CHARACTERIZATION OF PROFLAVINE METABOLITES IN RAINBOW TROUT

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ABSTRACT:

Proflavine [3,6-diaminoacridine] has potential for use as an antiinfective in fish, and its metabolism by rainbow trout was therefore studied. Fourteen hours after intraarterial bolus administration of 10 mg/kg of proflavine, three metabolites were found in liver and bile, and one metabolite was found in plasma using reversed-phase HPLC with UV detection at 262 nm. Treatment with hydrochloric acid converted the three metabolites to proflavine, which suggested that the metabolites were proflavine conjugates. Treatment with β-glucuronidase and saccharic acid 1,4-lactone, a specific β-glucuronidase inhibitor, revealed that two metabolites were proflavine glucuronides. For determination of UV-VIS absorption and mass spectra, HPLC-purified metabolites were isolated from liver. Data from these experiments suggested that the proflavine metabolites were 3-N-glucuronosyl proflavine (PG), 3-N-glucuronosyl, 6-N-acetyl proflavine (APG), and 3-N-acetylproflavine (AP). The identities of the metabolites were verified by chemical synthesis. When synthetic PG and AP were compared with the two metabolites isolated from trout, they had the same molecular weight as determined by matrix-assisted, laser desorption ionization, time-of-flight MS. In addition, they coeluted on HPLC under different mobile phase conditions. Finally, the in vitro incubation with liver subcellular preparations confirmed this characterization and provided the evidence that APG can be formed by glucuronidation of AP or acetylation of PG.

Proflavine (3,6-diaminoacridine; fig. 1) was used as a topical antiinfective in the two World Wars. Although proflavine is no longer approved for human use because of its toxicity, it has potential for use in fish. Its metabolism has not been characterized previously in any species. The acridine ring system could be oxidized by acridine dehydrogenase to form acridone (1). Conjugation at the two amino groups by a number of endogenous substances—such as GA, sulfite, and amino acids—is also possible. In addition, the acridine ring nitrogen is vulnerable to conjugation.

The objective of the present study was to explore the metabolism of proflavine by fish. We characterized the metabolites of proflavine to understand better its biochemical fate, pharmacokinetics, and total residue depletion in proflavine-exposed fish. Proflavine was administered to rainbow trout via intraarterial bolus injection. This route of administration was selected to produce adequate amounts of metabolites. Proflavine is slowly absorbed by fish from exposure water, and relatively small concentrations of proflavine and its metabolites were observed in plasma after water exposure. After isolation and purification of the proflavine metabolites, acid and enzymatic hydrolysis, spectrophotometric analysis, TOF-MS, and chemical synthesis were used to identify the structures of the metabolites. Finally, an in vitro study was used to elucidate the metabolic pathways.

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Materials and Methods

Reagents. Proflavine (3,6-diaminoacridine hemisulfate) was purchased from Janssen Chimica (New Brunswick, NJ). HPLC-grade acetonitrile, methanol, and sodium chloride were obtained from Fisher Scientific Company (Fair Lawn, NJ). Acetic acid and hydrochloric acid were purchased from EM Science (Gibbstown, NJ). Triethyamine was purchased from Aldrich Chemical Company (Milwaukee, WI). Sodium acetate was obtained from J. T. Baker Chemical Company (Phillipsburg, NJ). Cyanogen tubes were purchased from Supelco (Bellefonte, PA). β-Glucuronidase, saccharic acid 1,4-lactone, N-acetyl GA, UDP-GA, and acetyl CoA were purchased from Sigma Chemical Company (St. Louis, MO). BCA protein assay kits were from Pierce (Rockford, IL).

Experimental Animals. Rainbow trout (Oncorhynchus mykiss) were obtained from Fresh Water Fish Farm (Urbana, Ohio) and were transported to the vivarium in aerated water within 1 hr. Trout were acclimated to 12°C for a minimum of 2 weeks in a Living Stream Aquarium (Frigid Units, Inc., Toledo, OH), which contained 1000 liters of dechlorinated water. This aquarium was aerated, and the water was filtered through a charcoal filter with an average recirculation time of 1.5 min. Dechlorinated Columbus Municipal Water was added continuously to the holding aquarium at a rate of 1.5 liters/min. The aquarium was cleaned daily and water temperature, pH, and dissolved oxygen were measured daily; acceptable ranges for these parameters were 11°–13°C, 6.5–8.0 and >9.6 mg/liter (>80% saturation), respectively. Daily monitored values were within these ranges. The vivarium was windowless and had an automatically timed 07:00–19:00 photoperiod. Fish were fed a commercially available soft, moist food obtained from Rangen, Inc. (Buhl, ID), with a pellet size of 5/32 inch. Fish were not fed on the day preceding and during their experimental use.

Metabolite Isolation. Trout were anesthetized with tricaine methanesulfonate (0.1 g/liter) and then were fitted with a cannula [28-G thin-wall Tetlon tubing (Zeus Industrial Products, Orangeburg, SC)] in the dorsal aorta (2, 3), which allowed intraarterial bolus injection and blood sampling from free swimming fish. After 24 hr of recuperation, each fish was administered a single injection of proflavine (10 mg of proflavine/kg body weight, 10 mg/ml in 80% v/v dimethyl sulfoxide and 20% v/v water). Sixteen hours after dosing, blood samples were removed from the cannula. The blood was collected in a heparinized Eppendorf tube, centrifuged, and the plasma portion was separated and stored in a −20°C freezer until analyzed by HPLC. After blood with-
drawal, the fish was killed immediately. Bile and liver samples were then collected and stored at −20°C until analysis. Sample clean-up procedures were the same as described previously (4). Metabolites were separated by HPLC, and fractions of each metabolite were collected. The mobile phase component was evaporated under a stream of nitrogen or using a vacuum centrifuge evaporator. The purified metabolites were stored at −20°C.

**HPLC Analysis.** The HPLC system consisted of model 125 programmable binary gradient pump, model 166 UV-VIS detector set at 262 nm, Pharmacia model FRAC-100 fraction collector, IBM model 555X PC computer, and a System Gold control system (Beckman Instruments, Irvine, CA). Samples were introduced into a 100-μl sample loop. Separation was achieved on a 4-μm Nova-Pak C18 column 3.9 mm × 300 mm (Waters, Millipore Corporation, Milford, MA) and eluted by a gradient mobile phase: starting from 5 min of 100% system A, followed by an 8-min linear gradient to 75% system A (24% system B), and then continuation for 5 min before returning to 100% system A. The flow rate was 0.8 ml/min. Mobile phase A consisted of 93.23% v/v water, 5.00% v/v acetonitrile, 1.40% v/v acetic acid, and 0.37% v/v triethylamine. Mobile phase B consisted of 4.91% v/v water, 95.00% v/v acetonitrile, 0.07% v/v acetic acid, and 0.02% v/v triethylamine. Detection was by a UV detector at 262 nm.

**Acid Incubations.** Metabolites were dissolved in water. To a 200 μl aliquot of each solution was added 10 μl of 1 N HCl (final acid concentration: 0.05 N). The solution was incubated for 30 min at 45°C, and the reaction was terminated by an addition of 10 μl of 1 N NaOH. An aliquot of the solution was then injected onto the HPLC.

**Enzyme Incubations, β-Glucuronidase.** Metabolites were dissolved in 0.1 M sodium phosphate buffer (pH 6.8). To a 200 μl aliquot of each metabolite solution was added 100 μl of β-glucuronidase solution (1000 units/ml in sodium phosphate buffer) and 100 μl of the sodium phosphate buffer. After the mixture was incubated at 37°C for 24 hr, the reaction was terminated by processing the sample using an SPE tube. The eluate was analyzed by the previously described HPLC procedure. A control experiment was also performed in which 100 μl of the sodium phosphate buffer was added to the metabolite solution instead of the enzyme solution. To inhibit β-glucuronidase, 100 μl of 100 mM saccharic acid 1,4-lactone solution was added before the addition of the β-glucuronidase solution to the reaction mixture (5).

**Absorbance Spectra.** Spectra were obtained on a Kontron Uvikon 860 Spectrometer (Kontron Instruments, Everett, MA). Extracted metabolites were dissolved in 0.1 M sodium phosphate buffer at pH 7.0 and pH 12; pH was adjusted using NaOH. A 0.5 ml aliquot of each solution was placed in a cuvette, and the absorbance spectrum between 200 and 600 nm was determined.

**MS.** TOF mass spectra were obtained on a Compact MALDI III mass spectrometer (Kratos Analytical, Ramsey, NJ). The extracted metabolites (−50–100 pmol) were reconstituted with water and loaded onto a 20-well sample slide for TOF-MS analysis. After the samples were dried, 3 μl of saturated sinapinic acid in 30:70 (v/v) acetonitrile:water was added to each sample well. The slide was dried with a stream of air and placed into the TOF-MS sample compartment. A laser power of 60–80 mV was used with the positive-ion mode to obtain the molecular ion peak.

**Chemical Synthesis.** AP. Sodium acetate (10 mg) was dissolved in 5 ml of 0.1 mM proflavine aqueous solution. To the solution was added 200 μl of acetic anhydride, and the mixture was incubated at 60°C for 2 hr. After dilution, a 100 μl aliquot was injected onto the HPLC. The AP fraction was collected, and the mobile phase component was evaporated under a stream of nitrogen. The residue was subjected to TOF-MS analysis as before.

**PG.** d-GA (0.64 g, 0.0033 mol) and proflavine (0.37 g, 0.0015 mol) were placed in a round-bottom 200 ml flask equipped with a reflux condenser and a magnetic stirrer. A mixture of 13.5 ml acetonitrile and 1.5 ml of water was added with vigorous stirring. The suspension was heated to 45°C, and a 0.1 ml aliquot of concentrated hydrochloric acid was added slowly. After 10 min, the reaction mixture was placed into an ice bath for 1 hr, and the precipitate was isolated and dissolved in 3 ml of water. The solution was mixed with 20 ml of acetone, and the precipitate was filtered and washed with 2 ml of ethylacetate, followed by 1 ml of ether. Approximately 50 mg of product was obtained (6). Approximately 1 μg of this product was dissolved in water and was injected onto the HPLC. The PG fraction was collected, the mobile phase component was evaporated under a stream of nitrogen, and the residue was subjected to TOF-MS analysis. It was also mixed with the metabolites extracted from liver, and the mixture was injected onto the HPLC at three different HPLC mobile phase conditions: mobile phase B gradient up to 10%, 15%, and 25%.

**In Vitro Metabolism of Proflavine.** Preparation of the Liver Subcellular Fraction. Fresh trout liver was minced and mixed with 10 mM potassium phosphate buffer (pH 7.4) that contained 1.15% (w/v) potassium chloride. The tissue was then homogenized on ice (Omnimixer Homogenizer). The homogenate contained 0.2–0.25 g of wet tissue/ml of buffer; it was centrifuged at 9,000g for 20 min. The supernatant (the S9 fraction) was separated from the
pellet. One aliquot of the S9 fraction was stored at −80°C, and another aliquot was used to prepare the cytosolic and microsomal fractions. The S9 fraction was centrifuged at 105,000g for 60 min to obtain the cytosolic fraction (supernate), and the microsomal fraction was obtained by reconstituting the pellet in 6 ml of KCl phosphate buffer (7). Both the cytosolic and microsomal fractions were stored at −80°C. The protein concentration was measured using the BCA method (8), with bovine serum albumin as standard. Protein concentrations of the S9, cytosolic, and microsomal fractions were 20.7, 13.5, and 19.5 mg/ml, respectively.

**Formation of AP, PG, and APG from Proflavine.** To a 0.2 ml aliquot of the S9 fraction was added 0.4 ml of 15 mM tromethamine HCl buffer (pH 7.4) containing 100 nM of proflavine, 0.2 ml of the same tromethamine buffer containing 3 mM of acetyl CoA, and 0.2 ml of the tromethamine buffer containing 5 mM of UDP-GA. The mixture was incubated at 12°C for 3 hr. Two controls were also prepared. Control 1 was the complete mixture without acetyl CoA and UDP-GA. Control 2 was the complete mixture without the S9 fraction. The controls and sample were incubated similarly. The reaction was terminated by SPE. All samples were analyzed by the HPLC procedures as described previously.

**Acid Incubation.** After acid treatment, proflavine was released from all three metabolites. Almost 100% of Met II was collected for MS analysis. Results showed that the [M + H]⁺ ion at m/z 252.5, which was the same as that of Met III. This fraction was collected for MS analysis. Results showed that the [M + Na]⁺ peak was at m/z 252.5, which was the same as that of Met III.

**Enzyme Incubation.** After a 24-hr incubation with β-glucuronidase, 87% of Met I was converted to proflavine. Interestingly, acid hydrolysis of Met II gave not only proflavine, but also a compound with the same retention time as Met III. This fraction was collected for MS analysis. Results showed that the [M + Na]⁺ value of AP was at m/z 252.5, which was the same as that of Met III.

**Absorbance Spectra.** Spectra of the three metabolites and proflavine showed two main absorption bands with λmax at 262 and 454 nm at pH 7.0. For all metabolites, the 454 nm band shifted to 395 nm at pH 12.0, as did proflavine. This shift corresponded to ionization of the acridine ring (pKₐ = 9.6). When the acridine ring is protonated, the low energy band is at 454 nm, which shifts to 395 nm when it is not protonated (9). Because the ring nitrogen appeared to protonate at low pH for all of the metabolites, none of the metabolites involved substitution on the acridine ring nitrogen.

**Chemical Synthesis.** AP. Approximately 80% of the proflavine was converted to AP. Upon HPLC analysis, the synthetic AP gave the same retention time as Met III isolated from fish. The identity was also verified by TOF-MS, which gave an MH⁺ ion at m/z 386.4. Met II gave an MH⁺ ion at m/z 428.3, whereas the calculated MH⁺ value of APG was at m/z 428.4. Met III gave an MH⁺ ion at m/z 252.5, essentially the same as 252.3 calculated for AP. In all of the cases, the observed values were within <0.1% of the calculated values (table 1). A representative TOF mass spectrum is shown in fig. 3.

**Results**

**HPLC Analysis.** The HPLC chromatograms of plasma, liver, and bile samples are shown in fig. 2. One metabolite was found in plasma, and it along with two other metabolites were found in liver and bile. According to their retention behaviors on HPLC, these three metabolites were arbitrarily named Met I, Met II, and Met III; only Met I was found in plasma. Met I and Met II had retention times shorter than that of proflavine, which suggested that they were more polar than proflavine. The retention time of Met III was similar to that of proflavine.

**Absorbance Spectra.** Spectra of the three metabolites and proflavine showed two main absorption bands with λmax at 262 and 454 nm at pH 7.0. For all metabolites, the 454 nm band shifted to 395 nm at pH 12.0, as did proflavine. This shift corresponded to ionization of the acridine ring (pKₐ = 9.6). When the acridine ring is protonated, the low energy band is at 454 nm, which shifts to 395 nm when it is not protonated (9). Because the ring nitrogen appeared to protonate at low pH for all of the metabolites, none of the metabolites involved substitution on the acridine ring nitrogen.

**Fig. 2.** Typical HPLC chromatograms of proflavine (P) metabolites extracted from (a) plasma, (b) liver, and (c) bile.
iment showed that, at three different mobile phase compositions, the synthetic PG coeluted with Met I isolated from fish liver (fig. 4). The molecular weight of the synthetic PG was determined by TOF-MS, which gave a MH$^+$ peak at m/z 386.3 (calculated 386.39) (table 1).

**In Vitro Metabolism.** In the presence of liver S9 fraction, UDP-GA, and acetyl CoA, all three metabolites were produced, with AP being the most abundant (fig. 5a). However, no metabolites were found for the two control experiments, which indicated that both the liver subcellular fraction and the high energy substrate (UDP-GA and acetyl CoA) were essential for the in vitro proflavine metabolism. APG was generated from AP by glucuronidation and from PG by acetylation (fig. 5, b and c).

**Discussion**

From the HPLC analysis, two polar and one less polar metabolites were observed from proflavine-treated fish. Acid hydrolysis of all of the metabolites released proflavine, which suggested that all the metabolites were proflavine conjugates. Moreover, only proflavine was released from Met I and Met III, but both proflavine and Met III were released from Met II after acid hydrolysis. This suggested that Met I and Met III were proflavine monoconjugates, and that Met II was a proflavine diconjugate that shared one common ligand with Met III.

Known xenobiotic conjugates found in fish include those formed with glucose, sulfate, amino acids, glutathione, acetate, and GA (10). Chemical synthesis of proflavine glucoside and comparison of its HPLC retention time with those of the metabolites revealed that the glucoside was not the isolated metabolite. Treatment of the metabolites with sulfatase did not release proflavine. Amino acid conjugation was not likely because proflavine does not have the prerequisite carboxyl group. Glutathione conjugation was also not possible because proflavine does not have the necessary electrophilic center. Among the usual conjugates, that left only glucuronidation and acetylation as candidates for proflavine metabolism.

Treatment of the metabolites with β-glucuronidase and its specific inhibitor, saccharic acid 1,4-lactone, provided the evidence that Met I and Met II were PG. That Met I was converted to proflavine after this treatment suggested that Met I was proflavine conjugated with GA. However, Met II was converted to Met III after enzymatic treatment, which indicated that Met III was a proflavine diconjugate in which one ligand was GA and the other was the same as the ligand of Met III. Possible conjugation sites of proflavine included the 3- and 6-amino groups. The ring nitrogen probably remained intact in all metabolites based on the pH dependence of their absorption spectra.

TOF-MS analysis of three metabolites identified Met I as PG (3-N-glucuronosyl proflavine), Met II was identified as APG (3-N-glucuronosyl,6-N-AP), and Met III was identified as AP (3-N-AP). Therefore, Met I and Met III were proflavine monoconjugates, and Met II was a proflavine diconjugate. These conclusions were supported by the results of acid hydrolysis and enzymatic treatments.

The identity of the metabolites was further verified by chemical
The synthesis of Met I and Met III. Based on HPLC and TOF-MS analysis, the synthetic AP was indistinguishable from Met III, and the synthetic PG was indistinguishable from Met I. The mass spectrum of Met III gave a molecular ion identical to that of synthetic AP, indicating that Met III was AP. PG from chemical synthesis was found to coelute with Met I at three different mobile phase compositions, and gave the same molecular weight by TOF-MS analysis, indicating that Met I was PG.

These three metabolites were also formed after in vitro incubation of proflavine with trout liver preparations in the presence of UDP-GA and acetyl CoA. Met II (APG) was formed Met I (PG) by acetylation, and from Met III (AP) by glucuronidation. Therefore, Met II can be formed from either pathway (fig. 1); i.e. glucuronidation of proflavine, followed by acetylation of PG, or acetylation of proflavine followed by glucuronidation of AP. Although tricaine methanesulfonate may have affected the metabolism of proflavine, the in vitro study found the same metabolites. Water exposure studies (data not shown) also showed the same metabolites, and oxidation or reduction products were not observed. It therefore seemed unlikely that the anesthetic qualitatively altered the metabolite profile.

In summary, the combined results of this study provide unequivocal evidence that the three metabolites of proflavine found in rainbow trout after its intraarterial administration were Met I (PG), Met II (APG), and Met III (AP). Moreover, the diconjugate was formed from either monoconjugate.

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