Glycerolysis of Acyl Glucuronides as an Artifact of in Vitro Drug Metabolism Incubations

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ABSTRACT:
During an investigation of the in vitro glucuronidation of benoxaprofen by human liver S-9 fraction, an unusual drug-related entity possessing a protonated molecular ion that was 74 mass units greater than the parent drug was observed. It was identified as the glycerol ester of benoxaprofen. Formation of this entity required inclusion of uridine diphosphoglucuronic acid (UDPGA) in the incubation, suggesting the formation of benoxaprofen acyl glucuronide followed by transesterification with the glycerol present in the incubation due to its presence as a stabilizer for liver subcellular fractions. Formation occurred during the sample work-up procedure while the samples were subjected to evaporation in vacuo, which does not remove glycerol. Conversion of purified benoxaprofen acyl glucuronide to the glycerol ester was demonstrated in glycerol at 37°C. Other drugs that are converted to acyl glucuronides in vitro (diclofenac, mefenamic acid, tolmetin, and naproxen) were also shown to form corresponding glycerol esters when incubated with human liver S-9 fraction and UDPGA. The potential formation of glycerol esters of carboxylic acid drugs undergoing acyl glucuronidation in vitro represents an experimental artifact to which drug metabolism scientists should be aware.

The metabolism of xenobiotics containing carboxylic acid groups to acyl glucuronides is a well known biotransformation reaction (recently reviewed in Skonberg et al., 2008; Stachulski et al., 2006). Acyl glucuronide metabolites have received considerable attention due to evidence suggesting that they are chemically reactive, form adducts with proteins, and potentially cause various toxicities via this mechanism (Yang et al., 2006; Skonberg et al., 2008). Adducts can possess a protonated molecular ion that was 74 mass units greater than the parent drug was observed. It was identified as the glycerol ester of benoxaprofen. Formation of this entity required inclusion of uridine diphosphoglucuronic acid (UDPGA) in the incubation, suggesting the formation of benoxaprofen acyl glucuronide followed by transesterification with the glycerol present in the incubation due to its presence as a stabilizer for liver subcellular fractions. Formation occurred during the sample work-up procedure while the samples were subjected to evaporation in vacuo, which does not remove glycerol. Conversion of purified benoxaprofen acyl glucuronide to the glycerol ester was demonstrated in glycerol at 37°C. Other drugs that are converted to acyl glucuronides in vitro (diclofenac, mefenamic acid, tolmetin, and naproxen) were also shown to form corresponding glycerol esters when incubated with human liver S-9 fraction and UDPGA. The potential formation of glycerol esters of carboxylic acid drugs undergoing acyl glucuronidation in vitro represents an experimental artifact to which drug metabolism scientists should be aware.

During the course of an examination of the in vitro metabolism of benoxaprofen, an unusual drug-related entity was observed on HPLC-MS that possessed 74 additional mass units from the parent drug. Further pursuit showed that it was a glycerol ester of benoxaprofen (Fig. 1) and that it could be an artifact. The term metabonate was coined by Beckett in 1971 (Beckett et al., 1971) to describe drug-related chemical entities observed in work-ups or storage of biological samples that can be mistaken for drug metabolites. These entities can arise by intramolecular rearrangements of metabolites (e.g., cyclization) (Erve, 2008) or by reaction of metabolites with constituents of sample work-up such as solvents (e.g., methanol) (Dickinson and King, 1989, 1991; Chabot and Gouyette, 1991). The objective of these studies was to characterize the formation of the glycerol ester of the carboxylic acid group of benoxaprofen, determine how this process arises, and determine whether other COOH-containing drugs can undergo this reaction and form metabonates.

Materials and Methods
Materials. [14C]Benoxaprofen was prepared by a custom synthesis by Nerviano Medical Sciences (Nerviano, Italy). The position of the radioactive atom was the 2-carbon on the benoxazole. Benoxaprofen was obtained from an in-house library of chemicals at Pfizer (Groton, CT). Alamethicin, uridine diphosphoglucuronic acid (UDPGA), diclofenac, tolmetin, and naproxen were obtained from Sigma-Aldrich (St. Louis, MO). Pentadeuterated glycerol was obtained from Isotec (Miamisburg, OH). Mefanamic acid was obtained from ICN (Aurora, OH). Pooled human liver microsomes and S-9 fraction were obtained from BD Gentest (Woburn, MA).

In Vitro Incubation Procedures. Incubations consisted of benoxaprofen (50 μM) and pooled human liver S-9 fraction containing 20% glycerol (5 mg/ml) with alamethicin (50 μg/ml), MgCl₂ (5 mM), and UDPGA (5 mM) in 100 mM potassium phosphate buffer at pH 7.5. Alamethicin was mixed with

ABBREVIATIONS: UDPGA, uridine diphosphoglucuronic acid; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; R₄, retention time.
S-9 and allowed to stand on ice for 15 min before addition of other components. Incubations were commenced with the addition of UDPGA and run at 37°C in a shaking water bath for 1 h. (Based on this procedure, the concentration of glucuronide in the incubation is 5%.) Incubations were terminated with five volumes of CH3CN, the precipitated materials were removed by centrifugation (1700 g, 5 min), and the supernatant was evaporated in vacuo in a Genevac vacuum centrifuge (Genevac, Valley Cottage, NY) operated with the settings at “HPLC Fraction” and a temperature that did not exceed 40°C. The residue was reconstituted in HPLC mobile phase (0.2 ml). This incubation procedure was also done for naproxen, diclofenac, mefameric acid, and tolmetin.

Synthesis of Benoxaprofen Glycerol Ester. Benoxaprofen (50 mg) was added to glycerol (10 ml) and heated to 80°C and stirred. Five drops of sulfuric acid were added, and the reaction mixture was stirred for 2 h. The mixture was added to 300 ml of ice-cold water and extracted with CHCl3 (2 volumes CH3CN, 10 ml). The combined extracts were dried under nitrogen, and the residue was reconstituted in 0.3 ml of H2O/CH3CN/HCOOH (50:50:0.05). This mixture was injected onto a Waters Novapak C18 (Waters, Milford, MA) semipreparative column (7.9 x 300 mm; 7 μ) equilibrated in aqueous 0.1% HCOOH containing 40% CH3CN at a flow rate of 5 ml/min. The eluent was divided into 1-min fractions, and the fractions containing benoxaprofen 1-glycerol ester were combined and lyophilized to yield 10 mg product. 1H NMR (600 MHz; DMSo-d6): δ 8.80 (d, 2H), δ 7.75 (s, 1H), δ 7.74 (d, 1H), δ 7.69 (d, 2H), δ 7.39 (d, 2H), δ 4.86 (s, OH, 1H), δ 4.59 (s, OH, 1H), δ 4.09 and 4.02 (m, 2 × 0.5H), δ 4.00 (m, 1H) δ 3.97 and 3.90 (m, 2 × 0.5H), δ 3.61 (m, 1H), δ 3.28 (d, 2H), δ 1.48 (d, 3H), 4.59 (s, OH, 1H).

Biosynthesis of Benoxaprofen Acyl Glucuronide and Reaction with Glycerol. Benoxaprofen (20 μM) was incubated with pooled human liver microsomes (2 mg/ml), alamethicin (0.04 mg/ml), UDPGA (6.9 mM), and MgCl2 (5 mM) in 10 ml of potassium phosphate buffer at pH 7.5. The incubation was carried out at 37°C for 1 h followed by addition of CH3CN (10 ml) and centrifugation at 1700g to remove precipitates. Formic acid in water (0.1%; 100 ml) was added to the supernatant, and the resulting mixture was spun in a centrifuge at 40000g to clarify the mixture. The supernatant was loaded onto a Varian Polaris C18 column (4.6 × 250 mm; 5 μ) at 0.8 ml/min using a Jasco (Tokyo, Japan) PU-980 HPLC pump. After loading the entire sample, the column was moved to a Thermo-Finnigan Surveyor quaternary HPLC pumping system (Thermo Fisher Scientific, Waltham, MA) at 0.8 ml/min, and a gradient was applied starting at 30% CH3CN/0.1% HCOOH for 5 min followed by a linear increase to 90% CH3CN at 25 min. The effluent was collected into 20-s fractions, and the material eluting between 19.0 and 19.7 min contained benoxaprofen acyl glucuronide and was shown to be pure by HPLC. The solvent was removed in vacuo in a conical glass tube. After drying, glycerol (0.05 ml) was added and the tube was sonicated. A small aliquot was removed for immediate HPLC analysis, a second portion was removed, sealed, and placed at −20°C, and the remaining material was sealed and placed in a 37°C water bath. At various time points up to 4 days, aliquots were removed and analyzed by HPLC for the formation of benoxaprofen glycerol ester.

HPLC-MS/MS. The HPLC-mass spectrometry system consisted of a Surveyor quaternary pump, degasser, autoinjector, and photodiode array detector coupled to a Thermo-Finnigan LTQ ion trap mass spectrometer (Thermo Fisher Scientific). Separation was effected on a Varian Polaris C18 (Varian, Inc., Palo Alto, CA) column (4.6 × 250 mm; 5μ) using a mobile phase of 0.1% HCOOH in water (“A”) and CH3CN (“B”) at a flow rate of 0.8 ml/min. The mobile phase composition was held at 30% B for 5 min followed by a linear gradient to 90% B at 25 min. The eluent was split, with ~0.06 ml/min introduced into the source of the mass spectrometer operated in the positive ion mode. Instrument settings and potentials were adjusted to optimize the signal for the protonated molecular ion for benoxaprofen. Using these conditions,
benoxaprofen, benoxaprofen acyl glucuronide, and benoxaprofen glycerol ester eluted at 25, 20, and 22 min, respectively.

Results

**Metabolism of \([\text{¹⁴C}]\)Benoxaprofen to the Acyl Glucuronide and Glycerol Ester.** Incubation of \([\text{¹⁴C}]\)benoxaprofen in pooled human liver S-9 fraction in the presence of UDPGA yielded the acyl glucuronide with approximately 25% of the starting material converted to this metabolite in 60 min (Fig. 2). Two peaks corresponding to the acyl glucuronide were observed with a protonated molecular ion of \(m/z\) 478, an addition of 176 mass units. Fragment ions include \(m/z\) 460 (loss of water) and \(m/z\) 302 (loss of glucuronide) (Fig. 3B). In addition, another metabolite was observed at retention time (Rt) 22 min (Fig. 2). It had a protonated molecular ion of \(m/z\) 376 and was clearly related to the parent drug because of the isotope pattern of chlorine (Fig. 4A) and elution of radioactivity. Collision-induced dissociation yielded fragment ions of \(m/z\) 358 (loss of water), 302 (loss of 74 mass units to benoxaprofen), and \(m/z\) 256 (loss of 120 mass units). This metabolite is an ester of benoxaprofen and glycerol, and the spectral and chromatographic properties matched a chemically prepared authentic standard. The ion at \(m/z\) 302 represents the neutral loss of glycerol and \(m/z\) 256 the neutral loss of glycerol formate. When the incubation was conducted with \([\text{²H₅}]\)glycerol added, the protonated molecular ion was at \(m/z\) 381 (Fig. 4B), five mass units greater than, and confirming the structure of, the glycerol adduct. The formation of the glycerol ester required the presence of UDPGA, suggesting the obligatory formation of benoxaprofen acyl glucuronide, which reacts with glycerol present in the incubation. When \([\text{²H₅}]\)glycerol was added to the incubation after termination, the same relative amount of pentadeuterated benoxaprofen glycerol ester was observed, suggesting that the metabolite was formed by spontaneous glycerolysis of the acyl glucuronide during sample work-up. Thus, it is an artifact of the glycerol present in in vitro incubations (as a stabilizing agent for freezer storage of liver subcellular fractions), rather than an enzyme-generated metabolite (i.e., a metabonate).

**Formation of Benoxaprofen Glycerol Ester from Benoxaprofen Acyl Glucuronide.** Benoxaprofen acyl glucuronide was biosynthesized by incubation with human liver microsomes, alamethicin, and UDPGA, and isolated by HPLC. This material was dissolved in stabilizing agent for freezer storage of liver subcellular fractions), rather than an enzyme-generated metabolite (i.e., a metabonate). When \([\text{²H₅}]\)glycerol was added to the incubation after termination, the same relative amount of pentadeuterated benoxaprofen glycerol ester was observed, suggesting that the metabolite was formed by spontaneous glycerolysis of the acyl glucuronide during sample work-up. Thus, it is an artifact of the glycerol present in in vitro incubations (as a stabilizing agent for freezer storage of liver subcellular fractions), rather than an enzyme-generated metabolite (i.e., a metabonate).

**Generation of Glycerol Esters of Other Carboxylic Acid Drugs.** To determine whether the formation of the glycerol ester was specific for benoxaprofen, several other drugs known to form acyl glucuronides were incubated with pooled human liver S-9 fraction and UDPGA in the presence of added \([\text{²H₅}]\)glycerol to facilitate identifi-
cation of glycerol esters through the observation of an isotope pattern five mass units apart. Diclofenac, mefenamic acid, naproxen, and tolmetin all demonstrated formation of glycerol esters (Fig. 6), indicating that other carboxylic acid drugs besides benoxaprofen can form this artifact in in vitro incubations. Diclofenac glycerol ester showed protonated molecular ions at m/z 370 and 372 as well as the ester to the pentadeuterated glycerol at m/z 375 and 377. Mefenamic acid and tolmetin yielded glycerol esters with protonated molecular ions at m/z 316 and 332, respectively, along with the corresponding pentadeuterated analogs. For naproxen, the signal was weaker and the largest ion for the glycerol ester was the ammonium adduct ion (m/z 322 and 327 for glycerol and [2H5]glycerol, respectively), whereas the protonated molecular ions were present at a lower abundance (m/z 305 and 310). Each of these ions is 74 (or 79 for pentadeuterated glycerol) mass units greater than the corresponding parent drug ions indicative of glycerol esters. Thus, glycerol esters can be formed from acyl glucuronides of drugs other than benoxaprofen.

Discussion

In drug metabolism studies, investigators must be wary of the formation of metabonates: drug-related entities that arise from specific conditions of sample storage or work-up. Several examples of this phenomenon exist in the scientific literature and include oximes (Beckett et al., 1971), imidazolines (Li et al., 2006), phenothiazines (Beckett and Navas, 1978), esters (Dickinson and King, 1989), and many entities arising from internal cyclization reactions (reviewed in Erve, 2008). In the course of the study of the glucuronidation of [14C]benoxaprofen in human liver S-9 fraction, in addition to observation of the expected acyl glucuronide metabolite, an unusual drug-related entity was also observed (Fig. 2). It possessed a molecular ion that was 74 mass units greater than the parent drug. Further pursuit of this compound showed that it was the glycerol ester of benoxaprofen formed by transesterification (i.e., glycerolysis) of the acyl glucuronide metabolite. It is well known that esterification of carboxylic acid-containing drugs and metabolites can occur when alcohols (e.g., methanol, ethanol) are used in sample work-up and/or analysis, and this phenomenon makes the use of simple alcohols less preferred in drug metabolism studies. This process is the first description of a glycerol ester formed as an artifact in drug metabolism studies.

Glycerol (at a concentration of 20%) is used as a stabilizer for the long-term frozen storage of subcellular fractions. It has been used to stabilize cytochrome P450 enzymes and will increase stability even at high temperatures (Ichikawa and Yamano, 1967; Metelitza et al., 1982). Thus, in vitro metabolism studies in which liver S-9 or microsomes are used will contain a small amount of glycerol, with the final percentage depending on the concentration of the enzyme source used in the incubation. (In these incubations, glycerol was present at 5%). This low percentage is probably not enough to cause glycerol ester formation in situ. However, when the incubation samples are processed by precipitation of the protein followed by evaporation, the concentration of glycerol will increase because it is not volatile and therefore not removed in vacuo. After evaporation of in vitro samples, a small sticky residue of material remains and probably contains a high content of glycerol. Thus, the generation of the glycerol ester can occur throughout the evaporation process as the glycerol concentration increases. When [2H5]glycerol was added versus before the incubation, the glycerol ester observed had the same ratio of pentadeuterated and nondeuterated material, consistent with the formation occurring during sample work-up and not during the incubation. Furthermore, isolation of benoxaprofen acyl glucuronide and incubation in glycerol yielded the glycerol ester, and this reaction occurred at 37°C but not at −20°C.

The formation of benoxaprofen glycerol ester required UDPGA, indicating that it could not form spontaneously between glycerol and benoxaprofen. Instead, activation to the acyl glucuronide was required as a first step (Fig. 1). Thus, this type of metabonate should not be observed in incubations that do not use conditions required for uridine diphosphoglucuronosyltransferase activity, such as those used to study...
cytochrome P450 activity. It is possible that other types of activation of carboxylic acids, such as esterification with coenzyme A, could ultimately be subject to glycerolysis, but this has not yet been studied. Furthermore, formation of this metabonate should not occur with ether or amine glucuronides, because these are not reactive metabolites. In addition, if glucuronidation is being studied in hepatocyte incubations or in incubations with subcellular fractions stored with sucrose as the stabilizer instead of glycerol, the glycerol ester should not be observed. Glycerol esters of carboxylic acid drugs should not be observed in in vivo biological matrices, such as plasma or urine, because there should be no glycerol present. Finally, if samples are not processed by evaporation, which effectively increases the glycerol concentration, but instead are analyzed directly after termination of in vitro incubations, then glycerol esters should not be observed. Thus, this type of artifact is restricted to specific types of substrates, in vitro incubations, and work-up procedures.

The formation of the glycerol ester metabonate was not restricted to benoxaprofen but was observed for tolmetin, mefenamic acid, diclofenac, and naproxen as well. In each of these cases, the acyl glucuronide metabolite was readily formed, making it possible to form the glycerol ester in the same manner as that used for benoxaprofen. Thus, the formation of glycerol ester metabolites should be possible for any carboxylic acid that undergoes acyl glucuronidation in vitro. In conclusion, these data demonstrate that drug metabolism scientists need
to be aware of the generation of glycerol esters of carboxylic acid drugs and metabolites as artifacts in in vitro investigations.

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