Routine Analysis of Porphyrins in Urine

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Procedures are described that utilize an anion exchange resin for preliminary purification of porphyrins from highly colored urine specimens. Following purification, porphyrins may be quantitatively determined by a spectrophotometric method. A procedure is outlined for routine screening of porphyrins in the urines of hospital patients.

Although a number of procedures are available for quantitative determinations of porphyrins in urine, most of them involve extractions with organic solvents and are not applicable to screening of urinary porphyrins in a large number of patients. The direct spectrophotometric procedure of Rimington and Sveinsson (1) is rapid and works very well for most normal urine specimens. However, urine specimens encountered in clinical laboratories are often highly colored. One of the reasons for this is the increasing use of a wide variety of drugs, many of which absorb light in the visible and near-ultraviolet region of the spectrum. As much as 10 per cent of urine specimens brought to a clinical laboratory for porphyrin determinations may be too highly colored, as evidenced by optical density readings on the Beckman DU spectrophotometer, for application of the spectrophotometric procedure directly to the urine specimen.

Sveinsson et al. (2) have described a procedure for preliminary purification of urinary porphyrins by adsorption on calcium phosphate. With and Peterson (3) have utilized this procedure in the...
determination of porphyrins in urine specimens from a large number of hospital patients. Other workers have used talc (4, 5) and lead salts (6) in preliminary purification procedures. The very fine results obtained with preliminary absorption of porphobilinogen on an anion exchange column have been described by Granick and Mauzerall (7). These results suggested the application of a similar preliminary separation of porphyrins with the use of an anion exchange resin. The work described in this article indicates that anion exchange procedures are effective in the preliminary separation of porphyrins from highly colored urine specimens. A comparison of the results obtained by these procedures with those obtained by another commonly used procedure, calcium phosphate precipitation, is included in this report.

**Materials and Methods**

Dowex 1-X8 anion exchange resin (20/50 mesh, chloride form) was used as it came from the container without any preliminary treatment. This resin is distributed by J. T. Baker Chemical Co.

All spectrophotometric readings mentioned in this paper were made with a Beckman DU spectrophotometer. Presumably, a Beckman Model B spectrophotometer could also be used satisfactorily.

**Purification Procedures**

**Calcium Phosphate Precipitation**

This procedure was carried out as described by Sveinsson et al. (2) with the use of a 4-ml. sample of urine. After the calcium phosphate precipitate was washed with 0.1N NaOH and water, the precipitate was dissolved in 10 ml. of 0.5N HCl prior to spectrophotometric determination of porphyrins.

**Dowex Batch Method**

One gram of Dowex 1 anion exchange resin† (20/50 mesh, chloride form) is added to 5.0 ml. of urine in a test tube 2.0 cm. in diameter. The test tube is shaken vigorously for about 30 sec. and the anion exchange resin is allowed to settle to the bottom of the tube. The supernatant fluid is carefully poured off and discarded. The resin

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*In this laboratory, the anion exchange procedure of Granick and Mauzerall (7) is always used as a confirmatory test for porphobilinogen when a positive Schwartz-Watson test (8) is obtained.

†Moist resin, containing 39–45 per cent water.
is then washed with 20 ml. of water by mixing and pouring off the supernate. This water wash is also discarded. If the urine is particularly dark, the wash should be repeated. The porphyrins are then eluted from the resin by adding 10 ml. of 3N HCl and mixing for 30 sec. After the resin has settled the HCl solution is poured off. An additional 10 ml. of 3N HCl is added to the resin, and the shaking is repeated. This second HCl eluate is added to the first, and optical density readings are made at 380, 403, 406, and 430 m\(\mu\). The reading at 403 m\(\mu\) is taken to show whether appreciable coproporphyrin is present. When the absorption maximum is at 403–404 m\(\mu\), the urinary porphyrins are predominantly coproporphyrin. The total time required for purification of the urine sample by this procedure and for the spectrophotometric determination of porphyrins is about 15 min. per sample.

**Dowex Column Method**

Dowex 1 anion exchange resin (20/50 mesh, chloride form) is added to a glass column (Fig. 1), which is partially filled with water, to give a resin bed 1 cm. in diameter and 6 cm. in height. Since some highly colored urinary components cannot be completely re-

![Fig. 1. Ion exchange column.](image)

Water may be rapidly forced through this type of column, if desired, by applying pressure to the top of the column with a rubber bulb (W. H. Curtin No. 18654C). Addition of solutions to this column may stir up the top of the resin bed. Since the 20/50 mesh resin settles rapidly, the type of separation described is not adversely affected. The chromatography column may be obtained from the Special Apparatus Section of Corning Glass Works, Corning, N. Y.
moved from the ion exchange resin even with concentrated HCl, it is best to prepare a fresh resin bed for each determination. A column may be prepared in 2 min. or less, since the resin settles very rapidly. The flow rate of the column with 10 ml. of water above the resin should not exceed 4 ml./min. Five milliliters of the urine specimen is added above the column and allowed to drip through the resin until flow from the column ceases. With this large mesh resin, flow through the column ceases abruptly when the liquid level above the column reaches the surface of the resin. The column is washed with approximately 20 ml. of water. If the urine specimen is very highly colored, an additional wash may be required. These column washes may be discarded. Thirty milliliters of 3N HCl is then added to the column in 15-ml. portions. The optical density of the combined HCl eluates is determined at 380, 403, 406, and 430 mμ.

Calculations

Porphyrins are determined in the various acid solutions from the optical density readings as described by Rimington and Sveinsson (1), With (9), and Rimington (29). The following equation is used to correct for nonporphyrin compounds that are present:

\[ P_{\text{max}} = \frac{2 D_{\text{max}} - (D_{380} + D_{430})}{1.84} \]  

where \( P_{\text{max}} \) represents the optical density at the absorption maximum due to porphyrin, and \( D_{380} \) and \( D_{430} \) represent observed optical densities at the indicated wavelengths. \( D_{\text{max}} \) represents the observed optical density at the porphyrin absorption maximum. For most instruments, this wavelength would be 405-406 mμ for uroporphyrins in 0.5N HCl or 406-407 mμ in 3N HCl, but it should be checked for the particular instrument used. With coproporphyrins, the absorption maximum is at 400-401 mμ in 0.5N HCl and at 403-404 mμ in 3N HCl.

Since a uroporphyrin concentration of 1 μg./ml. in 0.5N HCl has an optical density (1, 9) at its absorption maximum of 0.652 (1-cm. light path), the uroporphyrin concentration in micrograms per milliliter of acid solution is equal to \( P_{\text{max}} / 0.652 \). This equation was utilized for the direct spectrophotometric analysis of urines (1:5 dilution of the urine specimen with 0.5N HCl) and for the determination of uroporphyrins following the preliminary calcium phosphate precipitation procedure. In 3N HCl, the absorption maximum of uro-
Porphyins is shifted to a higher wavelength (about 1 m\(\mu\)), and the optical density at the absorption maximum is 89 per cent of that in 0.5N HCl. Consequently, the uroporphyrin concentration in micrograms per milliliter in 3N HCl is equal to \(P_{\text{max}}/0.580\). This equation was utilized for the spectrophotometric analysis of the 3N HCl eluates obtained with the Dowex procedures. In this case, Equation 1 is used to determine \(P_{\text{max}}\).

For the determination of coproporphyrins, Equation 1 is applicable. Coproporphyrin, at a concentration of 1 \(\mu g./ml\). in 0.1N or 0.5N HCl, has an optical density (29, 30) of 0.747 (1-cm. light path). Accordingly, the coproporphyrin concentration in micrograms per milliliter in 0.5N HCl would be \(P_{\text{max}}/0.747\). In 3N HCl, the coproporphyrin absorption maximum is shifted to 403-404 m\(\mu\) and the maximal optical density is 90 per cent of that in 0.5N or 0.1N HCl. Consequently, in 3N HCl, the coproporphyrin concentration in micrograms per milliliter is equal to \(P_{\text{max}}/0.674\).

For the determination of total urinary porphyrins, neither of these equations is strictly applicable, since one is dealing with mixtures of porphyrins. The equations used for uroporphyrins, however, come very close to being correct for mixtures if uroporphyrins make up 50 per cent or more of the total. For samples containing equal amounts of uroporphyrins and coproporphyrins, the total porphyrin values obtained with these equations would be within 1 per cent of the true value in 3N HCl solutions and would be about 7 per cent low in 0.5N HCl solutions. Hence, one should calculate porphyrins in most cases with the use of the values for uroporphyrins. The values for coproporphyrins should be used only when it is reasonably certain that coproporphyrin constitutes 80 per cent or more of the total porphyrin. If the values for coproporphyrin are used in other cases, the error may be as high as 20 per cent.

**Results: Recovery of Porphyrins by the Dowex Procedures**

The Dowex procedures were first applied to the determination of porphyrins in a pathologic urine specimen that contained uroporphyrin, a very small amount of coproporphyrin, but no porphobilinogen. No tests were carried out on this urine specimen to detect the presence of porphyrins with five to seven carboxyl groups, nor were tests carried out to determine the type of uroporphyrin. The results of these porphyrin analyses are shown in Table 1. It will be noted
Table 1. Recovery (μg./ml.) of Uroporphyrins from Porphyrin Urine*

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Dowex 1 “batch”</th>
<th>Dowex 1 “column”</th>
<th>Calcium phosphate</th>
<th>Direct spectrophotometric</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.71 (3.86)†</td>
<td>3.48 (3.54)†</td>
<td>3.73</td>
<td>4.14</td>
</tr>
<tr>
<td></td>
<td>3.05 (3.34)†</td>
<td>3.47 (3.59)†</td>
<td>3.37</td>
<td>4.09</td>
</tr>
</tbody>
</table>

*The analyses were carried out on the same urine specimen on different days. Each value represents the average of duplicate analyses.
†Additional amount recovered in these instances by repeating the elution with 3N HCl.

that the recovery of uroporphyrin by the Dowex procedures compared favorably with the uroporphyrin recovery by the calcium phosphate precipitation procedure. The latter procedure has been shown to give good recoveries of uroporphyrin from pathologic urine specimens (2).

In the subsequent recovery studies, a portion of the uroporphyrin-containing urine specimen was added to 15 different highly colored urine specimens.

The initial uroporphyrin content of the specimens was not determined since it is very difficult to determine quantitatively very small amounts of porphyrins under these circumstances. In several cases the recovered uroporphyrins amounted to a little more than the added uroporphyrins. This may indicate that small amounts of uroporphyrins were initially present in the highly colored urine specimens.

Although no attempt was made to determine the cause of the color in all of the urine specimens, it was noted that specimen No. 1 contained considerable bilirubin and that No. 22 and 23 contained blood. The amount of uroporphyrin recovered subsequently from these urine specimens by application of the different purification procedures is shown in Table 2. The initial optical density of the urine specimen at 380 mμ is used as an indicator of color intensity, while the optical density at 380 mμ for the different acid extracts serves as an indicator of the success of the procedure in eliminating various interfering materials.

The calcium phosphate precipitation procedure was reasonably effective in removing interfering materials from 9 of 15 urine specimens. In each of the 9, uroporphyrin recovery was reasonably good. In the other 6 specimens, the interfering substance was not removed, and no porphyrins were recovered (as judged by the speci-
Table 2. Recovery of Uroporphyrins after Addition to Highly Colored Urine Specimens

<table>
<thead>
<tr>
<th>Urine specimen No.</th>
<th>Initial $D_{400}$ of urine</th>
<th>Procedure</th>
<th>Dowex 1 &quot;batch&quot;</th>
<th>Calcium phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$D_{400}$ Porphyrin (μg./ml.)</td>
<td>$D_{400}$ Porphyrin (μg./ml.)</td>
<td>$D_{400}$ Porphyrin (μg./ml.)</td>
<td>$D_{400}$ Porphyrin (μg./ml.)</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>0.40</td>
<td>0.706</td>
<td>0.75</td>
</tr>
<tr>
<td>5</td>
<td>6.3</td>
<td>0.57</td>
<td>0.477</td>
<td>0.77</td>
</tr>
<tr>
<td>8</td>
<td>&gt;8</td>
<td>0.38</td>
<td>0.724</td>
<td>0.68</td>
</tr>
<tr>
<td>12</td>
<td>2.7</td>
<td>0.57</td>
<td>0.380</td>
<td>0.90</td>
</tr>
<tr>
<td>13</td>
<td>&gt;8</td>
<td>0.70</td>
<td>1.50</td>
<td>0.70</td>
</tr>
<tr>
<td>14</td>
<td>4.2</td>
<td>0.31</td>
<td>0.370</td>
<td>0.72</td>
</tr>
<tr>
<td>15</td>
<td>3.0</td>
<td>0.51</td>
<td>0.612</td>
<td>1.02</td>
</tr>
<tr>
<td>16</td>
<td>2.2</td>
<td>0.23</td>
<td>0.368</td>
<td>0.14</td>
</tr>
<tr>
<td>17</td>
<td>2.4</td>
<td>0.32</td>
<td>0.400</td>
<td>0.68</td>
</tr>
<tr>
<td>18</td>
<td>3.7</td>
<td>0.81</td>
<td>0.572</td>
<td>0.69</td>
</tr>
<tr>
<td>19</td>
<td>2.4</td>
<td>0.45</td>
<td>0.450</td>
<td>0.59</td>
</tr>
<tr>
<td>20</td>
<td>5.6</td>
<td>0.54</td>
<td>0.470</td>
<td>0.56</td>
</tr>
<tr>
<td>21</td>
<td>3.0</td>
<td>0.70</td>
<td>0.522</td>
<td>0.67</td>
</tr>
<tr>
<td>22</td>
<td>1.9</td>
<td>0.60</td>
<td>0.380</td>
<td>0.52</td>
</tr>
<tr>
<td>23</td>
<td>&gt;8</td>
<td>0.40</td>
<td>0.298</td>
<td>0.56</td>
</tr>
</tbody>
</table>

*Observed value (in 0.4N HCl) times a dilution factor of 5.
†Observed value (in 3.0N HCl) times a dilution factor of 4.
‡Observed value (in 3.0N HCl) times a dilution factor of 6.
§Observed value (in 0.5N HCl) times a dilution factor of 2.5.

With the Dowex procedures, the interfering compounds were reasonably well removed from all 15 urine specimens. The average uroporphyrin recovery from the 15 specimens with the Dowex column procedure (0.66 mg. per liter) was approximately the same as the recovery with the calcium phosphate procedure (0.62 mg. per liter) in the 9 cases in which the latter could be successfully applied. The Dowex batch procedure was somewhat more effective than the column procedure in removing interfering color, but average recovery of uroporphyrin with the batch procedure was only 76 per cent of that obtained with the column procedure. Recovery of uroporphyrin from urine specimen No. 16 was low with both Dowex procedures. This analysis was repeated several times, with very poor recoveries in each case. At present, no explanation can be presented for the poor recoveries from this specimen.

Discussion

These studies indicate that purification procedures using an anion exchange resin may be successfully applied to the detection of por-
phyrins in porphyria urines and in highly colored urine specimens. The column procedure gives better recoveries and should be utilized when quantitative significance is to be attached to the results. On the other hand, the batch procedure is applicable when only semi-quantitative assays are desired. The batch procedure is probably the easiest to set up in laboratories in which only an occasional analysis is required.

For routine analyses in a clinical laboratory, assay of a single urine specimen would appear to be justified. The uroporphyrin level in urine must be increased at least 10-fold above the normal before it can be considered pathologic, and so variations in urine volume or in the output of porphyrins with the time of day would be relatively minor. It would be advisable, however, to check the urine specific gravity in cases in which the urine specimen is very dilute or very concentrated, and to adjust the porphyrin values accordingly (10). Urine specimens should be examined first by the direct spectrophotometric procedure. If the urine specimen, after a 1:5 dilution with 0.5N HCl, has an optical density of 0.25 or below at 380 m\mu, this assay will readily detect porphyrins at a level of 0.3 \mu g. per milliliter of urine. If the optical density of the diluted urine specimen at 380 m\mu is above 0.25, one of the purification procedures described in this paper may then be applied prior to spectrophotometric analysis. The combination of these procedures should effectively screen out all urine specimens in which the porphyrin content is in the normal range. In most laboratories, this would probably eliminate 95–99 per cent of the urine specimens from further consideration. When urinary porphyrins, as determined by these preliminary screening procedures, are above the normal level, the Dowex column procedure may be utilized for more accurate studies by analyzing 24-hr. urine specimens. The total porphyrin content of the urine specimen, together with an indication as to whether the predominant porphyrins are uroporphyrins or coproporphyrins, would be of great help to clinicians in the detection of cases of porphyria or porphyrinuria. In addition to the tests described above, more definitive tests may be used as desired. If the position of the absorption maximum indicates the presence of coproporphyrin, a quantitative coproporphyrin test may be carried out using ether extraction procedures (11, 30). If the procedure of Askevold (11) is used, the calculations should be made as described by Rimington (29). Recent
studies have shown that penta- and hexacarboxylic porphyrins, if present, are also extracted by ether (12). An additional test that might be utilized for uroporphyrins is the cyclohexanone extraction procedure of Dresel et al. (13). Fluorescence tests (either visual or those utilizing instruments) may be used to confirm results of these spectrophotometric procedures (4, 14, 15). Tests to determine the presence of Type I and Type III porphyrins (16, 17) or of porphyrins with five to seven carboxyl groups (12, 17, 18) are probably too time consuming to be applied in most clinical laboratories.

Many of the older procedures utilized for determination of uroporphyrins include a heating step, either in an acid solution (1, 19) or at pH 5.5 (11, 19), to convert porphobilinogen to uroporphyrin. Since porphobilinogen has been characterized and may be quantitatively determined by another procedure (7), there would appear to be no justification for continuing to heat urine specimens prior to analysis. The yield of porphyrin from porphobilinogen using either of these procedures is very low, and a mixture of other products is produced in addition to Type I and Type III uroporphyrins (19, 20).

Various workers have shown that porphyrins may be excreted in part as colorless porphyrinogens (21, 22, 25). If a 24-hr. urine specimen is collected, most of the porphyrinogens are probably oxidized by air to porphyrins prior to analysis. Iodine is very effective in bringing about oxidation of porphyrinogens to porphyrins (23). For most accurate results, therefore, it is advisable (21) to treat the urine specimen with a small amount of iodine (1 ml. of 0.02% iodine per 10 ml. of urine), prior to application of any of the analytic procedures described in this paper.

Normal values for urinary uroporphyrins are probably in the range of 10–50 µg. per day (13, 24). In an examination of urine specimens from 250 hospital patients, With and Peterson found 16 with porphyrin (other than coproporphyrin) excretions of 50–500 µg. per day (3). At the present time, however, no particular clinical significance can be attached to uroporphyrin values of this order of magnitude. In the porphyrias, uroporphyrin excretion may range from 2 mg. per day to as much as 100 mg. per day (26–28). The acute intermittent type of porphyria is an exception to this since excessive amounts of porphobilinogen are excreted in this condition with relatively small amounts of uroporphyrins. Uroporphyrin excretion values of 500 µg. to 2 mg. per day may indicate a true por-
phyria in remission, or they may possibly indicate a latent por-
phyria. Normal coproporphyrin excretion in urine ranges from 60
to 280 μg. per day (26). Coproporphyrinuria (300 μg. to 4 mg. of
coproporphyrin per day) may occur in a wide variety of conditions
(26). The most noteworthy of these are hepatic disorders, alcohol-
ism, and lead poisoning.

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