Glucuronidation of Prodrug Reactive Site: Isolation and Characterization of Oxymethylglucuronide Metabolite of Fosphenytoin

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Background: This investigation was undertaken to identify the structure of a novel immunoreactive metabolite derived from fosphenytoin that has been hypothesized previously as present in sera from renally impaired patients receiving this prodrug.

Methods: The metabolite was isolated from uremic sera using solid-phase extraction and HPLC. Structural analysis was performed using HPLC–tandem mass spectrometry, nuclear magnetic resonance (NMR), deuterium exchange, and chemical derivatization. Immunoreactivity was evaluated using a fluorescence polarization immunoassay.

Results: The metabolite had a parent ion at m/z 457 in the negative-ion mode and fragmented to yield the m/z 251 of phenytoin, as well as other mass fragments of phenytoin. Mass fragments associated with glucuronic acid were also present. The chromatographic peak corresponding to this metabolite demonstrated immunoreactivity sufficient to lead to falsely increased reported values for phenytoin immunoassays. The observed immunoreactivity was also proportional to the relative concentration of the metabolite in collected fractions. Analysis by NMR indicated the presence of phenyl groups with chemical shifts identical to those of phenytoin, as well as the presence of a methylene bridge, which was consistent with the same methylene bridge present on the phosphate ester of fosphenytoin. Comparative analysis of serum samples from renally impaired patients receiving phenytoin vs fosphenytoin using multiple reaction monitoring quantification demonstrated that this metabolite was associated with fosphenytoin administration.

Conclusions: A unique immunoreactive oxymethylglucuronide metabolite derived from fosphenytoin has been isolated from sera from uremic patients receiving this prodrug.

A major problem with the use of many classes of therapeutic drugs has been the challenge to deliver the agent to the desired site of action safely and effectively. In some cases, the active drugs are poorly absorbed after oral delivery and thus have variable bioavailability. In other cases, the drugs are insoluble in aqueous matrices and thus cannot be used effectively as intravenous or intramuscular agents in critical settings. Some drugs may be rapidly metabolized and thus do not circulate long enough to accumulate at the desired site of action.

The concept of “prodrug” development has been introduced as a solution to these types of problems. Prodrugs are structural analogs or derivatives of the desired active drug that endow the compound with properties that help mediate the delivery, pharmacokinetics, or action of the drug. Examples of recently introduced compounds include drug forms of platelet-activating factor antagonists (1), antiviral nucleoside analogs (2), cytoprotective agents (3), antineoplastic derivatives (4–6), neuroprotective drugs (7), and anti-influenza virus drugs (8).

One approach to increasing the solubility of drugs containing hydroxyl groups, or drugs in which a hydroxyl group can be attached to a chemical bridge, has been the addition of a phosphate group (6, 9, 10). Fosphenytoin (Fig. 1) is a phosphate ester prodrug of phenytoin that provides improved efficacy and safety when given intravenously or intramuscularly (11). After systemic administration, the phosphate moiety of fosphenytoin is rapidly
and H-D, hydrogen-deuterium.

cleaved (12) to form an unstable intermediate that breaks down to yield formaldehyde and the active drug phenytoin. Subsequent metabolism and renal clearance should follow the same hydroxylation and conjugation pathways known to exist for phenytoin. A recent report (13) presented data that supported the hypothesis of a unique fosphenytoin metabolite found in renally impaired patients receiving fosphenytoin. This purported metabolite demonstrated substantial cross-reactivity in numerous commercial phenytoin immunoassays, leading to falsely increased reported values for phenytoin.

In this study, we confirmed that a novel metabolite of fosphenytoin does indeed exist. We were able to initially identify, using gradient reversed-phase HPLC–tandem mass spectrometry (HPLC–MS–MS),4 a compound with a m/z 251 phenytoin ion and a parent m/z 457 ion. This metabolite was subsequently isolated and characterized by chemical derivatization, HPLC, MS–MS, exact mass analysis, isotope exchange, and nuclear magnetic resonance (NMR) spectroscopy. The metabolite is immunoreactive in the most widely used immunoassay for monitoring phenytoin and has been structurally identified as the N-3′-oxygenmethylglucuronide of phenytoin. To our knowledge, this also illustrates the first example of direct glucuronidation of the reactive site on a prodrug.

**Materials and Methods**

**SERUM SPECIMENS**

The serum samples used for the isolation and characterization of fosphenytoin metabolites from uremic patients receiving fosphenytoin were the same as those used in a prior Institutional Review Board-approved study (13). HPLC analyses (14) confirmed that these specimens contained no fosphenytoin that could account for the cross-reactivity attributed to a novel fosphenytoin metabolite (15). Sera from uremic patients receiving phenytoin were obtained from specimens used in a separate approved study (16).

**DRUGS AND REAGENTS**

Phenytoin, 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH), and N-3′-hydroxymethylphenytoin were obtained from Pfizer Pharmaceutical. Phenytoin- N-glucuronide and HPPH-glucuronide were synthesized as described previously (17). TDx phenytoin and free phenytoin reagents were purchased from Abbott Laboratories and were used according to the manufacturer’s instructions. The methylating reagent BF3–methanol was obtained from Pierce Chemical. Acetonitrile was purchased from Mallinkrodt.

Deuterated solvents were from Cambridge Isotope Laboratories Inc. Oasis HLB 1-mL extraction columns were obtained from Waters Corporation.

**PURIFICATION OF METABOLITE FROM SERUM**

Two 1.0-mL aliquots of pooled sera from uremic patients receiving fosphenytoin (14) were placed into two separate polypropylene tubes. Acetonitrile (4 mL) was added to each tube with vigorous vortex-mixing for 60 s. The tubes were centrifuged, and the acetonitrile supernatants from the two tubes were combined and dried, in small additions, under nitrogen in a 1.5-mL polypropylene microcentrifuge tube. The contents of the microcentrifuge tube were reconstituted into 100 μL of methanol and vortex-mixed, followed by the addition of 1 mL of water with the final vortex. The liquid was transferred to a 30-mg Oasis extraction column that had been activated with 100 μL of methanol and rinsed with water. The column was washed with 300 μL of water–acetonitrile (95:5 by volume). Phenytoin metabolites were eluted from the column with four serial additions of water–acetonitrile (80:20 by volume). The individual eluates were analyzed qualitatively for the presence of the m/z 457 metabolite using a Perkin-Elmer Series 200 autosampler and pump connected to a Micro-HPLC AVP 3000QQ QQQ mass spectrometer. Fractions containing the metabolite were pooled and dried under nitrogen. The pooled solid-phase-extracted fractions containing the metabolite were reconstituted in methanol–water (10:90 by volume), and the m/z 457 compound was isolated chromatographically using a 2.1 × 150 mm Zorbax RX-C18 column with a mobile phase of acetonitrile–1 mL/L acetic acid in water (22:78 by volume) at a flow rate of 0.3 mL/min. Fractions (1 min; 0.3 mL) were collected in 1.5-mL polypropylene microcentrifuge tubes and analyzed by MS–MS for phenytoin, HPPH, HPPH-glucuronide, phenytoin-N-glucuronide, and the fosphenytoin metabolite. Phenytoin-N-glucuronide and HPPH-glucuronide were monitored in the positive-ion mode because of better response, and the other compounds were monitored in the negative-ion mode for the same reason. Fractions containing the fosphenytoin metabolite were pooled, evaporated, and reconstituted in a small volume of methanol–water (10:90 by volume). Further purification was performed using a 2.0 × 100 mm Betamax Acid column, using a mobile-phase of acetonitrile–1 mL/L acetic acid plus 10 mmol/L ammonium acetate in water (85:15 by volume) at a flow rate of 0.3

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4 Nonstandard abbreviations: MS–MS, tandem mass spectrometry; NMR, nuclear magnetic resonance; HPPH, 5-(p-hydroxyphenyl)-5-phenylhydantoin; and H-D, hydrogen-deuterium.
mixing volume, were placed into a 0.5-mL polypropylene microcentrifuge tube and evaporated under nitrogen. The residue was reconstituted with 150 µL of water. The solvents containing acetonitrile were removed to mobile phase containing acetonitrile. The dried tube was reconstituted with 150 µL of 9 g/L NaCl, vortex-mixed, and analyzed on the TDx instrument.

**Immunoassay Reactivity**

During chromatographic separation and purification of the m/z 457 fosphenytoin metabolite, the 1-min (0.3 mL) fractions were analyzed using the TDx free phenytoin fluorescence polarization immunonoassay. A 150-µL aliquot of each eluate fraction was placed in a 0.5-mL polypropylene microcentrifuge tube and evaporated under nitrogen to remove mobile phase containing acetonitrile. The dried tube was reconstituted with 150 µL of 9 g/L NaCl, vortex-mixed, and analyzed on the TDx instrument.

**Exact Mass Analysis**

Exact mass analysis was performed on a Varian Inova 600 HPLC-NMR, using the VNMR 6.1B program, equipped with a 1H-[15N, 13C] flow cell with a 60-µL active volume, in series with a Waters ZMD single quadrupole mass spectrometer. To alleviate the effects of exogenous impurities, HPLC isolation of the m/z 457 metabolite for online NMR was performed on a Zorbax XDB-C8 column (2.1 mm×15 cm) in series with an Upchurch Uptight guard column (Upchurch Scientific) packed with C18 pellicular (40 µm particle size). Separation was achieved using a mobile phase of deuterioacetic acid (1 mL/L) in deuterium oxide–deuterioacetonitrile (80:20 by volume; flow rate, 0.3 mL/min). The peak corresponding to the metabolite was monitored using m/z 461 (negative-ion mode; four exchangeable protons by 2H). NMR data were acquired at the apex of the peak. The 1D NMR spectra were generated using fast Fourier transformation of the free induction decay, which were the sums of 18 352 transients. Each transient was induced using a nonselective 90° 1H pulse with a selective presaturation pulse applied during the relaxation decay to the HDO resonance. The resulting time-averaged free induction decay was multiplied by an exponential decay function (line broadening, 3 Hz) to enhance the signal-to-noise ratio.

**Measurement of Metabolite in Serum**

Relative amounts of the metabolite were assayed in sera from 12 uremic patients receiving fosphenytoin and 12 uremic patients receiving phenytoin. Uremic patient serum (3 µL) plus blank plasma (10 µL), to increase the mixing volume, were placed into a 0.5-mL polypropylene microcentrifuge tube. This was followed by the addition of 300 µL of acetonitrile with vigorous vortex-mixing for 30 s. After centrifugation for 5 min, the acetonitrile supernatant was transferred to a second 0.5-mL tube and evaporated under nitrogen. The residue was reconstituted with 5 µL of methanol, vortex-mixed, and 45 µL of water was added for a total volume of 50 µL. After vortex-mixing, the contents were transferred to a glass injection vial, and 10 µL was analyzed by HPLC-MS-MS. Chromatography was performed on a 2.1 × 150 mm Zorbax RX-C18 column, with a mobile phase of acetonitrile–1 mL/L acetic acid in water (15:85 by volume) at a flow rate of 0.3 mL/min. The integrated area response of the metabolite was monitored in the multiple reaction monitoring mode with the transition of m/z 457 to 193 using the Micromass Quattro Ultima tandem mass spectrometer.

**Deuterium Exchange Experiments**

Individual solutions (50-µL aliquots) of phenytoin, HPPH, HPPH-glucuronide, phenytoin-N-glucuronide, and the purified fosphenytoin metabolite were incubated with 200 µL of deuterium oxide for 1 h at ambient temperature. After this incubation, 10-µL volumes were injected directly into the Micromass Quattro Ultima mass spectrometer, using the Perkin-Elmer autosampler, in a mobile phase of acetonitrile–deuterium oxide (50:50 by volume). No analytical column was used because no separations were needed.

**Carboxyl Group Methylation**

A solution (10 µL) containing purified fosphenytoin metabolite was incubated overnight with an excess of BF3 in methanol. The mixture was then evaporated, reconstituted in 100 mL/L methanol, and injected onto a Betamax Column acid using a mobile phase of acetonitrile–1 mL/L acetic acid plus 10 mmol/L ammonium acetate in water (85:15 by volume) at a flow rate of 0.3 mL/min.

**Results**

Analysis of the fosphenytoin metabolite by exact mass analysis demonstrated a m/z 457.11 compound in the negative-ion mode. Masslynx software was used to predict chemical formulas that would be consistent with this mass. Fosphenytoin is a prodrug derivative of phenytoin, and we assumed that the metabolite would possess some core phenytoin-like component. Criteria were put into the elemental composition software to exclude chemical formulas that would not be consistent with a phenytoin-type structure. These included the following: (a) a minimal number of nitrogen, carbon, and hydrogen molecules; (b) 10–30 double-bond equivalents because phenytoin contains multiple double bonds; and (c) a mass tolerance of 0.04 atomic mass units. Elemental composition yielded 67 formulae, of which 7 met the specified tolerances. One formula, C22H21N2O9, matched the elemental composition predicted by our structural studies.
A complete mass spectrum of the isolated fosphenytoin metabolite, obtained with the Micromass Ultima MS-MS system, is shown in Fig. 2. As illustrated in Fig. 2, there are several special features to this mass spectrum: The parent \( m/z \) 457 ion matches the accurate mass observed with the Mariner time-of-flight instrument. In addition, several of the daughter masses can be attributed to phenytoin via comparison with reference spectra generated for phenytoin using the same tandem mass spectrometer. These include \( m/z \) 251, 131, 103, and 77. The \( m/z \) 193, 175, and 113 fragments cannot be demonstrated to be products of phenytoin, but they can be attributed to parent and daughter fragments known to exist for glucuronic acid.

**CHROMATOGRAPHIC CHARACTERISTICS**

The retention properties under several different chromatographic conditions were also consistent with the proposed structure of the fosphenytoin metabolite. Using conventional reversed-phase chromatography on a C18 column, the metabolite demonstrated a shorter retention time similar to the polar phenytoin glucuronide metabolites (all times <7 min). In contrast, phenytoin was highly retained on the column with a retention time of 14 min.

Retention properties were also checked using a Betamax Acid column. This column uses a special embedded polar group in a long alkyl chain and is designed to have a special affinity for carboxylic acid groups. As expected, phenytoin and HPPH did not interact strongly with the column. In contrast, the fosphenytoin metabolite, HPPH-glucuronide, and phenytoin-\(N\)-glucuronide were observed to have stronger interactions and longer retention times. Retention of the fosphenytoin metabolite and phenytoin-\(N\)-glucuronide, which have a free carboxylic acid (18, 19), were very close; these compounds were also the last eluting peaks.

**IMMUNOREACTIVITY OF FOSPHENYTOIN METABOLITE**

The immunoassay response plotted against chromatographic time for a serum specimen from one of the uremic patients receiving fosphenytoin is shown in Fig. 3. The chromatography time was expanded to maximize separation. The retention times of the various hydantoin compounds are also indicated in Fig. 3, illustrating that two predominant peaks with measurable immunoreactivity were observed. The first peak (10–14 min) was identified as phenytoin, and the second peak (26–32 min) was identified as the \( m/z \) 457 fosphenytoin metabolite. This demonstrates that the fosphenytoin metabolite has a readily observed cross-reactivity in this particular immunoassay.

During various stages of purification of the metabolite from uremic sera, as well as during analyses of acetonitrile precipitates of serum samples, chromatographic eluate fractions containing the metabolite were assayed to demonstrate a correlation between the relative concentration of the metabolite and the phenytoin concentration as measured by immunnoassay. A direct correlation exists and confirms that the metabolite does indeed cross-react with the phenytoin immunoassay, as illustrated by Fig. 4.

**NMR STRUCTURAL ANALYSIS**

The NMR data obtained for the metabolite and two reference compounds are illustrated in Fig. 5. Phenytoin-\(N\)-glucuronide (top spectrum) demonstrated two salient features by NMR. The two phenyl rings showed two
distinct resonances at 7.3 and 7.4 ppm, whereas the glucuronide moiety produced the five resonances (1\textsuperscript{9} – 5\textsuperscript{9}) consistent with the presence of such a carbohydrate structure. For hydroxymethylphenytoin (Fig. 5, middle spectrum), the phenyl rings also showed the same resonance pattern because they were not modified or conjugated in any way. Because no glucuronide is present on hydroxymethylphenytoin, none of the resonances associated with the carbohydrate was observed. However, the added CH\textsubscript{2} group (peak A) produced a predicted single resonance at 5.0 ppm. The NMR pattern for the fosphenytoin metabolite (Fig. 5, bottom spectrum) showed resonances consistent with both of the other reference compounds. The resonance signals for the two phenyl rings are identical at 7.3 and 7.4 ppm, showing that the point of conjugation is not on either of the phenyl rings. The resonances 1\textsuperscript{9}–5\textsuperscript{9} between 3.0 and 4.5 ppm were consistent with what would be expected for a glucuronide. The CH\textsubscript{2} bridge (peak A) was present, but was split because the two hydrogens were under the influence of two different elements: the hydantoin ring and the glucuronide ring.

DEUTERIUM EXCHANGE
The results of hydrogen-deuterium (H-D) exchange helped confirm the identity of the fosphenytoin metabolite. Because a hydrogen (in this case deuterium after exchange) molecule usually is lost in the negative-ion mode, one of the exchanged deuteriums will be lost; therefore, the observed \(m/z\) for the compound will be 1 mass unit less than the actual \(m/z\) value. It should be noted that C=H bonds are not readily exchanged, and the major H-D exchanges will be observed for N=H and O=H bonds. The data listed in Table 1 show that the predicted and observed changes in mass composition because of deuterium exchange are all consistent with the structures of the hydantoin compounds, including the proposed structure of the fosphenytoin metabolite.

CARBOXYL GROUP DERIVATIZATION
Our chromatographic studies demonstrated that the fosphenytoin metabolite was retained on the Betamax Acid HPLC column, which was presumed to result from the presence of a carbonyl group on the molecule. Derivatization with BF\textsubscript{3}–methanol, which selectively methylates any carbonyl group present, led to an observed loss of the \(m/z\) 457 metabolite at the expected chromatographic retention time and the appearance of a new compound with a \(m/z\) of 471 that eluted near the void volume. This change of 14 mass units was consistent with conversion of a single COOH group to COOCH\textsubscript{3}.

METABOLITE CONCENTRATIONS IN UREMIC PATIENT SERA
The comparison of sera from uremic patients receiving phenytoin vs fosphenytoin is summarized in Table 2. Sera samples (\(n = 12\)) in each group were all from different patients. The metabolite was found in the sera from the patients who had received fosphenytoin, but was not readily identifiable above the background response in the sera from phenytoin-treated patients.

Discussion
The initial identification of a fosphenytoin metabolite required some serendipity. We initially attempted to isolate metabolites of phenytoin and fosphenytoin using an immunologic approach. Sera from patients were incubated with 2- to 5-mL volumes of the Abbott polyclonal antisera reagent. After treatment with protein A-Sepharose and the subsequent release of antibody-bound compounds, the supernatants were evaluated using HPLC-MS-MS. Unfortunately, the higher concentrations of phenytoin in these sera, plus the primary specificity
toward phenytoin, yielded isolates that clearly showed only phenytoin. As a result, we were forced to screen serum extracts, using HPLC-MS-MS, to identify fosphenytoin metabolites. Assuming that the metabolite possessed the phenytoin or HPPH core as part of its overall structure, we performed reversed-phase chromatography with a shallow acetonitrile gradient, monitoring \( m/z \) 251 and 268 in the negative-ion mode. We were fortunate to observe two \( m/z \) 251 peaks, which were then further characterized by identifying the parent ions of the 251 fragment. One was identified as a compound with a parent \( m/z \) of 457, and the second was identified as phenytoin. With this information, we proceeded to isolate the \( m/z \) 457 metabolite according to the protocols outlined earlier. Solid-phase extraction was required as a first step in the purification. High concentrations of salts were present in the residue from the acetonitrile precipitation of patient sera. These salts suppress signals during mass spectral analysis and thus should be eliminated if possi-

### Table 1. H-D exchange data.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mass</th>
<th>Predicted H-D mass</th>
<th>Observed H-D mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenytoin</td>
<td>251</td>
<td>252</td>
<td>252</td>
</tr>
<tr>
<td>Phenytoin-N-glucuronide</td>
<td>427</td>
<td>431</td>
<td>431</td>
</tr>
<tr>
<td>HPPH-glucuronide</td>
<td>443</td>
<td>448</td>
<td>448</td>
</tr>
<tr>
<td>HPPH</td>
<td>267</td>
<td>269</td>
<td>269</td>
</tr>
<tr>
<td>Fosphenytoin metabolite</td>
<td>457</td>
<td>461</td>
<td>461</td>
</tr>
</tbody>
</table>

\( * m/z, \) negative-ion mode.

### Table 2. Comparison of relative metabolite concentrations in uremic patients receiving phenytoin vs fosphenytoin.\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Phenytoin patients</th>
<th>Fosphenytoin patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxy methyl glucuronide</td>
<td>Mean ± SD, area units</td>
<td>280 ± 808</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>0–2825</td>
</tr>
<tr>
<td>Creatinine</td>
<td>Mean ± SD, mg/L</td>
<td>65 ± 37</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>16–143</td>
</tr>
</tbody>
</table>

\( * \) Separate patients from each group (n = 12); comparable range of serum phenytoin concentrations in each group.
ble. After application of the crude serum extracts onto a column, the salts could be removed by elution with water. Compounds of interest were subsequently eluted in fractions of increasing organic solvent content, evaporated, and purified on the chosen analytical columns.

Although HPLC-MS-MS is a valuable tool for obtaining structural information, H-D exchange methods are useful for the determination of exchangeable functional groups on parent and metabolite structures and therefore as an aid in structure validation (20). In our experiments, deuterated solvents were used in the HPLC mobile phase to prevent back-exchange. Phenytoin has two labile hydrogen atoms at the secondary amino groups and will exchange these for two deuterium atoms. With the loss of one of these during negative-ion-mode analysis, the m/z value for phenytoin would be predicted to change from 251 to 252 after H-D exchange, which is what we observed. If the fosphenytoin metabolite were a hydroxymethylglucuronide, we would predict a change of 5 mass units from exchangeable hydrogen atoms (4 on the glucuronide, 1 on the hydantoin ring). The loss of one deuterium atom in the negative-ion mode would yield a mass of 461, which was exactly what we observed.

The H-D exchange experiments were consistent with our hypothesis that the fosphenytoin metabolite was a unique glucuronide conjugate. These experiments did not, however, demonstrate whether the conjugation was through the hydroxyl or the carboxyl group of glucuronic acid. This question was solved through our chromatography data, as well as through the BF$_3$–methanol derivatization data. If the carboxyl group were intact, then it would be methylated with this carboxyl-derivatizing reagent, yielding a compound with a parent mass 14 units higher. Additionally, if the carboxyl group were derivatized, then the glucuronide would be poorly retained on the Betamax Acid HPLC column. The results of our experiment showed that (i) before BF3 derivatization, the m/z 457 metabolite was retained on the Betamax Acid column with a retention time similar to that of phenytoin–N-glucuronide; (ii) after derivatization, the m/z 457 peak disappeared, and a compound of m/z 471 was observed; and (c) the m/z 471 compound was poorly retained on the column. These observations were consistent with the conjugation involving one of the hydroxyl groups on glucuronic acid.

With the HPLC-MS-MS, H-D exchange, exact mass, elemental composition, and derivatization data, we were able to account for the presence of both phenytoin and glucuronic acid as part of the structure of the fosphenytoin metabolite. These two entities would account for 444 mass units from the overall 458 mass for the complete metabolite, leaving 14 mass units to be explained. The possibilities allowed by the elemental composition software calculations showed that only a CH$_2$ and not an N, could account for this mass. The NMR data were invaluable in confirming that the two phenyl groups were not conjugated and that the CH$_2$ was present on a bridge between the phenytoin and glucuronide.

Because this is a new metabolite and insufficient purified reference material was available for true quantification, we were only able to measure the relative amount of the oxymethylglucuronide metabolite in sera from uremic patients receiving fosphenytoin vs phenytoin. Our data demonstrate that the metabolite is present in the sera from patients receiving fosphenytoin. These results, in conjunction with our NMR data showing that the methylene bridge from fosphenytoin is still present on the metabolite, make us confident that fosphenytoin is the source of the new metabolite.

Considering the lower cross-reactivity of HPH-glucuronide in commercial immunoassays, why would this metabolite seem to be sufficiently immunoreactive to cause the large false increases reported for phenytoin (13) in some patients receiving fosphenytoin? One contributing factor may be the fact that, for this novel metabolite, the glucuronide is conjugated onto the hydantoin ring of phenytoin rather than the phenyl ring. Another possibility is that the ester bridge may allow a better structural separation of the glucuronide and phenytoin portions of the compound, thus allowing a better conformational rotation and better reactivity with the antibody.

The implications of our identification of this novel metabolite cross several areas, including analytical effects (13), drug metabolism, and pharmacodynamics. This study demonstrates the direct glucuronidation of a prodrug reactive site. This same sort of reactive-site glucuronidation (or other type of conjugation) could theoretically occur with other prodrugs, yielding a route of metabolism that differs from conventional predictions. There currently are several types of chemical derivatives (e.g., prodrugs) of existing drugs that could be candidates for the same type of glucuronidation. In the area of anticancer therapy, benzyl phosphate derivatives of α-N-heterocyclic carboxyaldehyde thiosemicarbazone ribonucleotide reductase inhibitors have been synthesized and used in clinical investigations (21). In a manner similar to that for fosphenytoin, there is a proposed bioactivation pathway for these anticancer drugs where cleavage of the phosphorus–oxygen bond leads to an unstable intermediate, followed by a cascade fragmentation effect where quinone methide and CO$_2$ are lost to yield the desired active drug species.

Phosphate monoesters, such as fosphenytoin, are not the only group of prodrugs that contain a reactive site that could be chemically modified. For drugs that have a primary or secondary amine, aromatic amine, phenol, or sterically hindered secondary alcohol, phosphoryloxy-methyl carbonate and carbamate prodrugs have been synthesized (22). These compounds actually yield two potentially reactive intermediates after hydrolysis by phosphatase enzymes. The first intermediate, a reactive
hydroxymethyloxycarbonyl group that has a terminal hydroxyl group, decomposes to yield formaldehyde and a second unstable intermediate containing a terminal oxycarbonyl group. This secondary spacer group is believed to ultimately release CO₂ and the parent drug. Yet another approach to developing a hydrolyzable promoiety has been the development of acyloxymethyl and carboxyalkyl substituents (23, 24). Reactive intermediates can arise from prodrugs that contain ionizable promoieties other than phosphate (24, 25). Sulfonamidomethyl ester prodrugs of benzylenepicolloates have been synthesized and characterized for reactivity and chemical hydrolysis, and a dissociative mechanism for sulfonamide ester hydrolysis has been proposed (24).

From a pharmacodynamic perspective, an important question relates to whether this novel glucuronide metabolite possesses any pharmacologic activity. Generally, glucuronidation is believed to yield inactive metabolites that should be cleared via renal excretion. However, this is not always the case. An acylglicuronide conjugate of mycophenolic acid has been identified (26) that possesses pharmacologic potency comparable to that of the parent drug (27). Morphine-6-glucuronide is considered to be even more active than the parent drug (28). Thus, a definitive answer awaits synthesis of sufficient compound to perform these experiments for our described metabolite.

In summary, we have identified a unique metabolite isolated from sera from uremic patients receiving fosphenytoin. Using multiple analytical techniques, we have verified the structure as a glucuronide conjugate of the parent drug (28). Thus, a definitive answer awaits synthesis of sufficient compound to perform these experiments for our described metabolite.

References


