A Rapid Assay for Urinary Porphyrins by Thin-Layer Chromatography

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A procedure has been developed for the assay of urinary porphyrins. Total porphyrins are extracted from acidified urine into n-butanol. The fluorescence of this solution under ultraviolet illumination gives a preliminary indication of abnormal levels of porphyrins. They are then separated and identified by thin-layer chromatography. Uroporphyrin and coproporphyrin can be qualitatively estimated by visual inspection or accurately quantitated. For quantitation, the section of silica gel containing each porphyrin is scraped from the plate, suspended in glycerol solution, and the fluorescence measured directly without elution of the porphyrins from the silica gel. This assay, which yields either qualitative or quantitative information, is suitable as a diagnostic procedure in the clinical laboratory.

A differential diagnosis of the porphyrias, inborn imperfections of metabolism with defects in porphyrin biosynthesis, requires knowledge of the uroporphyrin and coproporphyrin content of urine (1, 2). Certain other diagnoses, e.g., lead poisoning, are also aided by an indication of abnormal porphyrin excretion.

Numerous methods have been developed for the quantitative assay of porphyrins, but new methods or modifications continue to appear, attesting to the continuing need for improvement. Porphyrin analyses and the underlying chemistry involved have been reviewed and discussed by Schwartz et al. (3) and Falk (4). The most frequently used procedures for urinary porphyrin assays are based upon solvent extraction and separation of uroporphyrin and coproporphyrin, with subsequent measurement of the porphyrin in hydrochloric acid solution by fluorometry or spectrophotometry. The common extraction procedures and the instrumentation employed have recently been eval-

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uated by Fernandez et al. (5). Although porphyrin assay by extraction can be satisfactory, the method suffers from being tedious and time-consuming as well as requiring skill in obtaining clean separations and quantitative recoveries.

A variety of chromatographic methods for the separation and preparation of porphyrins and their derivatives have been described (3, 4, 6). While many of these procedures have been useful for research experiments, they have proved to be impractical in the routine clinical chemistry laboratory because of their complexity or the time required to perform them.

Described below is a rapid, simple procedure for the separation and determination of urinary uro- and coproporphyrin employing thin-layer chromatography and fluorometry. The procedure may be used in three steps: (1) as a rapid, gross screening assay for detecting elevated total urinary porphyrins; (2) as a qualitative assay for separated uro- and coproporphyrin; and (3) as a quantitative assay for uro- and coproporphyrin. The method can be performed in a routine manner by personnel having limited experience.

**Materials and Methods**

**Reagents**

Analytical grade reagents were used throughout the procedures, without further purification.

The chromatographic medium was silica gel G (manufactured by E. Merck AG, Darmstadt, and obtained from Brinkman Instruments, Inc., Westbury, N. Y.). The chromatography solvent consisted of a mixture of chloroform:methanol:ammonium hydroxide:water (12:12:3:2). For fluorometric readings, the silica gel was suspended in a glycerol:water solution (2:1).

**Porphyrin Standards**

The uro- and coproporphyrin standards used in developing the assay were obtained by purification from urine specimens having elevated concentrations of these porphyrins. The standard for routine fluorometric measurements was chromatographically pure coproporphyrin dissolved in 1.5N HCl at a concentration of 0.05 μg./ml.

**Fluorometric Equipment**

For visualization of porphyrin spots on thin-layer chromatograms, a high-intensity ultraviolet lamp containing a 100-w mercury vapor spot bulb (Ultra Violet Products, Inc., San Gabriel, Calif.) was used. The assay method was developed using a Farrand Model A fluorometer.
The primary filter was Corning No. 5113 and the secondary filter was Eastman Kodak No. 29 A. Comparable results were obtained using a Turner Model 111 fluorometer fitted with a high-sensitivity door and the same filter combination.

Chromatographic Plates

Standard 20 cm. × 20 cm. or 20 cm. × 5 cm. glass plates coated with silica gel G of about 250 μ thickness were prepared according to Stahl (7). The plates were air dried overnight and used directly, or stored in a desiccator cabinet until needed. Activation of the coated plates prior to use was unnecessary.

Precoated silica gel plates (Machery-Nagel Polygram Sil S and Eastman chromatogram) have also been used with satisfactory results; however, rate and quality of separation varies with the different products.

Preparation of Urine Sample

To prepare the sample, 8.0 ml of urine were placed in a 12-ml centrifuge tube; 0.2 ml of 8N HCl were added to insure solution of all salts; then the mixture was adjusted to pH 3 with saturated sodium acetate (0.2-0.3 ml) as determined with pHydrion test paper. Two ml of n-butanol was added and the mixture was shaken vigorously and centrifuged for 10-15 min. to break the emulsion and to clearly separate the phases. This procedure removed most water-soluble fluorescing substances and concentrated the porphyrins. The upper butanol layer was then 1.5 ml in volume. Examination of the n-butanol layer under ultraviolet light indicated the presence of elevated porphyrins and was useful as a rapid screening test; normal urine gave a barely perceptible porphyrin fluorescence. Urine specimens known to contain a high concentration of porphyrins should be used in smaller amounts and the volume made to 8.0 ml with water; potential difficulties in subsequent steps will be eliminated and complete recovery of the porphyrins assured by making this initial dilution.

Chromatography

The n-butanol phase was used directly for chromatography. Spotting of the butanol solution and all further steps in the procedure had to be performed in subdued light—away from direct sunlight and high intensity fluorescent bulbs—to assure that no decomposition of porphyrins occurred. Sixty μl. samples were spotted 2.0-2.5 cm. from the edge of the plate. The spot size was kept equal to or less than 7 mm. in diameter. Drying of the spot was aided by the use of a stream of warm air from a hair dryer. Complete dryness of the spot before develop-
ment was essential to prevent the coproporphyrin from streaking during chromatographic separation.

The prepared plates were placed in chromatography jars containing the solvent mixture, which was allowed to ascend a distance of 10 cm. This normally required 30–45 min. at room temperature. The plates were removed and placed for 30 min. in a forced-air hood to dry. Complete dryness was essential, but prolonged standing (90 min. or longer) resulted in loss of fluorescence.

**Measurement of Porphyrins**

Visualization of the separated porphyrins was achieved by illumination with a high-intensity ultraviolet lamp and their location marked. The uroporphyrin remained at the origin and the coproporphyrin was a sharp band at Rf 60–65. This clean separation of the porphyrins offered a refined screening test for elevated excretion where quantitative information was not desired.

For quantitation, the silica gel containing the porphyrin spot (~ 1 sq. cm. 10–20 mg.) was carefully scraped from the plate and placed in a 10 × 75 mm. test tube used for fluorometric measurement. A similar-sized area of silica gel containing no fluorescence was removed from an area below the origin and used as a blank. Two ml. of the glycerol solution was added. Reproducible readings were obtained by shaking each tube vigorously to disrupt and suspend all silica particles, allowing the tubes to stand 15–20 min., then shaking gently just before placing in the fluorometer. It is important to wait 15 min. before reading the fluorescence to allow the background fluorescence contributed by the silica gel to stabilize at a minimum value. A porphyrin standard containing 0.05 μg. of coproporphyrin in 1.5N HCl was used as a full scale reference for adjusting the instrument.

**Calculations**

Since the porphyrins adsorbed on the silica were not sufficiently stable to be used as a standard, the fluorometer reading obtained in a silica-glycerol suspension must be related to the coproporphyrin-HCl standard. By chromatographing known amounts of pure uro- and coproporphyrin, one can determine a conversion factor for each porphyrin. In our laboratory 0.1 μg. of coproporphyrin in 2.0 ml. glycerol solution gave a reading of 10.0 and a uroporphyrin reading of 12.4, with the coproporphyrin standard set at 10.0 (Fig. 1).

To calculate urinary porphyrin excretion:

\[
\frac{\text{Galvanometer reading}}{\text{Conversion factor}} \times 0.1 \times \frac{24 \text{ hr. vol. in ml.}}{0.06} \times \frac{1.5}{8.0} = \mu g. \text{ porphyrin/24 hr.}
\]
This procedure yields true quantity of uroporphyrin even though coproporphyrin was used as a standard. When the prescribed quantities are used, the calculations can be reduced to:

\[
\text{Copro-} \quad \text{Reading} \times 24 \text{ hr. vol.} \times 3.12 \times 10^{-2} = \mu g. \text{ coproporphyrin/24 hr.}
\]

\[
\text{Uro-} \quad \text{Reading} \times 24 \text{ hr. vol.} \times 2.52 \times 10^{-2} = \mu g. \text{ uroporphyrin/24 hr.}
\]

**Solvent Extraction Method**

A typical type of solvent extraction procedure was used for evaluating and comparing the TLC method being developed. The urine specimen was added to acetate buffer, pH 4.5. This mixture was extracted with ethyl acetate, which removed the coproporphyrin and left the uroporphyrin in the aqueous phase. Last traces of uroporphyrin were washed out with 1% sodium acetate. The coproporphyrin was then extracted from the ethyl acetate with 1.5N HCl for fluorometric measurement. The combined aqueous solutions containing uroporphyrin were adjusted to pH 2.6–2.8, the uroporphyrin extracted into ethyl acetate, and finally re-extracted with 1.5N HCl for fluorometric measurement.

**Results**

**Porphyrin Fluorescence**

The fluorescence intensity of porphyrins after separation by thin-layer chromatography and suspension in glycerol was linear when plotted against porphyrin concentration (Fig. 1). The plot was linear from 0 to 0.1 \( \mu g./2 \) ml. (and is known to extend to at least twice this figure). The upper limit of this linear response was not determined, but porphyrin fluorescence is known to be linear to a concentration of 10 \( \mu g./ml \) when measured in HCl. The lower limit of detection by this procedure is 0.002 \( \mu g. \) of either uro- or coproporphyrin.

**Reproducibility**

Multiple determinations on the same specimen were reproducible within \( \pm 5\% \) of the mean value, which was comparable to the usual extraction methods.

**Recovery**

Recovery on single samples of uroporphyrin added to urine specimens ranged from 80 to 85\%. Single samples of coproporphyrin gave a higher recovery figure, varying from 105 to 111\% on different days.

**Effect of Silica on Fluorescence**

The amount of silica suspended in glycerol solution was not critical, as it contributed little fluorescence and no apparent quenching. Silica
within the 5-30-mg. range gave, on a scale of 10, a galvanometer deflection of 0.025 U./mg. of silica. A typical determination contained 9-16 mg. of silica, depending upon plate thickness and the precise area scraped.

**Fig. 1.** Relationships between porphyrin concentrations and galvanometer readings (fluorescence). Points at which curves intersect ordinate corresponding to concentration of standard (0.10 µg./2 ml.) are equivalent to conversion factors used in calculating porphyrin content of specimen.

**Urinary Values**

For normal persons this method gave results comparable to the extraction procedure with uroporphyrin in the range of 10–50 µg./24 hr. and coproporphyrin 50–200 µg./24 hr. Table 1 contains typical results obtained using the TLC method in three stages: as a screening test; for qualitative visual estimates of urinary porphyrins after TLC separation; and for quantitation by fluorescence measurement. These over-all results compared favorably with those obtained by a solvent extraction method. The one noticeable difference was revealed by summing the uro- and coproporphyrin values, in which case the TLC method compared to the extraction method consistently yielded equal or higher values.

Table 1 also contains comparative data obtained by assay of urine specimens from 3 normal individuals and 6 porphyria patients. The latter were selected merely to illustrate application of the method to...
Table 1. **Comparison of Porphyrin Analytic Data**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Screening test*</th>
<th>Qualitative assay</th>
<th>Quantitative assay (µg·24 hr.)</th>
<th>Solvent extraction assay</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>Uro</td>
<td>Copro</td>
<td>Uro</td>
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<tr>
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<td>0</td>
<td>Tr</td>
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<td>0</td>
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<td>Tr</td>
<td>2+</td>
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</tr>
<tr>
<td>Acute intermittent</td>
<td>3+</td>
<td>4+</td>
<td>1+</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>Acquired</td>
<td>3+</td>
<td>4+</td>
<td>1+</td>
<td>4900</td>
</tr>
</tbody>
</table>

Figures in parentheses represent the sums of uroporphyrin and coproporphyrin.  
*Visual fluorescence in n-butanol extract.  
^Visual fluorescence of porphyrin spots on TLC plate.  
\*Measured fluorescence of porphyrin spots from TLC plate.  
\$Typical extraction method described in Materials and Methods.

**Comments**

In some diseases of porphyrin metabolism, notably acute intermittent porphyria, porphyrins may be excreted in the reduced, nonfluorescent form, as porphyrinogens. Unlike the extraction procedures, an oxidation step is not included here; however, handling of the specimens prior to chromatography would be expected to result in oxidation of the porphyrinogens. The behavior of porphyrinogens during chromatography has not been observed.

The possibility for isomer separation by this technic was not evaluated, since such information is not essential in routine diagnosis. Indications were that the copro isomers I and III have slightly different Rf values in the one-dimensional system and could be further separated by two-dimensional procedures.

Because of the refined separations possible by thin layer chromatography, it is not uncommon to find multiple fluorescent spots or bands. Generally these are minor components which may be clumped near the uro- or the coproporphyrin areas. These probably represent isomer separation or traces of porphyrins having other than 8 (uro) or 4 (copro) carboxyl groups. Since such porphyrins would be recovered with the uro- or coproporphyrin following solvent extraction, they can for routine assays be included with these porphyrins when scraping the plates. In a few cases, significant amounts of hepta-, hexa-, specimens having widely varying concentrations and ratios of uro- and coproporphyrin.
and pentacarboxylic porphyrins may be present. These will appear as additional fluorescent bands between the uro- and coproporphyrin, but for purposes of diagnosis, they usually can be ignored. Occasionally the presence of drugs or drug metabolites will produce highly fluorescent spots; however, these have not been found to interfere following the butanol extraction.

If commercially prepared plates are to be used, several brands should be evaluated to find the type yielding the best results with the prescribed conditions.

References