL. The column temperature was maintained at 23°C and the effluent was monitored using a diode array detector at a wavelength of 260 nm.

In Vitro Formation of Creatinine Conjugates

Because of the suspected *ex vivo* formation of creatinine conjugates in urine samples, the following experiments were conducted. In the first experiment, hydroxy cyclic amide, α-keto cyclic amide, and α-keto acid were incubated with control urine samples at 37°C for 16 hours. In the second experiment, each of these reference standards was incubated in 0.5 mol/L ammonium acetate buffer (pH 5) with and without 1 mol/L creatinine at 37°C for 16 hours. After incubation, an aliquot of each sample was analyzed by HPLC with ultraviolet (UV) detection and LC/MS. In the third experiment, the hydroxy cyclic amide was reduced by stirring 1.7 mg of the standard in 5 mL of tetrahydrofuran and 0.22 mg of sodium borohydride for 8 hours at room temperature. After removing the solvent, the reaction product was incubated in buffer with and without creatinine to produce the ketone reduced creatinine conjugate of the hydroxy cyclic amide.

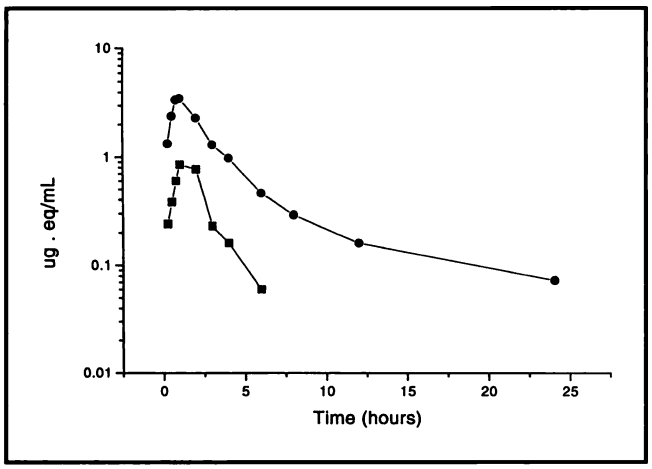

Figure 2. Mean concentrations of total radioactivity in plasma (●) and whole blood (■) after a single 50-mg (50 μCi) oral dose of ^{14}C-bromfenac to six male subjects.

Pharmacokinetic Calculations

Values for C_{max}, time to C_{max} (t_{max}), and points selected for the determination of the terminal elimination phase were determined by visual inspection of total radioactivity concentrations in plasma. Other pharmacokinetic parameters, such as $t_{1/2}$ and area under the concentration–time curve extrapolated to infinity ($AUC_{0-\infty}$), were calculated by noncompartmental methods using the LAGRAN program.[14] Because of the small sample size (n = 6), no formal statistical analysis was performed. Data are reported as mean ± standard deviation (SD).

RESULTS

Plasma and Whole Blood Radioactivity

The mean concentrations of radioactivity in whole blood and plasma for six subjects after administration of ^{14}C-bromfenac are presented in Figure 2. Mean ± SD pharmacokinetic parameters of radioactivity in plasma for all six subjects are shown in Table I.

^{14}C-Bromfenac was rapidly absorbed, with a t_{max} of 1.0 ± 0.5 hours. The average C_{max} for total radioactivity was 4.9 ± 1.8 μg·equiv/mL. The elimination of radioactivity from plasma was rapid. The 24-hour time point after administration was the last time point at which radioactivity was observed at measurable concentrations. The average was $t_{1/2}$ 4.5 ± 0.6 hours. The ratios of whole blood to plasma radioactivity concentrations were between 0.2 to 0.3 during the collection period, indicating no uptake into erythrocytes.

Table I Pharmacokinetic Parameters of Radioactivity in Plasma after Oral Administration of 50 mg ^{14}C-Bromfenac to Humans

Pharmacokinetic Parameters	Mean ± SD
C_{max} (μg · equiv/mL)	4.8 ± 1.8
t_{max} (hrs)	1.0 ± 0.5
AUC (μg · equiv · hr/mL)	12.5 ± 2.4
$t_{1/2}$ (hrs)	4.5 ± 0.6

C_{max}, maximum plasma concentration; t_{max}, time to C_{max}; AUC, area under the concentration–time curve; $t_{1/2}$, elimination half-life.

Excretion

Mean percent recovery of radioactivity in the excreta of four subjects (subjects 2, 3, 4, and 6) after administration of ^{14}C-bromfenac as the sodium salt is shown in Table II. The excretion of radioactivity into the urine was low (48.9%) in one subject (subject 1) and high (105.1%) in one subject (subject 5). In these subjects, the creatinine and radioactivity concentrations for some of the urine samples treated with citric acid and those not treated with citric acid were dissimilar, indicating that samples were not adequately mixed before splitting or that an error was made during sample handling. Accordingly, data from subjects 1 and 5 were not included in the determination of the mean values.

After administration of ^{14}C-bromfenac, an average of 82.4% and 13.2% of dose was recovered in the urine and feces, respectively, for a mean of 95.6% over the 4-day collection period. The majority of excretion (66.6%) occurred in the first 8 hours (Figure 3). The majority of dose (91.9%) was recovered by 24 hours after administration.

Table II Recovery of Radioactivity in Excreta after a 50-μCi Dose of ^{14}C-Bromfenac to Healthy Volunteers

Time after Administration (hrs)	Mean Percent of Dose Recovered	
	Urine	*Feces*
0–2	23.3	—
2–4	26.1	—
4–8	17.1	—
8–12	5.5	—
12–24	6.6	1.5
24–48	2.9	4.5
48–72	0.6	7.8
72–96	0.2	0.5
Total*	82.4	13.2

* Total recovery in urine and feces was 95.6%.

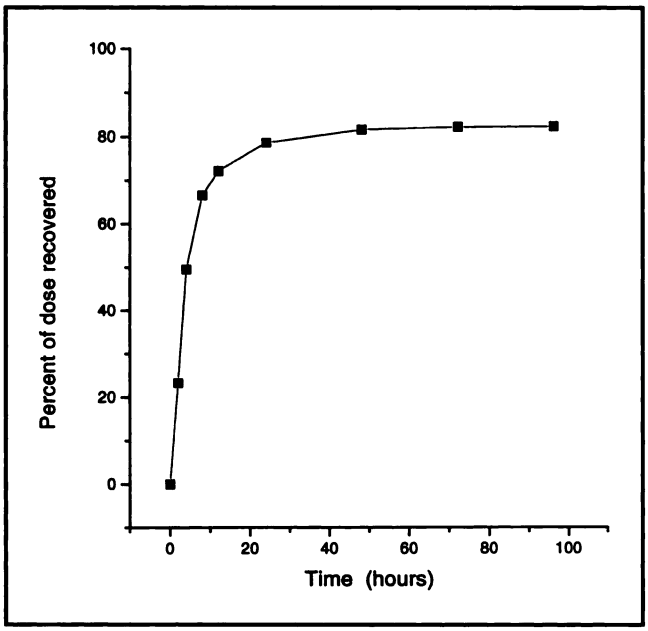

Figure 3. Urinary excretion of radioactivity after a 50-μCi dose of ^{14}C-bromfenac to healthy volunteers.

Plasma Metabolite Profiles

Plasma metabolite patterns were examined 1, 2, 3, and 4 hours after administration of ^{14}C-bromfenac, because these samples contained the highest concentration of radioactivity. After 4 hours, plasma radioactivity concentrations were too low to permit metabolite profiling with radiodetection. In all subjects, bromfenac was essentially the only component detected in plasma through 4 hours after dose administration. The plasma metabolite profile of a sample taken 1 hour after administration in subject 2 is shown in Figure 4 as an example.

Urinary Metabolite Profiles

The patterns of bromfenac metabolites were examined in the citric acid treated urine samples obtained for each subject during the periods from 0 to 2, 2 to 4, 4 to 8, and 8 to 12 hours after administration of ^{14}C-bromfenac sodium. A representative radiochromatogram showing the metabolite profiles is displayed in Figure 5. Five major peaks were detected in these samples. Four of the peaks (peaks 1–4) were more polar than bromfenac and the remaining peak (peak 5) eluted later than bromfenac at the retention time of the cyclic amide of bromfenac. The first four peaks eluted as two pairs of peaks; the second peak of each pair (peaks 2 and 4) eluted closely to and was always smaller than the first peak. Urine samples collected from 0 to 2 and 2 to 4 hours after administration contained cyclic amide as the major metabolite. At 4 to 8 and 8 to 12 hours, peak 5 (cyclic amide) was negligible and the major metabolites were peaks 1 through 4. In the nonacidified samples, the first four peaks were smaller than those in acidified urine, due to instability of the metabolites at neutral pH. Further metabolite identification experiments were performed only with the acidified urine.

On enzyme hydrolysis, the polar metabolites (the first four peaks) either disappeared completely or diminished in size. A chromatographic peak corresponding to the retention time of cyclic amide and two new peaks were seen in the hydrolyzed samples. No change in the profile was observed in the presence of d-saccharic acid, 1,4-lactone, demonstrating that the conjugates are glucuronides.

Structure Elucidation of Metabolites

Urinary metabolite profiles in monkeys given 3 mg/kg ^{14}C-bromfenac were similar to those in human samples. The five major metabolite peaks whose retention times matched those of the metabolites in human samples were observed. When monkeys received 3 mg/kg of ^{14}C-cyclic amide, the cyclic amide was the only major radioactive peak present in plasma samples, and five major urinary metabolite peaks, whose retention times matched those obtained after administration of ^{14}C-bromfenac, were observed. Urine samples were then collected for LC/MS analysis from cynomolgus monkeys receiv-

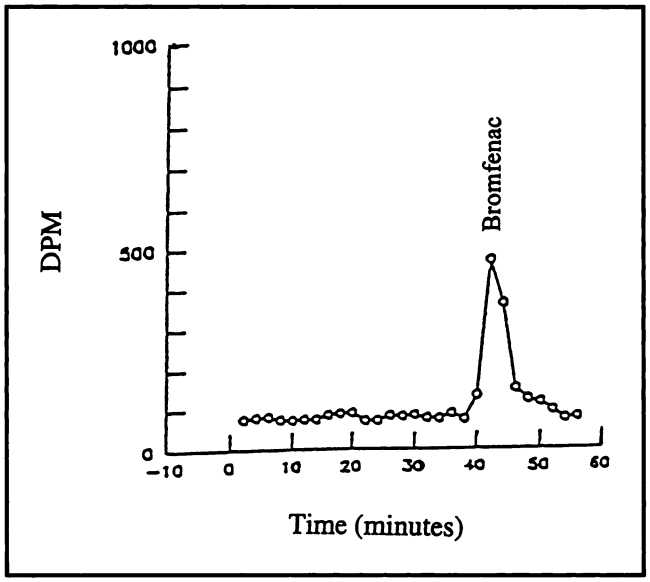

Figure 4. High-performance liquid chromatographic (HPLC) profiles of radioactivity in plasma at one hour for a subject receiving a 50-mg (50 μCi) dose of ^{14}C-bromfenac. DPM, disintigration per minute.

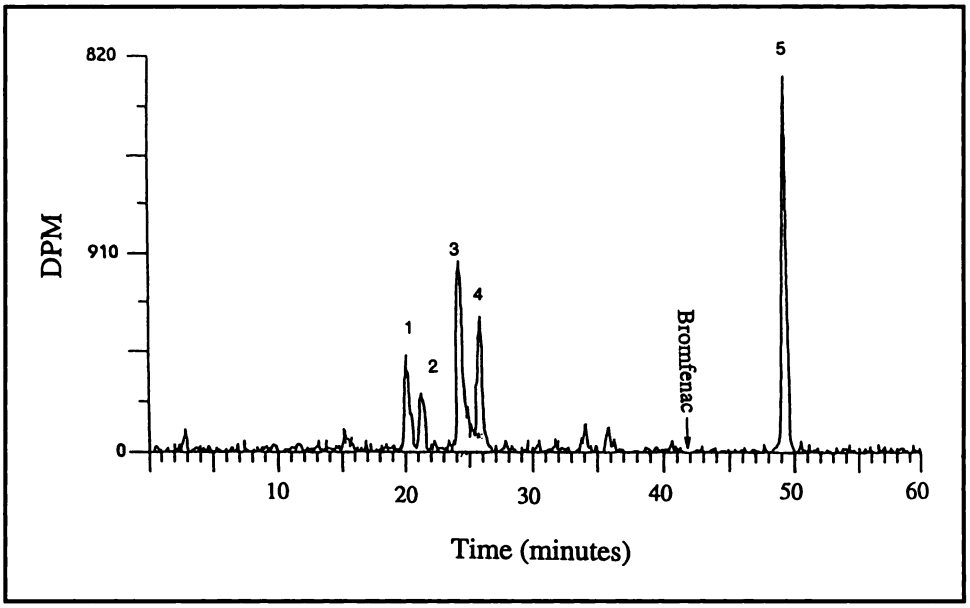

Figure 5. High-performance liquid chromatographic (HPLC) profiles of radioactivity in urine from 0 to 2 hours in a subject receiving a 50-mg (50 μCi) dose of ^{14}C-bromfenac. DPM, disintigration per minute.

ing a high dose (30 mg/kg) of unlabeled bromfenac, because these samples contained high concentrations of the same metabolites that were seen after a low dose of ^{14}C-bromfenac.

Cochromatography, matching (UV) spectra from diode array detection, and on-line LC/MS techniques were used to elucidate the structures of urinary metabolites. Results from electrospray LC/MS analysis of a monkey urine sample indicated that the four polar metabolites (peaks 1–4) were two pairs of isomers whose molecular weights were 508 and 510 daltons. The two pairs of isomers were confirmed to be glucuronide conjugates by the presence of an ion at m/z 193 in LC/MS/MS experiments. The LC/MS/MS data also suggested that the molecular weights of the aglycones were 332 and 334. Attempts were made to isolate the glucuronides for complete structure elucidations. However, these attempts were unsuccessful due to instability of the metabolites.

Cochromatography with reference standard and LC/MS/MS experiments confirmed that one of the peaks in the chromatograms of enzyme hydrolyzed samples was hydroxy cyclic amide. Because the hydroxy cyclic amide contains a chiral center, conjugation with glucuronic acid could produce a diastereomeric pair of conjugates. When the synthetic reference standard of hydroxy cyclic amide was subjected to chiral analysis, two peaks with identical UV spectra were produced, confirming the diastereoisomeric nature of this aglycone. When the synthetic hydroxy cyclic amide reference standard was incubated with UDP-glucuronyl transferase and necessary cofactors, the formation of two glucuronides with identical retention times as those of peaks 3 and 4 occurred. The UV spectra of these two synthetic glucuronides were identical to each other and to those of peaks 3 and 4, confirming that peaks 3 and 4 were two diastereomeric glucuronide conjugates of hydroxy cyclic amide.

On hydrolysis by Glusulase, the urine sample contained two bromfenac-related components. The LC/MS analysis showed that the molecular weight of these two components were 443 and 445. That they were related to bromfenac was confirmed by the detection of the bromine isotopes in the MS experiments. MS/MS data suggested that 113 daltons had been added to a bromine-containing species with formula weight of 330 and 332. The 113 daltons could be a creatinine moiety, because there is high abundance of it in urine. The bromine-containing species could be degradation products of bromfenac metabolites. To test this hypothesis, several of the reference standards of potential bromfenac metabolites were incubated with control urine and in hydrolysis buffer with and without creatinine. The results clearly showed that the species with a molecular weight of 443 was due to the *ex vivo* formation of a conjugate between creatinine and the α-keto acid, which was readily formed from the hydroxy cyclic amide by oxidation of the hydroxyl group to its keto form and opening of the five membered ring (Figure 6). Additional experiments, which involved reduction of the hydroxy cyclic amide with sodium borohydride and then incubating the reaction product, indicated that a similar creatinine conjugate with a molecular weight of 445 could be generated *ex vivo*. However, the exact structure of this

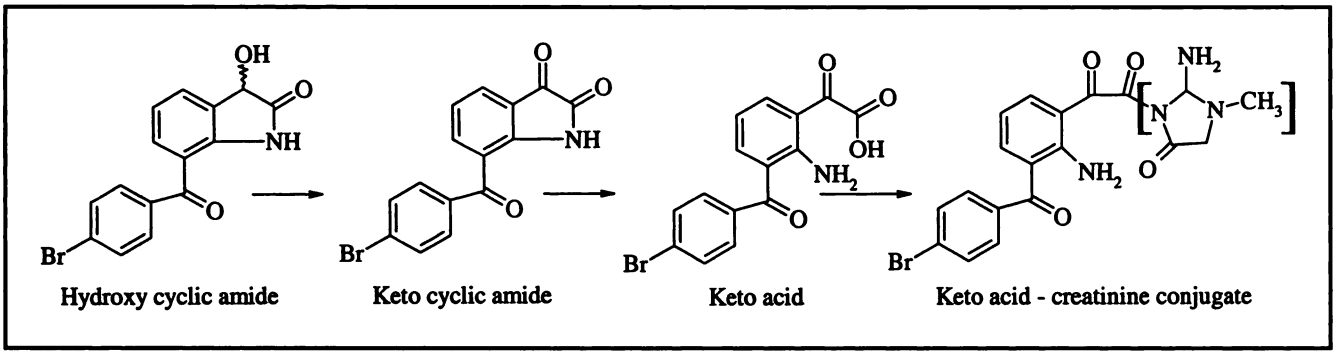

Figure 6. Proposed scheme for the ex vivo *formation of conjugates between creatinine and the α-keto acid derived from the hydroxy cyclic amide.*

compound could not be elucidated, because the compound was highly unstable and a mixture of products was formed by the reaction.

DISCUSSION

^{14}C-Bromfenac was rapidly absorbed and eliminated from human volunteers; this is consistent with data reported for unlabeled bromfenac.[1] In humans, as in rats and monkeys, the whole blood/plasma ratio of radioactivity averaged 0.2, suggesting only slight uptake into formed blood elements. The ratio did not increase with time, indicating no accumulation in the formed blood elements.

Virtually all of the radioactivity in plasma was due to unchanged drug. No cyclic amide or other metabolites were detected in plasma. Approximately 80% of the administered dose was excreted in urine. Excretion into urine was rapid, as most of the urinary radioactivity was excreted in the first 24 hours. Unchanged drug was not excreted into urine. The cyclic amide was the major metabolite in urine 0 to 4 hours after administration. Over the 4- to 12-hour time intervals, the cyclic amide peak decreased in size and four polar glucuronides predominated.

The metabolite patterns of bromfenac in humans and monkeys are similar. This similarity permitted the use of samples obtained from monkeys after a high dose of bromfenac for structure elucidation experiments. Attempts to isolate intact metabolites for structure identification were unsuccessful due to instability of the metabolites. Also, the aglycones from enzyme hydrolysis of urine readily reacted with creatinine *ex vivo* under the hydrolysis conditions used in this study. However, LC/MS experiments were successfully used to determine structures of the metabolites. The presence of bromine in bromfenac and its metabolites was useful in determining the structures of metabolites, because abundance of its isomer was used as a marker to follow the metabolites in LC/MS experiments. Results from these experiments led to the identification of hydroxy cyclic amide and the ketone reduced hydroxy cyclic amide (dihydroxy cyclic amide) as the aglycone structures of the glucuronides in urine of monkeys and humans receiving bromfenac.

Bromfenac cyclizes in aqueous solutions to the cyclic amide at room temperature at neutral pH or under basic conditions. However, it is not known whether cyclization in the body is catalyzed by an enzyme. The *ex vivo* formation of creatinine conjugates of the aglycones is unusual. However, formation of creatinine conjugates of other xenobiotics have been noted before.[15] For example, guanabenz forms two isomeric forms of creatinine adducts. Again, LC/MS and a series of *in vitro* experiments were used successfully in determining the reaction sequence of the formation of these conjugates.

Diclofenac is a nonsteroidal antiinflammatory that has a similar chemical structure to bromfenac. With respect to route of excretion, bromfenac is similar to diclofenac. After oral administration of ^{14}C-diclofenac to humans, 61% of the radioactivity was recovered in urine and 30% was recovered in feces.[15,16] At least 5 hydroxylated and conjugated metabolites of diclofenac that exhibited no pharmacologic activity were detected in urine.[15–17] In contrast to bromfenac, four hydroxylated metabolites of diclofenac were detected in plasma as major metabolites through 12 hours after administration, with total concentrations exceeding those of diclofenac.[15,16] These metabolites have no pharmacologic activity.

Bromfenac is rapidly absorbed after oral administration to healthy volunteers. It is primarily excreted as metabolites in urine. Because administration of cyclic amide led to the formation of same urinary metabolites as bromfenac, it is proposed that the metabolic pathways of bromfenac is through the cyclic amide. The major metabolites in urine are the cyclic amide and two pairs of diastereomeric gluc-

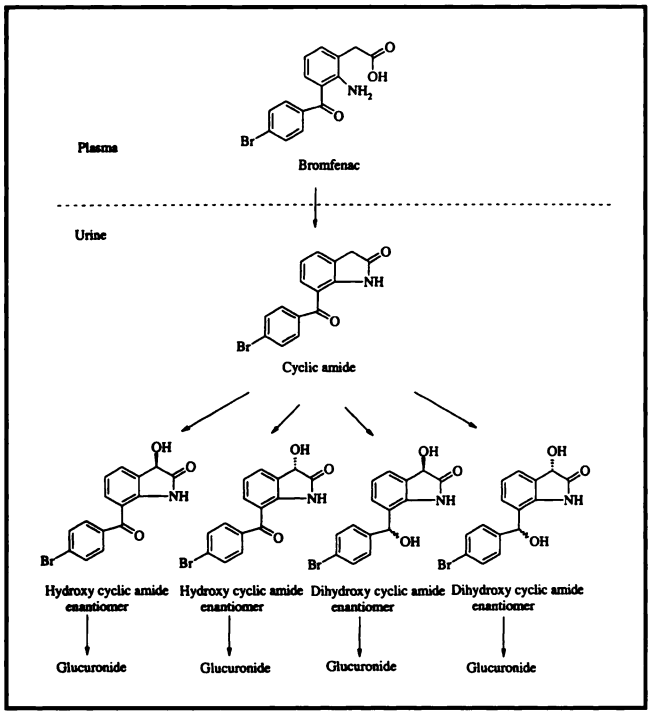

Figure 7. Proposed metabolic pathways of bromfenac in humans.

uronide conjugates of hydroxy cyclic amide and the ketone-reduced hydroxy cyclic amide (Figure 7).

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