Liquid-Chromatographic Assay of Urinary Porphobilinogen
Azim Jamani, Morris Pudek, and William E. Schreiber

This is a rapid (10 min per sample), highly sensitive procedure for quantifying urinary porphobilinogen (PBG). Interfering substances are removed by selectively adsorbing PBG onto an ion-exchange resin. After PBG is eluted with 0.5 mol/L formic acid, Ehrlich's reagent is added to produce the chromophore, which is then injected into a liquid chromatograph equipped with a diode-array detector. PBG is separated by a linear gradient (10% to 100%) of methanol in 10 mmol/L phosphate buffer, pH 3.0. Absorbance is monitored at 555 nm. Assay response varies linearly with PBG concentration over the range 0–110 μmol/L (0–25 mg/L). As little as 1.5 μmol/L (0.3 mg/L) can be detected. In prepared urine samples with known PBG concentrations, the within-run coefficient of variation (CV) ranged from 1.7% to 3.2%, the day-to-day CV from 3.5% to 6.1%. PBG concentrations in 24-h urine collected from 25 healthy subjects were all below the detection limit of the assay (<1.5 μmol/L). We used the new assay to measure PBG concentrations in the urine of two patients with latent porphyria. This method is more sensitive than spectrophotometric techniques currently used for measuring urinary PBG.

Additional Keyphrases: sample preparation · diode array spectrometry · reference interval · spectrophotometry compared

A unique feature of the three neurological porphyrias is the excretion of relatively large amounts (>150 μmol/L) of porphobilinogen (PBG) in urine during symptomatic periods (1). Qualitative screening tests for PBG such as the Watson–Schwartz or Hoesch tests (2–5), widely used to diagnose acute porphyria, are generally adequate for detecting large increases in urinary PBG. However, weak color development and (or) the presence of interfering substances, of which urobilinogen is the most common (6–8), may lead to problems in interpreting these screening tests. Because these interpretations are subjective, quantitative methods of PBG analysis are often used to confirm a diagnosis of acute porphyria.

The methods most commonly used involve passing a urine sample through an ion-exchange resin, followed by spectrophotometry (6–13). Unfortunately, these methods cannot accurately measure PBG at concentrations <18 μmol/L (4 mg/L), whereas the upper limit of normal is considered to be 4.5 μmol/L (1 mg/L) (9). In several published liquid-chromatographic methods, the PBG was either derivatized with o-phthalaldehyde and measured fluorometrically (14) or monitored directly at 240 nm (15). In our hands, neither of these techniques has proved adequate. Many compounds in urine react with the derivatizing agent and also fluoresce, masking any PBG. Ho et al. (14) did not address this point, their report being based on the use of standards rather than PBG in a urine matrix. Monitoring the absorbance at 240 nm was similarly fruitless: numerous other urinary components exhibit significant absorbance at this wavelength, making it difficult to isolate the PBG peak.

Our approach was to extract PBG from urine by using an ion-exchange resin, react the extract with Ehrlich's reagent, and then inject this mixture into a liquid chromatograph. This enabled us to separate PBG from interfering compounds in urine and to measure its concentration accurately and with high sensitivity. Here we describe our new assay, compare its performance with that of a standard spectrophotometric procedure, establish a reference interval, and validate the clinical usefulness of the assay by measuring the PBG concentrations in urines from two women with acute intermittent porphyria.

Materials and Methods

Reagents

Glass-distilled methanol was purchased from BDH Chemicals Canada Ltd., Vancouver, B.C. V5T 1E8; 0.20-μm (pore size) nylon filters from Rainin Instruments Co., Inc., Woburn, MA 01801; and 10-mL Econo-columns from Bio-Rad Laboratories, Richmond, CA 94804. Polybenzimidazole (PBI) resin ("Aurorez") was obtained from the Celanese Corp., Charlotte, NC 28232. Porphobilinogen and urobilinogen were supplied by Porphyrin Products, Logan, UT 84321. p-Dimethylaminobenzaldehyde was purchased from J. T. Baker Co., Phillipsburg, NJ 08865. All other chemicals were reagent grade.

Preparation of Solutions and Resins

Sodium phosphate, 10 mmol/L, pH 3.0: Dissolve 1.38 g of NaH2PO4·H2O in 950 mL of de-ionized water, adjust the pH to 3.0 with 1 mol/L phosphoric acid, and dilute to 1 L.

Sodium acetate, 5 mmol/L, pH 5.0: Pipet 0.71 mL of glacial acetic acid into 2440 mL of de-ionized water, adjust the pH to 5.0 with 2 mol/L NaOH, and dilute to 2.5 L.

Ehrlich's reagent: Mix 2.0 g of p-dimethylaminobenzaldehyde with 25 mL of concentrated HCl and 75 mL of glacial acetic acid. Store the solution in an amber-colored bottle at 4 °C. Under these conditions, the reagent is stable for four weeks.

PBG standard: Dissolve 1 mg of PBG in 10 mL of 5 mmol/L sodium acetate buffer, pH 5.0, using a 10-mL volumetric flask. Pipet 0.5-mL aliquots into polypropylene tubes, and keep them in the dark at −20 °C until use.

Polybenzimidazole (PBI) resin: Prepare the PBI resin (150- to 250-μm particle size), using the following steps: 1. Suspend 1 volume of PBI resin in two volumes of 1.0 mol/L NaOH for 5 min.
2. Filter the resin through a fritted-glass funnel and wash with 40 volumes of de-ionized water.
3. Resuspend resin in two volumes of 1.0 mol/L acetic acid for 5 min.
4. Refilter the resin through a glass-fritted funnel and wash it with 40 volumes of de-ionized water.
5. Store the resin in a glass bottle containing the acetate buffer.

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6. To prepare columns, pack 2.5 mL of resin into an Econo-column and wash with the acetate buffer.

Specimen Collection and Preparation
Collect urine for 24 h in a dark container to which 5 g of sodium bicarbonate has been added, to keep the specimen near neutral or slightly alkaline. Specimens can be stored at 4 °C for a week, or longer frozen at -20 °C, with little or no deterioration (16).

To 0.5 mL of urine add 8.0 mL of distilled water, adjust the pH to 5.0 with 1 mol/L acetic acid (approximately one drop), and run this through the PBG column (PBG is retained by the column). Wash the ion-exchange resin with 2.5 mL of water, followed by 2.2 mL of formic acid (this volume of formic acid will displace the water volume in the column). Add 5.0 mL of 0.5 mol/L formic acid to the column and allow to flow by gravity; collect the eluate, which contains PBG.

Chromatography System and Conditions
The liquid-chromatographic system consists of a Model 1090 chromatograph (Hewlett-Packard Co., Palo Alto, CA 94304) equipped with a diode-array detector. The pilot wavelength is set at 555 nm and the reference wavelength at 590 nm. The chromatograph is linked to a Hewlett-Packard 85B personal computer, 9121 disc drive, and 3392A integrator. The 5.6 mm × 25 cm analytical column (Whatman RP ODS-1) is packed with particles 10 µm in diameter (Whatman Inc., Clifton, NJ 07014). A universal reversed-phase guard-cartridge system (Whatman) is placed between the pump and the analytical column.

Sodium phosphate (10 mmol/L, pH 3.0) is channeled through pump A, methanol through pump B. To equilibrate the system, pump so that the mixture is 10% B, at 1.6 mL/min, until a steady baseline is achieved—about 15 min. Analyze standards and samples, using the following program:

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<th>Time, min</th>
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<tr>
<td>0</td>
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The gradient between 10% and 100% B is linear. At 6 min the analysis is finished, and the system then returns to initial conditions and is ready for the next injection. Total run time is 10 min per sample.

Procedure
Mix 0.5 mL of pretreated sample or standard with 0.5 mL of Ehrlich's reagent in a 2.0-mL liquid chromatography vial, cap the vial, and incubate it for 5 min at room temperature. Absorbance of the colored product is maximum at 5 min (Figure 1). Inject the mixture into the system at a flow rate of 1.6 mL/min and begin the gradient program. After the last sample is analyzed, switch to 100% B and allow this mobile phase to flow through the system for at least 20 min to clean any residual material out of the column. Typically, we inject 100 µL of sample or standard, but these volumes may be increased or decreased as necessary. Results are expressed in micromoles per liter or per 24-h urine.

We also analyzed samples by a spectrophotometric method (17), using a Lambda Array 3840 UV/Vis spectrophotometer, a Model 7300 professional computer, and a PR 200 printer (all from Perkin-Elmer Corp., Oak Brook, IL 60521). The same processed samples were used for both the liquid-chromatographic and spectrophotometric methods.

Results
Assay Development
During the development of the liquid-chromatographic method, we tried various isocratic and gradient programs. The 3-min linear gradient gave us the best peak definition and shortest run time. With the diode array detector's scanning capability, we were able to obtain a spectrum of the PBG peak as it passed through the detector cell. We chose to monitor absorbance at 555 nm (pilot wavelength), because the condensation product formed by PBG and Ehrlich's reagent absorbs maximally at this wavelength. By varying the reference wavelength during repetitive runs, we were able to determine which wavelength gave maximum sensitivity without distortion of the baseline or the peak of interest. This study led us to set the reference wavelength at 590 nm.

Figure 2 illustrates various chromatograms. Figure 2a shows the chromatogram of a "blank" composed of 0.5 mol/L formic acid (without PBG) and Ehrlich's reagent. The three peaks seen are observed in all other chromatograms and vary in size, depending on injection volume. A chromatogram of PBG standard is shown in Figure 2b; PBG appears at about 4.6 min. Urobilinogen, the major interfering substance in PBG analysis, is eluted at 5.2 min (Figure 2c). To demonstrate the effectiveness of the ion-exchange resin clean-up procedure, we assayed normal urine supplemented with both PBG and urobilinogen. Figure 2d shows the chromatogram of the untreated supplemented urine; the chromatogram in Figure 2e was generated after the urine had been through the ion-exchange resin. The results indicate that the ion-exchange step eliminates virtually all "Ehrlich's positive" substances from urine, with the exception of PBG.

Analytical Performance
The linearity of the assay was examined by analysis of urine supplemented with five different concentrations of PBG (0–110 µmol/L). The data fit the equation y = x(0.1539) - 0.1483, where x is the PBG concentration in micromoles per liter and y is the peak height in centimeters. The correlation coefficient (r) was 0.999. Analytical recovery of
PBG ranged from 95% to 100% throughout the linear range. Because of the excellent correlation, we routinely used only one working standard (11 µmol/L). We determined the sensitivity of this method by finding the lowest concentration of PBG giving a clear peak (signal/noise ratio ≥3/1) on the chromatogram. The lower limit of detection is 1.5 µmol/L (0.3 mg/L). We evaluated precision, using the urine samples containing 9.0 µmol (2.0 mg) and 39.0 µmol (9.0 mg) of PBG per liter. At the lower concentration, the CVs were 3.2% (within-run) and 6.1% (between-day); at the higher concentration, 1.7% (within-run) and 5.3% (between-day). These values are based on 20 replicate analyses in each case.

Comparison of Liquid-Chromatographic and Spectrophotometric Methods

Normal urines with added known amounts of PBG (9 to 200 µmol/L) were analyzed by both methods. The equation describing the relation was $y = x(0.9288) - 2.6989$, where $x$ = the liquid-chromatographic results and $y$ the spectrophotometric results ($r = 0.999$). In the spectrophotometric method, it is important to obtain an identifiable PBG spectrum, i.e., a major peak at 555 nm and a minor peak at 525 nm with a ratio of $A_{555}/A_{525}$ close to 0.83 (Figure 3). Most pigments that cause interference absorb at the lower wavelength and therefore increase the ratio. During our study, urines with a PBG concentration below 18 µmol/L (4 mg/L) did not meet this criterion. This is illustrated in Figure 4, which is a spectrophotometric scan of a urine, supplemented with 11 µmol (2.5 mg) of PBG per liter, that had been passed through the ion-exchange resin and reacted with Ehrlich’s reagent. At this concentration, there is no longer an identifiable PBG spectrum. However, the liquid-chromatographic assay (inset) clearly shows a PBG peak at a retention time of 4.7 min.

Reference Interval

To establish a reference interval for PBG, 24-h urine specimens, collected from 25 healthy individuals (13 men, 12 women), were analyzed for PBG by both the liquid-
chromatographic and the spectrophotometric method. In every case, the concentration of PBG was below the detection limit of either method, <1.5 μmol/L (<0.3 mg/L).

Porphyric Patients

The spectrophotometric scans for two asymptomatic patients (mother and daughter) with acute intermittent porphyria are illustrated in Figure 6. The daughter (PBG = 50 μmol/L) shows a scan that is readily identified as PBG (Figure 5). However, the mother (PBG = 18 μmol/L) has a spectrophotometric scan in which the two peaks are not well defined, and the $A_{520}/A_{655}$ ratio = 0.88 (Figure 5). In contrast, by liquid chromatography, both samples show a clear PBG peak at 4.6 min (Figure 6, a and b).

Discussion

This paper introduces a quantitative assay for PBG that is more than 10 times as sensitive as other methods. Our approach of measuring the condensation product of PBG with Ehrlich's reagent eliminates nearly all interfering substances. Extended gradient studies of urine by liquid chromatography show that only two peaks, corresponding to PBG and urobilinogen, are eluted in our chromatographic system. Because the two are completely resolved, the method could alternatively be set up to quantify urobilinogen at high sensitivity.

Initially, we thought that injection of the acidic Ehrlich’s reagent into a liquid-chromatographic system might damage the stainless-steel column and related components. We have performed over 300 injections to date and have not noticed any deterioration of the column or in the quality of the chromatograms.

We compared the Aurorez resin with other commonly used anion-exchange resins—e.g., Dowex 2-X8 and DEAE-cellulose—and found that Aurorez has several advantages. Its granular nature prevents it from adhering to the disposable Econo-columns, allowing it to be retrieved, regenerated, and then re-used. The large bead size also permits urine to flow through the column much faster than is true of the other resins. In addition, analytical recovery of PBG is quantitative.

Spectrophotometric determination of PBG followed the procedure of Galbraith et al. (13), except that PBG was eluted completely with 5.0 mL of 0.5 mol/L formic acid (rather than 10 mL). We found that PBG cannot be measured accurately below 18 μmol/L (4 mg/L). However, the reference interval for PBG, commonly regarded as <4.5 μmol/L (9), is below the lower limit of detection by conventional methods. With our liquid-chromatographic method, urinary PBG can be measured in a considerably lower concentration, <1.5 μmol/L (0.3 mg/L).

Recent studies suggest that the standard screening tests for PBG (Watson-Schwartz and Hoesch tests) are unreliable, giving false-negative results (17). In fact, both women with acute intermittent porphyria in our study gave negative results for screening tests for PBG. When we used the quantitative spectrophotometric technique (13), one of these patients was clearly positive, but results for the other were equivocal. Liquid-chromatographic analysis accurately measured the increased PBG in both of these patients. Therefore, the present method may enable clinical laboratories to detect latent porphyria, which will be especially useful in examining relatives of known porphyrins.

We thank Dr. J. E. Ramires (Celanese Corporation) for supplying Aurorez resin and Dr. B. F. Burnham (Porphyrin Products) for supplying synthetic urobilinogen.

References

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**Binding of Labeled Thyroxin Analog to Serum Proteins Evaluated after Radioimmunoassay of Free Thyroxin**

**Gaston Arevalo**

In ambulatory patients, assay of free thyroxin (FT₄) in serum correlates well with thyroid status and with results obtained by equilibrium dialysis. The validity of FT₄ results has been questioned mainly in euthyroid patients with altered concentrations of thyroid hormone-binding proteins, as in nonthyroidal illness, hereditary analbuminemia, familial dysalbuminemic hyperthyroxinemia (FDH), and the presence of idothyronine-binding antibodies. I present here a study of the binding of [¹²¹I]FT₄-derivative to serum proteins in the supernate, which is ordinarily discarded after determination of FT₄ by one-step radioimmunoassay with dextran-coated charcoal used to separate the free and bound fractions. The results are expressed as a ratio, with results for a normal serum pool as reference. The average ratio was high in hyperthyroid subjects, 1.26 (SD 0.12, n = 25), and in hypoalbuminemia, 1.20 (SD 0.10, n = 15), and low in FDH, 0.62 (SD 0.11, n = 9), and hypothyroid subjects, 0.90 (SD 0.06, n = 20). In normal individuals it was 0.98 (SD 0.05, n = 30). Determination of the analog-binding rate complements the FT₄ result and allows for the recognition of cases with abnormal binding by serum proteins, without recourse to other tests recommended for thyroid-function studies.

**Additional Keyphrases:** variation, source of • economics of laboratory operation

The concentration of free thyroxin (FT₄) in serum, determined by radioimmunoassay, correlates well with thyroid status (1) and with results obtained by equilibrium dialysis in ambulatory patients (2). However, in patients with nonthyroidal illness (2–5) or hereditary analbuminemia (6), low values for FT₄ have been reported, caused by factors that reduce T₄-binding to serum proteins (7). Moreover, high FT₄ values have been reported in familial dysalbuminemic hyperthyroxinemia (FDH) (8, 9), owing to increased T₄ affinity for albumin, and in subjects with immunoglobulin binding, as in patients with idothyronine-binding autoantibodies (10–12).

Although these conditions are rare, the very fact that they occur detracts from the validity of the assay. In medicine, no method is perfect, and determination of FT₄ is no exception. Consequently, for clinicians to interpret their results correctly, they must identify those cases in which an alteration of the normal T₄-binding to serum proteins distorts those results significantly.

Here I present a simple method of measuring the uptake of [¹²¹I]FT₄-derivative by dextran-coated charcoal, to study the binding of the analog to serum proteins. The result is expressed as a ratio, with reference to results for a normal serum pool, to minimize the effects of variations in the results. With this method, one can recognize those cases in which FT₄ values are distorted by abnormal binding of the [¹²¹I]FT₄-derivative to serum proteins.

**Materials and Methods**

**Samples.** Serum was obtained from 30 subjects (15 men and 15 women) with normal thyroid function, as the control group; 25 hyperthyroid patients, with total thyroxin (T₄) >150 µg/L and total triiodothyronine (T₃) >3000 ng/L, 10 of whom had a subnormal response of thyrotropin (TSH) to thyroliberin; 20 hypothyroid patients with TSH >20 milli-int. units/L; nine patients with FDH (three men and six women), all with normal thyroid function; and 15 patients with severe hypoalbuminemia, 10 of whom were hospitalized for treatment of chronic renal failure and five with liver failure.

Blood samples were collected in the morning after an overnight fast, and serum was promptly separated. Sera from FDH patients were stored at −18°C.

**Methods.** The following measurements were taken by radioimmunoassay: FT₄ (Amerlex-M; Amersham International, Amersham, U.K.); T₄ (International-Cis, St. Quentin-Yvelines Cedex, France): T₃ (Coat-A-Count; Diagnostic Products Corp., Los Angeles, CA 90045); and TSH (Abbott Laboratories, North Chicago, IL 60064). In addition, total protein and albumin were measured with an Abbott VP, with use of the biuret and bromcresol green dye-binding reagents recommended by the manufacturer.