A Method for Determining Nitrofurantoin in Urine in the Presence of Phenazopyridine Hydrochloride and Its Metabolites

R. D. Hollifield and John D. Conklin

The high urinary drug concentrations usually encountered after administering nitrofurantoin in the chemotherapy of urinary tract infections are often measured by the nitromethane-Hyamine method. We show here that, if the urinary tract analgesic, phenazopyridine hydrochloride, and its related metabolite(s) are in the urine, they interfere with this determination of nitrofurantoin. Nevertheless, the nitromethane-Hyamine method may be modified to determine nitrofurantoin quantitatively in urine collected from human subjects who have been treated with the analgesic and nitrofurantoin concomitantly.

**Nitrofurantoin** [1-(5-nitrofurfurylideneamino)-hydantoin; "Furadantin," Eaton Laboratories] is effective in the treatment of urinary tract infections and is usually excreted in large amounts in the urine after its administration. The nitromethane-Hyamine procedure has become the method of choice for determining nitrofurantoin in urine, since the method is readily used in a clinical laboratory. Certain other antimicrobials that may also be in the urine of patients with urinary tract infections reportedly do not interfere with this determination of nitrofurantoin.

Our results establish that the urinary tract analgesic, phenazopyridine hydrochloride (2,6-diamino-3-phenylazopyridine·HCl; "Pyridium," Warner-Chilcott), which is occasionally coadministered with nitrofurantoin, does interfere. However, data are also provided showing that the nitromethane-Hyamine procedure may be adapted for the accurate determination of nitrofurantoin in urine collected from human subjects receiving the analgesic and nitrofurantoin concomitantly. Some observations are also presented on phenazopyridine hydrochloride in urine.

**Materials and Methods**

A Hitachi Perkin-Elmer UV Spectrophotometer (Model 139) was used to measure absorbances.

**Reagents**

The reagents include crystalline nitrofurantoin (Eaton Laboratories); crystalline phenazopyridine hydrochloride (Nepera Chemical Co., Inc., lot no. 26A-8076); 0.2M glycine buffer (pH 10.6, glycine ammonia-free); sodium hydroxide, AR; 0.04 hydrochloric acid, AR; N,N-dimethylformamide, AR; xylene, practical grade; nitromethane, practical grade; aluminum oxide, neutral, activity grade I (Bodman Chemicals); and Hyamine hydroxide [p-(diisobutylresoxyethoxyethyl)dimethylbenzylammonium hydroxide], 1M in methanol (443 g/liter of methanol) (Packard Instrument Co., Inc.).

The Hyamine solution (1 ml) is diluted to 25 ml with absolute methanol to 0.04 mol/liter.

**Standard Solutions**

Exactly 50 mg of nitrofurantoin is dissolved in 25 ml of N,N-dimethylformamide, and 25 ml of water is added. Ten milliliters of this solution is diluted to 100 ml with water; this is a concentration of 100 µg/ml. This solution is diluted with water to obtain concentrations of 5, 10, 30, and 50 µg/ml. Reference standard solutions are prepared with 2 ml of urine, 2 ml of the standard solution, and 2 ml of the 0.2M glycine buffer. The usual standard solutions are prepared with 2 ml of the standard solution and 2 ml of the 0.2M glycine buffer. Adjustments are made by adding water to give a final volume of 8 ml of aqueous phase for each standard. When recovery experiments were performed, water and the urine used to prepare the corrected reference standards served as control samples.

Phenazopyridine HCl was dissolved in water with the help of moderate heat; volumes of 2 ml were used.

**Procedure**

*Removal of phenazopyridine HCl.* Mix 2 ml of urine and 4 ml of water; then add 2 ml of the glycine buffer and mix thoroughly. The mixture is

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vigorously shaken for 3 to 4 min with 10 ml of xylene, and centrifuged for 10 min. Aspirate and discard most of the organic (top) layer (at least 9 ml), which contains the phenazopyridine HCl.

Removal of phenazopyridine metabolite(s). Avoiding contamination with the remaining xylene, remove 7 ml of the aqueous phase (bottom layer) containing nitrofurantoin and the metabolite(s) of phenazopyridine HCl to another test tube. To this tube, add 1 ml of 0.6N hydrochloric acid followed by 8 ml of nitromethane. Shake the contents vigorously for 2 min and centrifuge for 10 min. Transfer 6 ml of the nitromethane (bottom layer) onto a column of alumnum oxide (see below). When the nitromethane has entered the column, elute with nitromethane until a total of exactly 10 ml of eluate is collected (requires about 5 min). To 4 ml of the eluate, add 0.5 ml of the 0.04M Hyamine solution, mix the contents, and allow it to stand for at least 1 min.

The columns are prepared by introducing exactly 1 g of the dry aluminum oxide into a glass column containing a plug of glass wool. Pass 5 ml of nitromethane through the column and discard. A glass column with an i.d. of 1 cm usually yields a wet column of aluminum oxide about 1-cm long.

Absorbance of the nitrofurantoin–Hyamine complex is determined by spectrophotometry at 400 nm vs. pure nitromethane. The absorbance of all samples should be determined within 30 min of the addition of the Hyamine solution (see Experimental).

Construct a standard curve for nitrofurantoin by plotting the absorbance at 400 nm vs. the amount of drug present. Subtract absorbance of the control sample from the absorbance of the unknown urine sample, and read the amount of nitrofurantoin directly from the standard curve. If the nitrofurantoin concentration in the urine is too high to determine the absorbance, the nitromethane eluate should be diluted with additional nitromethane before adding the Hyamine reagent. Since the aqueous standard curve and the reference urine standard curve are identical when each is corrected with its respective control, a curve prepared from standards in either water or urine is suitable for calculating the concentration of nitrofurantoin present.

Experimental

Absorbance Characteristics

The nitrofurantoin–Hyamine complex in nitromethane has an absorption maximum at 400 nm (3). Nitromethane eluates from the alumina columns, containing nitrofurantoin standard solutions previously added to human urine and treated according to the described method, also have an absorption maximum near 400 nm. Urine specimens collected from human subjects receiving either nitrofurantoin or nitrofurantoin and phenazopyridine HCl simultaneously, when subjected to the modified nitromethane–Hyamine method, have an absorbance maximum anywhere from 395 to 400 nm.

Standard Curves

A standard curve for the nitrofurantoin–Hyamine complex in water obeys the Beer-Lambert law below 100 μg/ml. As shown in Table 1, the absorbance values obtained with human urine plus nitrofurantoin, or human urine plus both nitrofurantoin and phenazopyridine HCl, indicate that these corrected reference standard curves are identical. The recovery data show that nitrofurantoin is completely extracted by the nitromethane.

Solubility and Stability

The reported solubility of nitrofurantoin in nitromethane, determined from the nitrofurantoin

<table>
<thead>
<tr>
<th>Recovered from</th>
<th>Range of control</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>50</th>
<th>100</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0.003 to 0.004</td>
<td>0.028</td>
<td>0.058</td>
<td>0.188</td>
<td>0.318</td>
<td>0.679</td>
<td>...</td>
</tr>
<tr>
<td>Human urine</td>
<td>0.009 to 0.010</td>
<td>0.028</td>
<td>0.053</td>
<td>0.192</td>
<td>0.329</td>
<td>0.679</td>
<td>99.2 ± 4.7</td>
</tr>
<tr>
<td>Water + phenazopyridine HCl</td>
<td>0.034 to 0.035</td>
<td>0.028</td>
<td>0.055</td>
<td>0.181</td>
<td>0.330</td>
<td>0.673</td>
<td>98.8 ± 3.5</td>
</tr>
<tr>
<td>Human urine + added phenazopyridine HCl</td>
<td>0.035 to 0.041</td>
<td>0.027</td>
<td>0.056</td>
<td>0.176</td>
<td>0.327</td>
<td>0.680</td>
<td>97.8 ± 3.6</td>
</tr>
<tr>
<td>Human urine after administration of phenazopyridine HCl</td>
<td>0.006 to 0.007</td>
<td>0.027</td>
<td>0.056</td>
<td>0.185</td>
<td>0.326</td>
<td>0.680</td>
<td>98.4 ± 2.6</td>
</tr>
</tbody>
</table>

* Control-corrected data based on mean of at least three samples.  
* Represents an absorbance range at 400 nm for at least three controls.  
* Based on nitrofurantoin concentrations in water.  
* Phenazopyridine HCl concentration of 1000 μg/ml.  
* Pooled urine collected from two subjects (0–8 h) following the oral administration of 200 mg of phenazopyridine HCl. Nitrofurantoin added to urine.
toin–Hyamine complex, is greater than 3.7 g/liter (3). Ten milliliters of xylene can extract at least 2 mg of the analgesic (200 mg/liter) under the described alkaline conditions.

Investigation showed that, during the procedure, there is no appreciable loss of nitrofurantoin if it is left in nitromethane for 24 h at room temperature. When the Hyamine reagent is added to a nitromethane solution containing nitrofurantoin, the absorbance of this mixture increases about 4% after 1 h. Urine specimens collected from a human subject receiving nitrofurantoin were used in this experiment. These results agree with data reported previously concerning the stability of nitrofurantoin during the various steps of the nitromethane–Hyamine procedure (3). The 0.04M Hyamine solution in methanol is stable for at least 90 days (11).

Specificity

Selected concentrations of nitrofurantoin were added to urine collected from humans treated with phenazopyridine HCl and the samples subjected to the described method. Since identical results were obtained for these reference standards and those presented for nitrofurantoin in water (Table 1), it appears that the modified method is appropriate for determining nitrofurantoin in urine in the presence of phenazopyridine HCl and its related metabolite(s). The method has a sensitivity of 5 μg/ml.

Results and Discussion

Phenazopyridine HCl, used as a urinary tract analgesic, causes difficulties in certain urinalyses (12). When the analgesic is added to human urine, there is interference with the determination of nitrofurantoin by the nitromethane–Hyamine method (3). For example, when 30 μg of nitrofurantoin was added per ml of urine, the absorbance at 400 nm was 0.230; when 50 μg of phenazopyridine was added per ml of urine, the absorbance at 400 nm was 0.257; and when both 30 μg of nitrofurantoin and 50 μg of phenazopyridine were added per ml, the absorbance was 0.490 (control corrected).

We found that it is possible to extract phenazopyridine HCl directly from alkaline urine containing nitrofurantoin into xylene, leaving the nitrofuran derivative behind. A subsequent extraction with nitromethane to obtain the nitrofurantoin still revealed spectrophotometric interference, apparently owing to the presence of a metabolite(s) of phenazopyridine HCl. Nitrofurantoin was readily separated from this metabolite(s) because the latter is (are) absorbed onto aluminum oxide. The nitromethane–Hyamine method was modified accordingly.

The modified method was used to determine nitrofurantoin in urine collected from the same subjects after oral therapeutic doses of nitrofurantoin or of the analgesic and nitrofurantoin concomitantly. The results (Table 2) show the reliability and specificity of the modified method.

Urine samples containing nitrofurantoin were analyzed by the modified method described and by the original nitromethane–Hyamine method (3). The data in Table 3 show that results of the two different methods agreed. Previously, agreement was obtained also when urine samples containing nitrofurantoin were analyzed by the nitromethane–Hyamine method and by a biological cup plate assay (3). The bacteriostatic and bactericidal activity anticipated with urines collected from humans treated with phenazopyridine HCl (13) suggests that the modified nitromethane–Hyamine method be used rather than a biological assay to measure nitrofurantoin concentrations in urine when both drugs are administered simultaneously.

Few reports have appeared concerning the measurement of phenazopyridine HCl in urine since its introduction as a urinary tract antiseptic and analgesic nearly 40 years ago (13). Since xylene extracts phenazopyridine HCl selectively

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Drug</th>
<th>Dose, mg</th>
<th>Drug concn, μg/ml</th>
<th>Urinary drug recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Nitrofurantoin</td>
<td>100g</td>
<td>182</td>
<td>44.0</td>
</tr>
<tr>
<td>B</td>
<td>Nitrofurantoin</td>
<td>100g</td>
<td>38</td>
<td>42.8</td>
</tr>
<tr>
<td>A</td>
<td>Nitrofurantoin +</td>
<td>100g</td>
<td>204</td>
<td>45.9</td>
</tr>
<tr>
<td></td>
<td>phenazopyridine HCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Nitrofurantoin +</td>
<td>100g</td>
<td>12</td>
<td>39.8</td>
</tr>
<tr>
<td></td>
<td>phenazopyridine HCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Phenazopyridine HCl</td>
<td>200g</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>B</td>
<td>Phenazopyridine HCl</td>
<td>200g</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

* Based on the total mg of nitrofurantoin administered and the total mg of nitrofurantoin recovered in the urine.

+ One 100-mg Furadantin tablet administered.

- Two 100-mg Pyridium tablets administered.

+ N.D. none detectable.
Table 3. Nitrofurantoin Concentrations in Human Urine, Determined with Two Methods

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Time, h</th>
<th>Drug concn, µg/ml</th>
<th>Drug concn, µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Modified method*</td>
<td>Original method*</td>
</tr>
<tr>
<td>A</td>
<td>0-4</td>
<td>182</td>
<td>165</td>
</tr>
<tr>
<td></td>
<td>4-8</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>B</td>
<td>0-4</td>
<td>38</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>4-8</td>
<td>146</td>
<td>143</td>
</tr>
</tbody>
</table>

* Determined by the modified nitromethane–Hyamine method, see Table 2.
* Determined by the original nitromethane–Hyamine method (2).
* N.D., none detectable.

Table 4. Determinations of Phenazopyridine HCl in Human Urine

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Drug</th>
<th>Dose, mg</th>
<th>Drug concn, µg/ml 6-8 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Phenazopyridine HCl</td>
<td>200‡</td>
<td>1.1</td>
</tr>
<tr>
<td>B</td>
<td>Phenazopyridine HCl</td>
<td>200‡</td>
<td>1.0</td>
</tr>
<tr>
<td>A</td>
<td>Phenazopyridine HCl</td>
<td>200‡</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>+ nitrofurantoin</td>
<td>100‡</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Phenazopyridine HCl</td>
<td>200‡</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>+ nitrofurantoin</td>
<td>100‡</td>
<td></td>
</tr>
</tbody>
</table>

‡ Two 100-mg Pyridium tablets administered.
‡ One 100-mg Furadantin tablet administered.

and adequately, we could evaluate an analytical procedure with potential specificity for the analgesic alone: direct extraction of phenazopyridine HCl from urine in glycine buffer into xylene, and its spectrophotometric determination at 405 nm, vs. an appropriate control.

A standard curve for phenazopyridine HCl in water determined by this procedure obeys the Beer-Lambert law below 50 µg/ml. Absorbance values obtained when phenazopyridine HCl (1–50 µg/ml) was added to human urine showed that standard curves corrected with either the standard or reference control are identical. Recovery data (mean, 101%; sd, 3.2%) indicate that the phenazopyridine HCl is completely extracted by the xylene. Phenazopyridine HCl, 1 µg/ml water, yields an absorbance of 0.018 at 405 nm.

The described procedure was used to measure phenazopyridine HCl in urine collected from human subjects receiving the analgesic at a therapeutic dose. As shown by the results in Table 4, only traces of phenazopyridine HCl (about 0.1% of the dose) were detectable in the urine within 4 h and none later, after an oral dose of 200 mg of the drug. Phenazopyridine HCl extracted into xylene has an absorption maximum at 405 nm. When urine from humans treated with phenazopyridine HCl was extracted with xylene, the extracts also absorbed maximally at 405 nm.

If it is accepted that the described procedure is specific for the determination of phenazopyridine HCl, then it appears that very little unchanged phenazopyridine HCl is excreted in human urine under these conditions. In contrast, Johnson and Burba (14) reported that about 45% of a 600-mg dose of the orally administered analgesic was recovered as phenazopyridine HCl in human urine within 24 h after dosage. Unfortunately, they do not describe the analytical methodology used or indicate its specificity. The earlier procedure (14) evidently measures other substances than unaltered phenazopyridine HCl.

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References