Quantitation of Urinary Porphyrins by Use of Second-Derivative Spectroscopy

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We describe a simple, rapid procedure for measuring porphyrins in urine. After acidification of a urine specimen with hydrochloric acid, the second derivative of the absorption spectrum is recorded in the region of the Soret band maximum. The amplitude of the deflection is linearly related to porphyrin concentration, and the point at which the second derivative minimum occurs provides an estimate of the ratio of uroporphyrin to coproporphyrin in the sample. Non-specific interference is not significant and reproducibility is excellent. Porphyrin excretion was studied in 24-h urine collections from men and non-pregnant as well as pregnant women. Median daily porphyrin excretion (nmol) was: men, 65.0; non-pregnant women, 60.1; and third-trimester pregnant women, 175.0. Distribution of these data was significantly skewed; upper and lower 2.5 percentile limits are given.

Additional Keyphrases: excretion by normal men and women and by pregnant women - uroporphyrin/coproporphyrin ratio - results by anion-exchange compared - electronically generated first- and second-derivative spectra

Quantitation of excreted porphyrins is a central feature of the diagnosis of various disorders of porphyrin metabolism, but procedures in current use are either useful only as screening tests or are time-consuming. Urinary porphyrins exist as mixtures of two major fractions, uroporphyrin (with eight carboxyl groups) and coproporphyrin (with four carboxyl groups); minor components with seven, six, and five carboxyl groups are also present. Porphyrinogens may also be present, but are readily oxidized to the corresponding porphyrins. For diagnostic purposes, the total urinary porphyrin content as well as the relative concentrations of the individual components, especially uroporphyrin and coproporphyrin, must be determined.

Porphyrins in urine are usually measured after extraction procedures designed to separate "uroporphyrins" and "coproporphyrins" (1) or, as recommended by Doss (2), urines may be screened by extracting the porphyrins with ion-exchange resin and pathological samples quantified by using thin-layer chromatographic procedures, performed on derivatized porphyrins similarly extracted from the urines. Complete extraction from urine and separation and quantitation of material with the wide range of polarity shown by uroporphyrin and coproporphyrin pose problems and necessarily are costly.

We have previously reported a method for quantitation of urine porphyrins based upon the optically generated first derivative of the ultraviolet-visible absorption spectrum of acidified urine (3). This method is both rapid and accurate, avoids troublesome extraction procedures, and provides an estimate of the ratio of uroporphyrin to coproporphyrin in the solution. Its major drawback is the requirement for a dual-wavelength spectrophotometer. Recently, however, units have become available that generate first- and second-derivative spectra electronically from the output of scanning spectrophotometers. Whereas the first derivative generated by a dual-wavelength spectrophotometer approximates to dE/dλ where E is extinction and λ is wavelength, this type of attachment differentiates with respect to time (t). The first and second derivatives generated are, therefore, dE/dt and d²E/dt². Wavelength is established as a function of time by the spectrophotometer scan speed.

Using such equipment, we have evaluated the use of second-derivative spectroscopy to measure unextracted urinary porphyrin and we found that it offered several advantages, including increased sensitivity and facilitated interpretation of the spectra. The purpose of this paper is to describe a rapid, accurate, and inexpensive method for quantitation of urinary porphyrins by use of electronically generated second-derivative absorption spectra. The method permits "uroporphyrin" and "coproporphyrin" fractions to be reported quantitatively, as is done by use of older solvent extraction procedures, but does not resolve porphyrins with seven, six, and five carboxyl groups.

Materials and Methods

Instrumentation

Scanning Spectrophotometer, Model 200 with derivative accessory (Perkin-Elmer Corp. Limited, Toronto, Ontario M3N 1X3, Canada).

We prepared coproporphyrin III (as its methyl ester) from an ultrafiltrate of Corynebacterium diphtheriae culture kindly donated by Connought Laboratories, P.O. Box 1755, Station A, Willowdale, Ontario M2N 5T8, Canada. Uroporphyrin I methyl ester was purchased from Dr. Bruce Burnham (Porphyrin Products Limited, P.O. Box 31, Logan, UT 84321). Other chemicals were of reagent grade from Fisher Scientific Co., Limited, Don Mills, Ontario M3A 1A9, Canada.

Preparation of Porphyrin Standards

After hydrolysis of porphyrin methyl esters in a minimum volume of HCl (7.5 mol/L) for 24 h, solutions were diluted

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Fig. 1. A 2.10 μmol/L solution of coproporphyrin in HCl (1.0 mol/L) was scanned for zero-order (a), first-derivative (b), and second-derivative (c) absorbance output, with instrumental conditions as described in the text. Arrows indicate corresponding points in the spectra

appropriately with HCl (1.0 mol/L) and the concentrations were established spectrophotometrically by use of the physical constants of Falk (4).

Procedure

To obtain second-derivative spectra we used the following instrument settings: spectrophotometer in the absorption mode; slit width, 1 nm; scan speed, 240 nm/min; derivative attachment at mode = 6; second derivative output. (The "mode" setting controlled instrument gain, but also influenced phase shift due to parallel capacitance introduced to limit noise inherent in analog differentiators.)

For urine specimens a 1.0-mL aliquot of urine was diluted with 4.0 mL of HCl (1.25 mol/L) containing the sodium salt of EDTA (1.0 mmol/L). One drop of H$_2$O$_2$ solution (300 g/L) was added. The resulting solution was placed in a 1.0-cm pathlength cuvette and scanned from 430 to 380 nm, with air as the reference.

Results

Interpretation of Spectra

The zero-order, first-derivative, and second-derivative spectra obtained for coproporphyrin (2.10 μmol/L) with the above instrument settings are contrasted in Figure 1. From this it may be seen that (a) the amplitude of the signal was increased by each successive phase of differentiation; indeed, the characteristic deflection obtained in the second-derivative spectrum of the porphyrin Soret band was over 10 times as large as the zero-order absorbance; (b) the second-derivative spectrum resulted in a trough corresponding to a maximum of the zero-order spectrum; the corresponding point in the first-derivative spectrum occurred at the intersection with a reconstructed baseline; and (c) there was an apparent shift in the absorption peak 8.5 nm toward the blue end of the spectrum on use of derivative spectroscopy. This shift was constant at any given instrument settings and was attributable to a phase shift inherent in the circuitry of the derivative attachment necessary to optimize signal-to-noise ratio.

The size of the derivative deflection obtained was a linear function of concentration to at least 10 μmol of porphyrin per liter of urine. However, the apparent "absorptivity" is not a physical constant as it is with zero-order spectra but rather is a function of the instrumental conditions used. These figures were derived experimentally by scanning samples of known porphyrin concentrations under the precise optical and electrical conditions used for the assay. Results obtained did not vary from day to day.

Figure 2 shows the second-derivative spectra of the Soret band region for various ratios of solutions of coproporphyrin and uroporphyrin containing identical total molar concentrations of porphyrin (125 nmol/L). The wavelength at which the second derivative minimum occurred was a function of the ratio of uroporphyrin to coproporphyrin in the solution, as was the exact magnitude of the deflection. Thus, once the wavelength was known at which the second derivative minimum occurred for any mixture of uroporphyrin and coproporphyrin, the ratio of uro- to coproporphyrins and the second derivative "absorptivity" required to calculate the porphyrin molar
concentrations was also known. Table 1 shows the “absorptivities” and ratio of uroporphyrin to coproporphyrin which occurred at various minima for \(d^2a/dt^2\).

**Analytical Recovery and Precision**

Addition of porphyrin to urine before measurement produces the same deflection (when measured against an acidified urine reference) as an identical concentration of porphyrin in HCl (using air as the reference). The within-run and between-run precision of the assay was determined by repeatedly assaying urines containing various amounts of porphyrin. Two samples with low concentrations from normal subjects and two specimens with high concentrations from patients with porphyria cutanea tarda were examined. Each sample was assayed 20 times within a single day and 16 times during the next three weeks. For storage, the urine was diluted with HCl-EDTA as described for the assay and kept at -20°C. The results are reported in Table 2.

**Comparison with an Anion-Exchange Chromatographic Method**

Urine from 10 normal subjects and two patients with porphyria cutanea tarda was assayed for porphyrin by second-derivative spectroscopy as described above and by the method of Doss (5) in which porphyrin is extracted from urine onto anion-exchange resin and eluted with HCl. Absorbance of the eluate is determined at 380 nm, at 430 nm, and at the Soret maximum with correction for background absorbance according to With (6). The results are given in Table 3.

Since the second-derivative method avoided losses owing to incomplete extraction, the results for normal urine were found to be generally higher than with the ion-exchange method. Where there were significant differences, the error appeared to be attributable to the fact that the rising baseline found in the zero-order spectrum absorption after ion-exchange chromatography was not necessarily linear. In consequence, corrections as described by With (6) were inadequate.

**Urinary Excretion Range**

We analyzed aliquots of urine from 67 24-h specimens collected from adult patients with diagnoses other than porphyria. In addition, 158 24-h urine collections from pregnant women were also examined, to establish a normal range for this group of patients. The resulting values are summarized in Table 4. The porphyrin content of the urine from pregnant women was significantly higher than that of urine from men or non-pregnant women, but the percentage of coproporphyrin did not differ significantly among the groups (median = 75%, range = 20% to >95%).

**Discussion**

Detailed analysis for porphyrins and porphyrinogens in urine requires several steps, ending with quantitation by thin-layer chromatography or high-performance liquid chromatography. Such detailed analysis is seldom required for diagnostic purposes. Further, methods of this type that have been published recently (5, 7) give few details regarding analytical recovery from urine, precision, or accuracy.

Direct measurement of urine porphyrins by conventional absorptiometric techniques is not possible because impurities
confer nonspecific absorbance, which increases towards shorter wavelengths. Extraction of urine diminishes but does not eliminate such nonspecific absorption in the region of the porphyrin Soret band and quantitation requires correction of absorbance measurements, for example by use of the technique of With (6).

The problem of nonspecific absorbance can be overcome by using derivative spectroscopy because the first derivative of background absorbance over the narrow range of the porphyrin Soret maximum appears to be a constant and the second derivative is, therefore, zero. This makes it possible to obtain an undistorted second-derivative absorbance spectrum for porphyrins without extracting them from urine. The presence of a peak (albeit a minimum) in the second-derivative spectrum corresponding to the zero-order absorbance maximum makes interpretation of the second-derivative absorbance output easier than the corresponding first-derivative spectrum, which requires that a baseline be reconstructed (3). The method described above is sufficiently sensitive to detect excreted porphyrins down to 8.0 nmol/L. Also, because no extraction procedure is involved, the problem of variable losses is eliminated. Calibration of wavelength on the records of second-derivative spectra is clearly critical. An error of 0.5 nm in determining the trough of the second-derivative spectrum will cause a 5 to 20% error in uroporphyrin/coproporphyrin ratios. Use of a solution of coproporphyrin (0.1 μmol/L) would provide a rapid confirmation of wavelength accuracy.

The ease and rapidity of this quantitative method has made it preferable, in our laboratory, to the screening method of Eales et al. (8) for detecting pathological urines. In addition, the uroporphyrin/coproporphyrin ratio deduced from the derivative scan has allowed rapid differentiation of coproporphyrinuria owing to lead poisoning from porphyria cutanea tarda, in which highly carboxylated porphyrins predominate.

The consistent absence of baseline shift in the second-order derivative suggests that nonspecific interference from substances in urine did not occur in the 225 samples analyzed. Contamination of samples with blood was separately examined; addition of blood to urine to a concentration of 1 g of hemoglobin per liter did not interfere with the second-derivative porphyrin spectrum.

The lack of accepted standards for accessories to generate first- or second-derivative spectra does pose a problem for the laboratory wishing to establish this method. However, if data comparable with our results in Table 2 are recorded as part of the setting-up procedure, the laboratory will be independent of such instrumental variables.

We have summarized the daily excretion of uroporphyrin and coproporphyrin fractions as determined by second-derivative spectrophotometry. Non-porphyric men and women were studied; pregnant (third trimester) and non-pregnant women were evaluated separately. In all groups the distribution of total porphyrin was skewed towards higher values, but log transformation of data only resulted in a small reduction of the standard deviation. Neither parametric nor nonparametric tests suggested that porphyrin excretion differs significantly between men and non-pregnant women. However, the Mann-Whitney test did demonstrate that the increase in median porphyrin excretion recorded for pregnant women was highly significant. 95% confidence limits place the upper limit of normal for total urine porphyrin higher than is generally recognized. The greater part of this porphyrin is coproporphyrin.

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