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Short Communication

Isolation and Identification of Bromfenac Glucoside From Rat Bile

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ABSTRACT:

Bromfenac (Duract®), a drug approved for pain, was expected to be metabolized by the rat to an acyl glucuronide, a metabolite formed with most compounds of similar structure. During the investigation of metabolite profiles in rat bile following administration of 1 mg/kg iv doses of ^{14}C-bromfenac, an acid-labile metabolite was found that degraded to form ^{14}C-bromfenac. Isolation and characterization of this metabolite indicated that it is an unusual conjugate, bromfenac *N*-glucoside.

Bromfenac (Duract®, 2-amino-3-(4-bromobenzoyl)benzene acetic acid), a compound approved for analgesia, has both analgesic and anti-pyretic properties (Sancilio *et al.*, 1987). It is structurally similar to non-steroidal antiinflammatory drugs and thus contains a carboxyl group. Metabolism of these compounds generally includes formation of an acyl glucuronide at this carboxyl group, as is the case with the structurally similar tolmetin (Hyneck *et al.*, 1988) and ketoprofen (Upton *et al.*, 1980). Acyl glucuronides are labile compounds subject to hydrolysis in solutions of dilute alkali and at physiological pH conditions, yielding glucuronic acid and the aglycone (Ruelius *et al.*, 1985). In preliminary studies in the rat, bromfenac was observed in bile after rigorous base hydrolysis (18 hr, 0.1 N NaOH, 37°C), and it was first concluded that bromfenac was formed by hydrolysis of an acyl glucuronide, although the glucuronide had not been observed in any of the acid-stabilized biological fluids examined. In order to determine the stability of the putative bromfenac acyl glucuronide metabolite, the present studies were undertaken to isolate and characterize this putative metabolite. In rat bile, after administration of a 3 mg/kg iv dose of ^{14}C-bromfenac, only one metabolite was observed, which formed bromfenac at acid or basic pH. Isolation and characterization of this compound indicated that the metabolite was bromfenac *N*-glucoside.

Materials and Methods

^{14}C-Bromfenac, labeled in the keto carbon, was obtained from Amersham International, Buckinghamshire, England, as the sodium salt. Bromfenac and its analogs AHR-10240, (lactam analog), AHR-11665 (benzoic acid analog), and AHR-11779 (ethyl ester analog) were obtained from the Wyeth-Ayerst Research in-house compound bank. Trimethylsilylglucose (TMS-glucose[1]) and bis(trimethylsilyl)trifluoroacetamide were obtained from Sigma Chemical Company, St. Louis, MO. All other reagents and solvents were reagent-grade or better.

[1] Abbreviations used are: TMS-glucose, trimethylsilylglucose; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; GC/MS, gas chromatography/mass spectrometry; ESI, electrospray ionization; CI, chemical ionization; CID, collision-induced dissociation; 5-ASA, 5-aminosalicylic acid.

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Dosing and Sample Collection. Three studies were conducted. In the first study, male bile-duct cannulated Sprague-Dawley rats (330–375 g) were administered 3 mg/kg iv doses of ^{14}C-bromfenac (as the sodium salt) dissolved in sterile distilled water. Bile was collected for 12 hr over wet ice into tubes containing nothing or 0.6 ml of 1M citric acid (pH 3.5). In the second study, all conditions were similar to the first, except that bile was collected into tubes containing 0.5 ml of 1M sodium phosphate buffer, pH 7.5. The third study was conducted in the same manner as the second, utilizing nonradioactive bromfenac. All bile was stored frozen until analyzed.

Hydrolysis. Bile was adjusted to pH 11 with 1 N NaOH and incubated at room temperature for 15 min to hydrolyze any acyl glucuronides present. Bile was then injected directly onto the HPLC immediately after the incubation period.

HPLC. Bile metabolite profiles were determined by HPLC on a 5-micron Brownlee Spheri-5 RP-18 column (Applied Biosystems, Inc., Foster City, CA). The mobile phase was a linear gradient of 10% to 45% acetonitrile in ammonium acetate, pH 5, or sodium phosphate, pH 5. Detection of radioactivity utilized a Radiomatic Flo One β Model A500 flow detector and Ultima Flo M as scintillant (both from Packard Instrument Co., Meridan, CT). Ultraviolet absorbance was detected at 270 nm. A conjugate was isolated by HPLC using both small (2.1 mm) and standard (4.6 mm) bore columns. Conjugate peaks were collected directly from the ultraviolet detector and stored in mobile phase at −20°C until used.

pH Stability of Conjugate. After adjustment of pH to 4, 5, 6, 7, and 8 with sodium phosphate buffer, aliquots of the isolated conjugate were incubated for 2 hr at 37°C in a shaking water bath to assess stability. Samples were injected directly onto the HPLC system at the end of the incubation period.

Preparation of Bromfenac Glucoside Ethyl Ester. Approximately 0.04 μmol of isolated conjugate (97% pure, based on HPLC and detection at 270 nm) was evaporated to dryness and redissolved in 100 μl of dimethylformamide. After the addition of 20 μl of triethylamine and 0.5μl (~100-fold molar excess) of ethyl iodide, the reaction was stirred for 2 hr at room temperature. The reaction mixture was evaporated to dryness and the residue was redissolved in acetonitrile in ammonium acetate, pH 7.5 (50:50). Yield was 72%, based on HPLC analysis and detection at 270 nm, with 23% remaining as unreacted conjugate. The ester was isolated by HPLC as described above, using a linear gradient of 10 to 80% acetonitrile in ammonium acetate, pH 7.5, over 40 min.

Preparation of Isolated Conjugate for Mass Spectrometry. In addition to LC/MS analysis of the intact conjugate, the isolated conjugate was hydrolyzed to determine molecular weights of the aglycone and the conjugating moiety by mass spectrometry. For analysis of the aglycone, the conjugate solution in mobile phase was hydrolyzed by incubation for 15 min at pH 4 and 37°C. The

by LC/MS. For analysis of the conjugating moiety, the conjugate solution in mobile phase was adjusted to pH 4 and incubated at 37°C for 30 min. The solution was evaporated to dryness and the residue redissolved in pyridine (dried over KOH pellets), silylated by the addition of freshly opened bis(trimethylsilyl)trifluoroacetamide, and analyzed by GC/MS. Mass spectra were compared with those of standards of bromfenac and TMS-glucose.

Mass Spectrometry. The intact conjugate and the aglycone were analyzed by ESI LC/MS on a Finnigan MAT TSQ-700 mass spectrometer (Finnigan Corp., San Jose, CA). HPLC was performed on a 5-micron Supelco LC-18-DB column (Supelco, Inc., Bellefonte, PA). Mobile phase was a linear gradient of acetonitrile:ammonium acetate, pH 7.5. Mass spectra were obtained at a scan rate of 1 sec/scan over a range of *m/z* 250–750 and *m/z* 150–500 for the bromfenac conjugate and the aglycone, respectively. The heated capillary temperature in the ion source was 225°C. The conjugating moiety (glucose) was analyzed by CI GC/MS on a Finnigan MAT SSQ-70 mass spectrometer. GC was performed on a DB5-MS column, 0.25 mm x 15 m, coated as 0.25 μ film (J and W Scientific, Folsom, CA). GC injector temperature was 250°C. The column temperature program started at 80°C for 1 min, rose 20°C per min for 8.5 min, then remained constant at 250°C for 3 min, for a total run time of 12.5 min. Mass spectra were obtained in positive-ion chemical ionization mode using ammonia gas. Source temperature was 148°C.

The conjugate and its ethyl ester were analyzed by atmospheric pressure CI mass spectrometry on a Finnigan SSQ 710c mass spectrometer. A Michrom HPLC system (Michrom BioResources, Inc., Pleasanton, CA) was interfaced to the mass spectrometer with a mobile phase of methanol and water (80:20) pumped at a flow of 100 μl/min. The spectra were acquired at unit mass resolution by flow injection of sample in a solution of acetonitrile:water, 50:50 (500 ng in 5 μl). The sample was sprayed into the mass spectrometer at vaporizer and heated capillary temperatures of 450 and 150 °C, respectively. Spectra were obtained at a scan rate of 2 sec/scan over a range of *m/z* 200–800. The electron multiplier and the conversion dynode were set at 1.0 kV and −15 kV, respectively. The fragment ions were obtained using collision energy of 20 or 30 eV in the ion source.

Results

All attempts at preparation of a standard of bromfenac acyl glucuronide using rat, beagle dog, cynomolgus monkey, and human hepatic microsomes supplemented with uridine 5′-diphosphoglucuronic acid were unsuccessful, suggesting that the glucuronide does not form under physiological conditions.

Biliary Metabolite Profiles of Bromfenac and its Metabolites. HPLC metabolite profiles of bile collected in citric acid exhibited a large peak at the retention time of the lactam analog of bromfenac (AHR-10240), but no bromfenac. Bromfenac has been shown to degrade to the lactam under conditions of acidic pH but was demonstrated to be stable at pH 9 to 11 under conditions similar to those required for base hydrolysis of acyl glucuronides. A peak corresponding to a bromfenac conjugate and exhibiting a retention time of approximately 26–28 min was prominent in bile collected without pH adjustment at approximately pH 7 but was not present or present in small amounts in bile collected in citric acid at pH 3.5. In bile collected in phosphate buffer at pH 7.5, this peak at 26–28 min was a major component of the profiles.

Hydrolysis Studies. No bromfenac was observed after mild base hydrolysis of bile collected without pH adjustment, suggesting that an acyl glucuronide of bromfenac was not present. The peak at 26–28 min observed in bile collected without pH adjustment was not affected by these mild hydrolysis conditions. However, mild alkaline treatment of bile resulted in generation of large quantities of AHR-11665 in both acidified and non-acidified bile, suggesting the presence of an acyl glucuronide of AHR-11665, a benzoic acid metabolite of bromfenac (fig. 2). This AHR-11665 conjugate appeared to be at least moderately stable, as no AHR-11665 was apparent in bile collected without pH

Stability of the isolated ^{14}C-bromfenac glucoside incubated at various pH, from 4 to 8, for 2 hr at 37°C was investigated. At pH 4, the compound was almost completely degraded to bromfenac, with some lactam also evident. At pH 6, approximately 50% of the conjugate was degraded to bromfenac, while 50% remained unchanged. At pH 8, very little degradation was noted; the profile is similar to that of the untreated control.

Mass Spectrometry of Bromfenac Conjugate. In the ESI LC/MS spectrum of the ^{14}C-bromfenac conjugate, the base peak was the molecular ion and was observed at *m/z* 496/498, $[M\text{-}H]^-$. In the spectrum of the ^{14}C-aglycone resulting from hydrolysis of the conjugate, the molecular ion was also the base peak and was observed at *m/z* 334/336, $[M\text{-}H]^-$. The only other prominent ion pair in the spectrum, resulting from loss of the carboxyl group, was observed at *m/z* 290/292, $[M\text{-}COOH]^-$. The ESI LC/MS spectrum of a bromfenac standard was similar to that of the ^{14}C-aglycone; the base peak was the molecular ion, which was observed at *m/z* 332/334, $[M\text{-}H]^-$, and the loss of the carboxyl group was observed at *m/z* 288/290, $[M\text{-}COOH]^-$. As the specific activity of the ^{14}C-bromfenac utilized for this study was approximately 85%–90% of the theoretical maximum, an isotope effect was observed, resulting in a molecular ion for the radioactive compound 2 Da higher than that observed with the non-radioactive standard. With the exception of the 2-Da shift, the two spectra were identical, indicating that the aglycone from the isolated conjugate was ^{14}C-bromfenac. The GC retention times of the TMS-glucose standard and the silylated conjugating moiety after hydrolysis of isolated conjugate were similar at 10.4 and 10.3 min, respectively. The CI GC/MS spectra of the silylated standard and hydrolyzed conjugate were nearly identical, with the molecular ion observed in both spectra at *m/z* 558, $[M+NH_4]^+$, representing the ammonia adduct of fully silylated glucose. Ions were also observed in both spectra at *m/z* 468, *m/z* 378, *m/z* 288, and *m/z* 198, representing loss of 1, 2, 3, and 4 *O*-trimethylsilyl groups, respectively.

Mass Spectrometry of Ethyl Esters. The atmospheric pressure CI mass spectra of bromfenac, AHR-11779, the non-radioactive isolated conjugate (purity 97%, based on HPLC analysis with detection at 270 nm), and the ethyl ester of the isolated conjugate (purity >98%) are shown in fig. 1, panels A-D, respectively. In spectra (not shown) of the conjugate and its ethyl ester obtained without CID, the base peak in both spectra was the molecular ion, $[M+H]^+$, at *m/z* 496/498 and *m/z* 524/526, respectively, with very weak fragment ions. In the CID spectra of bromfenac standard (fig. 1, panel A), fragments were observed at *m/z* 316/318 and *m/z* 288/290, representing loss of water and loss of the carboxyl moiety, respectively. In the CID spectra of AHR-11779, (fig. 1, panel B), fragments were again observed at *m/z* 316/318 and *m/z* 288/290, representing loss of ethanol and loss of the ethyl ester moiety, respectively. The most prominent fragments in the CID spectra of the conjugate and its ethyl ester were observed at *m/z* 334/336 and *m/z* 362/364, respectively, representing loss of the glucose moiety, $[MH\text{-}C_6H_{11}O_5+H]^+$. This fragmentation pattern indicated that conjugation is through an *N*-linkage, as it is expected that loss of the glucose moiety in an *O*-linked glucoside would result in loss of an additional oxygen from the carboxyl moiety, $[MH\text{-}HOC_6H_{11}O_5]^+$ at *m/z* 320/322 and *m/z* 348/350, respectively, as was observed in the loss of ethanol from the AHR-11779 (fig. 1, panel B). The fragmentation patterns of the conjugate and its ethyl ester (fig. 1, panels C, D) indicate that in the reaction of the conjugate with ethyl iodide, the ethyl group was added to the carboxyl moiety and not to the amine, as the CID mass spectrum of the ethyl ester of the conjugate is nearly identical to that of the authentic standard AHR-

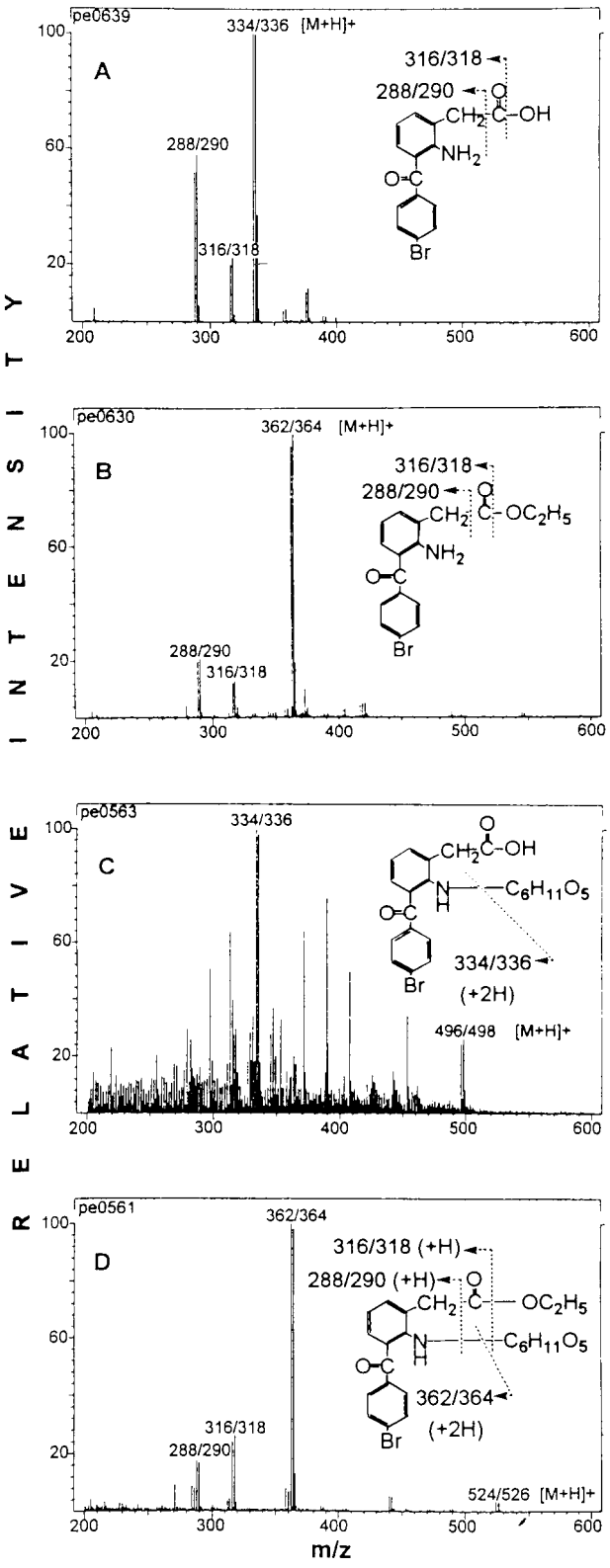

FIG. 1. *Mass spectra of bromfenac (A), AHR-11779, the ethyl ester of bromfenac (B), bromfenac glucoside (C), and the ethyl ester of bromfenac glucoside (D).*

Attempts to obtain supporting nuclear magnetic resonance data for the *N*-linkage of the glucose moiety were unsuccessful, as a result of

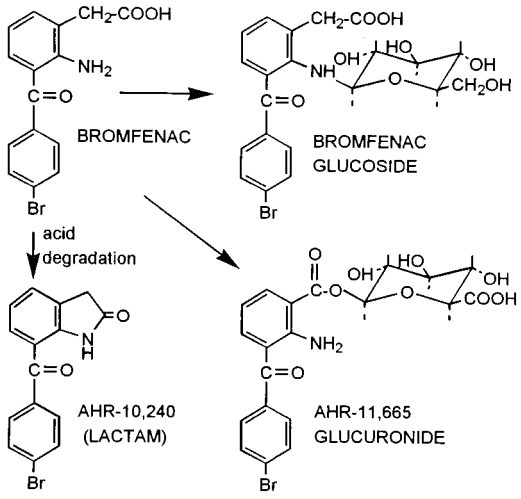

FIG. 2. *Metabolic pathways for bromfenac in rat bile.*

purify the conjugate. The lack of purity is evident in the mass spectrum of the conjugate (fig. 1, panel C).

Discussion

A survey of the literature indicated that approximately a dozen xenobiotics are known to form conjugates with glucose in mammalian systems (or models for mammalian systems), exhibiting both *N*- and *O*- linkages. The *N*-linked glucosides of 5-ASA (Tjørnelund *et al.*, 1989), amobarbital (Tang and Kalow, 1978), and phenobarbital (Tang *et al.*, 1979) were identified as major metabolites of these drugs in man. *N*-linkages were formed through primary and secondary amines, while *O*-linkages were formed through aromatic and aliphatic hydroxyl groups, as well as carboxyl groups. The *N*-linked glucosides of sulfamethazine (Paulson *et al.*, 1981), 4,4′-methylenebis(2-chloroaniline) (Duggan *et al.*, 1974), 3-(4-pyrimidinyl)-5-(4-pyridyl)-1,2,4-triazole (3,5 PPT; Manis and Braselton, 1986), and 5-ASA (Tjørnelund *et al.*, 1989) were all found to be unstable in acid, and those of 3,5 PPT and 5-ASA were stable in base. The *O*-linked glucosides of hopantenic acid (Nakano *et al.*, 1986) and furosemide (Hezari and Davis, 1993) were found to be stable in acid and that of furosemide was unstable in base. As has previously been noted (Tjørnelund *et al.*, 1989), *N*-glucosides, as well as *N*-glucuronides, exhibit varying degrees of instability under acidic conditions and stability under basic conditions. Bromfenac glucoside was shown to be unstable under acidic conditions, suggesting that the glucoside linkage is through the amine group rather than the carboxyl group. A carboxyl *O*-linkage would be expected to result in a pH stability profile for the compound similar to those observed with acyl glucuronides, *i.e.* stable in acid, unstable in base. The formation of the ethyl ester of bromfenac glucoside by reaction with ethyl iodide, as evidenced by a CID mass spectrum nearly identical to that of AHR-11779, the ethyl ester of bromfenac, provides additional evidence of the *N*-linkage for the glucose moiety. Proposed biotransformation pathways of bromfenac in rat bile are shown in fig. 2, although the metabolites presented are only a few of those observed in rat bile after administration of ^{14}C-bromfenac, as the purpose of the experiments was to identify the bromfenac conjugate.

The finding of a glucose conjugate of bromfenac, rather than the more common glucuronide conjugate, is unusual in two respects. First, compounds in this class are generally metabolized to glucu-

only nonsteroidal antiinflammatory drug found to form a glucoside conjugate, 5-ASA, is structurally similar to bromfenac in that both compounds contain a primary amine group near the carboxyl group. Many other compounds in this class contain nitrogen, but no others containing a primary amine moiety were noted in the literature. Interestingly, AHR-11665 is structurally more similar to 5-ASA than is bromfenac, as the carboxyl groups in both AHR-11665 and 5-ASA are linked directly to the phenyl ring, but AHR-11665 apparently forms an acyl glucuronide (while the other compounds do not) and not an *N*-glucoside.

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