Investigation of Protoporphyrin IX Standard Materials Used in Acid-Extraction Methods, and a Proposed Correction for the Millimolar Absorptivity of Protoporphyrin IX

Elaine W. Gunter,1 Wayman E. Turner, and Dan L. Huff

Erythrocyte protoporphyrin (EP) has been used for more than 30 years as an indicator of lead intoxication, iron deficiency, and porphyrias. Recently, numerous analytical problems associated with various EP methods have been reported, including a lack of consensus among investigators regarding the best calibration material or analytical procedure. We investigated commercially available protoporphyrin IX (PPIX) standard materials and measured the millimolar absorptivity (ε) of these materials, focusing on variables affecting the determination of their absorptivities. Among the five forms of PPIX available, PPIX dimethyl ester, when hydrolyzed to PPIX free acid, gave the most consistent and reproducible results. This work confirmed our earlier observations, made on more than 600 separate occasions during 12 years, that the ε of PPIX free acid in 1.5 mol/L HCl at the Soret maximum is 297 ± 1.3 L · mmol⁻¹ · cm⁻¹, 19% higher than the arbitrary value of 241 L · mmol⁻¹ · cm⁻¹ generally accepted by most investigators but based on unpublished data. We propose that the ε of 297 L · mmol⁻¹ · cm⁻¹ for PPIX be adopted and that PPIX dimethyl ester be used for the calibration of acid-extraction methods. A detailed protocol for the preparation and verification of PPIX from the dimethyl ester is available upon request.

Additional Keyphrases: standards · proposed new value for millimolar absorptivity · screening for lead poisoning · factors contributing to analytical error · iron deficiency

Protoporphyrin IX (PPIX) is the biosynthetic precursor of heme, the iron-containing moiety of hemoglobin and other hemoproteins.2 Determination of erythrocyte protoporphyrin (EP) has been widely used as a clinical indicator of lead intoxication and iron-deficiency states and for diagnosing porphyrias. Concentrations of erythrocyte zinc protoporphyrin (ZP) are increased when lead interferes with intracellular iron transport or blocks the final step of heme synthesis (catalyzed by the enzyme ferrochelatase, EC 4.99.1.1) (1). Inadequate iron stores also result in the formation of ZP. Increased concentrations of “free” or unchelated PPIX found in porphyrias are caused by disturbances in ferrochelatase synthesis (2).

Current methods for quantifying EP are based primarily on three techniques: (a) hematoforometry, in which ZP is measured directly in whole blood by a front-surface fluorometer; (b) acid-extraction, in which ZP and PPIX are extracted from whole blood in an ethyl acetate/acetate acid mixture and back-extracted into dilute hydrochloric acid, where ZP is converted to “free” PPIX and both forms are measured fluorometrically as “total” PPIX (and referred to as “free erythrocyte protoporphyrin”); and (c) “high-performance” liquid chromatography (HPLC), in which ZP and PPIX are extracted from whole blood into an organic solvent, separated by reversed-phase column chromatography, and detected fluorometrically.

The most frequent clinical application of EP determination has been for childhood lead-screening programs. Acid-extraction methods were used exclusively in these programs until the advent of hematoforometry, which is the most frequently used method today (3). HPLC has not been used routinely in these programs because of its higher cost per test and greater technical difficulty.

Recently, analytical problems associated with both hematoforometric and acid-extraction methods have been studied (2–9). Although hematoforometers are referenced to acid-extraction methods for comparability (3, 4, 10, 11), no calibration protocol has been uniformly adopted for acid-extraction methods. Various protoporphyrin materials and secondary standards, such as coproporphyrin, have been used, but few methods have actually made use of PPIX standards processed through the extraction steps of the procedure (2, 10, 12–15). Further complicating calibration of acid-extraction analyses are discrepancies in the ε of PPIX, which has been reported variously as 241, 282, and 275 L · mmol⁻¹ · cm⁻¹ in HCl solutions (13, 17–19).

For uniformity, most investigators have arbitrarily adopted 241 L · mmol⁻¹ · cm⁻¹ (17), because this value has long been used in establishing the current clinical database for EP concentrations. We have consistently observed, however, that the ε for PPIX is 297 ± 1 L · mmol⁻¹ · cm⁻¹ in 1.5 mol/L HCl (15, 16, 20), a discrepancy that could change reported PPIX concentrations by 19%.

We investigated commercially available PPIX standard materials, an important starting point in resolving some of these calibration problems. Although widely used in the

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2 Nonstandard abbreviations: PPIX, protoporphyrin IX; EP, erythrocyte protoporphyrin; ZP, zinc protoporphyrin; S-DME, Sigma protoporphyrin IX dimethyl ester; PP-DME, Porphyrin Products protoporphyrin IX dimethyl ester; PP-FA, Porphyrin Products protoporphyrin IX free acid, in bulk; PP-PFS, Porphyrin Products protoporphyrin IX fluorescence tube standards; PP-NaP, Porphyrin Products protoporphyrin IX disodium salt; MME, protoporphyrin IX monomethyl ester; HP, hematoporphyrin IX dimethyl ester; MP, mesoporphyrin IX dimethyl ester; me, millimolar absorptivity; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; DME, dimethyl ester; ppm, parts per million.

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past (1, 21, 22), ZP was not included in these studies, because it is no longer available in preweighed form and is very difficult to dissolve. We checked the purity of these commercial standards by spectrophotometric, spectrofluorometric, and HPLC techniques. In addition, we used thin-layer chromatography (TLC), nuclear magnetic resonance (NMR) spectroscopy, and elemental analyses to characterize several materials. Finally, we focused on measuring the me of these PPIX materials and on variables that may affect the reference value determined for me.

Materials and Methods

Reagents

Protoporphyrin IX dimethyl ester (S-DME), approximately 95% purity, lot no. 108C-0225, was from Sigma Chemical, St. Louis, MO 63178. All other porphyrin materials were from Porphyrin Products, Inc., Logan, UT 84321: protoporphyrin IX dimethyl ester (PP-DME), >97% pure, lot no. 12063; protoporphyrin IX dicarboxylic ("free") acid in bulk (PP-FA), >97% pure, lot no. 92785; protoporphyrin IX dicarboxylic acid, "5 µg/tube fluorescence standards" (PP-FTS), no purity stated, lot nos. 246, 248, 253, 255, 257, and 260; and protoporphyrin IX disodium salt (PP-NaP), no purity stated, lot 22685.

Additional porphyrins used for HPLC and TLC were protoporphyrin IX monomethyl ester (MME), lot no. 21483; hematoporphyrin IX dicarboxylic acid (HP), lot no. 517; and mesoporphyrin IX dimethyl ester (MP), lot no. 031386, all from Porphyrin Products. Reagent-grade formic acid (J. T. Baker Chemical Co., Phillipsburg, NJ 08865) was used to dissolve most materials. "Milli-Q" de-ionized water with a resistivity greater than 1 MΩ at 25 °C (Continental Water Co., Atlanta, GA 30345) was used to prepare all aqueous solutions.

HPLC-grade ethyl acetate and "Baker Analyzed" hydrochloric and glacial acetic acids (J. T. Baker Co.) were used for the EP acid-extraction analysis.

Kerosene (deodorized), reagent-grade chloroform (J. T. Baker), and HPLC-grade methanol (Fisher Scientific, Fair Lawn, NJ 07410) were mixed to make up the TLC solvent system in a 50/100/3 (by vol) ratio and used with precoated Silica Gel-60 plastic plates without fluorescent indicator (Curtin-Matheson Scientific, Inc., Houston, TX 77001) for TLC.

Deuterated chloroform (CDCl₃), 99.8% D, with 10 mL/L tetramethylsilane (Stohler Isotope Chemicals, Waltham, MA 02154), was used in the NMR investigations.

The HPLC mobile phase consisted of a mixture of HPLC-grade methanol, "Milli-Q" de-ionized water, and reagent-grade formic acid (94/6/0.5 by vol), degassed with nitrogen.

Apparatus

For absorbance measurements in me determinations, we used a Cary 219 spectrophotometer equipped with a thermostate and printer (Varian Associates, Palo Alto, CA 94303). Two circulating water baths (Polyscience Corp., Niles, IL 60648) were used for temperature-controlled experiments; bath no. 1 was set at 20 °C and used to keep water in the thermal isolator jacket at a constant temperature; bath no. 2 was used to heat or cool the turrets and the sample compartment (100 mL of methanol was added to the water in bath no. 2 to prevent freezing). For all absorbance measurements we used 1-cm (light path) quartz cuvettes. Spectrophotometric accuracy was verified with National Institute of Standards and Technology's Standard Reference Material No. 931a; wavelength accuracy was confirmed with a holmium oxide filter, with a scan of the ultraviolet lamp for peaks at 486.0 and 656.1 nm. For fluorometry we used a Model 650-10 spectrofluorometer (Perkin-Elmer Corp., Norwalk, CT 06856). Proton NMR spectra were obtained with a Varian XL-300 NMR spectrometer equipped with a 7.0-tesla superconducting magnet and the XL data system (Varian Associates).

The HPLC system consisted of the following: (a) a Model U6-K manual injector with 2-mL sample loop; (b) a Model 6000A solvent delivery system operating at 2 mL/min flow rate (Waters Associates, Milford, MA 01757); and (c) a Model 56 recorder, with 10-mV and 20-mm/min chart settings (Perkin-Elmer). For detection we used a Model 650-40 spectrofluorometer (Perkin-Elmer). Wavelength settings were 406 nm (excitation) and 626 nm (emission). We injected 10–15 µL of sample with a 25-µL glass syringe (Hamilton Co., Reno, NV 89510). A 3.9-mm (i.d.) ç 30-cm reversed-phase µBondapak column (Waters Assoc.) was used to separate the compounds of interest. All chromatographic runs were performed at ambient temperatures.

Procedures

Standard Preparations

PPIX standard solutions, 1 mg/L (1.78 £ 10⁻³ mmol/L) or 0.5 mg/L (8.9 £ 10⁻⁴ mmol/L), were prepared from the various PPIX materials and stored at 4 to 8 °C in foil-wrapped, acid-washed acetic glassware.

Prewashed fluorescence standards (PP-FTS), nominally 5 µg of FA per tube (23), were dissolved in 100 µL of "PROTO-SOLV," a solvating agent provided by the manufacturer. After 10 min, 10 mL of 1.5 mol/L HCl was added, with gentle mixing, to produce a PPIX solution with a nominal 0.5 mg/L concentration.

Fifty milligrams of PP-FA (662.27 g/mol) was dissolved in 3.0 mL of formic acid and diluted with 1.5 mol/L HCl to produce 0.5 and 1 mg/L PPIX solutions, respectively.

Both PP-DME and S-DME (590.73 g/mol) were hydrolyzed to the free acid by dissolving 42 mg of DME in 1 mL of formic acid and diluting to 200 mL with 7 mol/L HCl. The DME in these solutions underwent hydrolysis for 3 h (complete hydrolysis as confirmed by HPLC) (24) at ambient temperature while being mixed slowly with a magnetic stirrer. Twenty-five milliliters of the resulting hydrolysate was diluted to 500 mL with de-ionized water, producing a 10 mg/L intermediate stock in 0.35 mol/L HCl. A final 1:10 dilution of the intermediate stock was made with 1.62 mol/L HCl to produce a 1 mg/L PPIX standard in 1.5 mol/L HCl.

PP-NaP (606.23 g/mol) was prepared by drying 70 mg of the salt in a vacuum oven for 12 h at 25 °C. A 200 mg/L solution of PPIX was prepared by diluting 43.1 mg of PP-NaP to 200 mL with de-ionized water. This solution was further diluted with 1.5 mol/L HCl to produce a 1 mg/L solution (2). Unless otherwise stated, all solutions were brought to 20 ± 1 °C before spectrophotometry or fluorometry.

Methods

Spectrophotometric Comparisons

We initially compared the various PPIX standard materials by absorbance spectrophotometry. Three hydrolysates of each protoporphyrin IX dimethyl ester and three working stocks of PP-FA and PP-NaP were prepared. Three
1 mg/L solutions in 1.5 mol/L HCl were diluted from each stock. Tubes of each of five lots of 0.5 mg/L PP-FTS were prepared, and three aliquots from each tube were used. Identical sets of standards were prepared in ethyl acetate-saturated 1.5 mol/L HCl for comparison, because this protocol is used in some acid-extraction methods (11, 12). All solutions were scanned from 380 to 440 nm, and their respective spectra were recorded. Various spectrophotometric variables were evaluated for each material.

Fluorescence Comparisons

We compared PPIX solutions by excitation at 404 nm and scanning the fluorescent emission spectra from 500 to 700 nm. The peak height at 605 nm for each solution was arbitrarily set to read 90 relative fluorescent units at sensitivity setting "0.3." The 605 nm/658 nm peak height ratio was calculated after exciting the porphyrin molecules at 390, 404, and 412 nm in an effort to detect contamination by other porphyrins and/or degradation products such as MP or HP (25). The relative intensities of the 658-nm emission peak were measured in additional aliquots of these solutions, with the S-DME peak set to read 50 relative fluorescent units at the same sensitivity setting and with excitation at 404 nm.

HPLC

For qualitative HPLC of the PPIX standard materials we used the method of Bailey and Needham (20), with which they characterized PP-FTS, S-DME, and ZP. Reference standard solutions of individual porphyrins (HP, MP, DME, MME, and PP-FA) were prepared by dissolving about 5 mg of each compound in 2 mL of chloroform and diluting with mobile phase to give a final concentration of 10 μg/L. We also prepared a combined standard solution by combining equal volumes of these solutions. Solutions of the free-acid materials under investigation were prepared from both S-DME and PP-DME, as well as free-acid standards of PP-FTS (lot 280), PP-FA, and PP-NaP. The free acid and protoporphyrin IX dimethyl ester solutions were diluted with mobile phase to obtain approximately the same concentration as that of the standard solutions. Retention times were measured after injecting 15-20-μL aliquots. The combination standard solution was analyzed before and after the individual standard materials, to provide retention-time markers.

TLC

One-milligram to 2 mg of S-DME, PP-DME, and MME were each dissolved in 1 mL of chloroform, and 2-μL aliquots were applied to TLC plates with micropipets. The plates were developed for 25 min with the kerosene/chloroform/methanol solvent system and examined under ultraviolet light.

NMR

NMR spectra were recorded for the S-DME, PP-DME, and PP-FA. Measurements were made in CDCl₃ with a high-sensitivity-5 mm probe tuned at 300 MHz, with all chemical shifts reported relative to trimethylsilyl. Experimental conditions were: pulse 90 °C; acquisition time 2 s, with a 30-s delay between pulses; number of scans 32; and temperature 24 °C.

Elemental Analyses and Karl Fischer Water Titrations

Galbraith Laboratories, Inc. (Knoxville, TN 37921) used standard methods for elemental analyses and Karl Fischer water titrations on S-DME, PP-DME, and PA-FA as received from their suppliers.

Effects of HCl Concentration on Protoporphyrin IX Millimolar Absorbivity

Using 1 mg/L solutions of PPIX free acid, prepared from hydrolysates of PP-DME and S-DME as previously described, we examined the effect of various HCl concentrations on the absorbance spectra in the Soret region. Each solution was scanned from 400 to 415 nm. Final HCl concentrations in the solutions were 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mol/L, with corresponding HCl solutions used to blank each respective solution of protoporphyrin IX.

Effect of Temperature on Protoporphyrin IX Millimolar Absorbivity

Although it is well-documented that the fluorescence of porphyrins is quenched by increases in temperature (26), we found no specific reference to the effects of temperature on absorbance or me of PPIX. To determine this relationship, an aliquot of a 1 mg/L PPIX solution (from S-DME) was placed in the Cary 219 spectrophotometer, blanked against 1.5 mol/L HCl. Absorances were initially measured at 408 nm, the observed absorbance maximum (λmax or Soret peak) at 20 °C. The solution was then cooled to 2 °C and slowly warmed to about 85 °C during an hour. The chart drive was also programmed to plot absorbance with each chart unit equal to 1 °C, with absorbance and temperature of the solution printed at 30-s intervals. The 1.5 mol/L HCl solution, blanked against air, was treated similarly. A second aliquot of the 1 mg/L PPIX solution was exposed to the tungsten light source in the spectrophotometer at 408 nm at 20 °C for 1 h, and its absorbance was monitored to assess possible photodegradation. A third aliquot was cooled to 5 °C, then gradually heated to 75 °C during 1 h and scanned from 320 to 417 nm with every 5 °C increase in temperature.

Adherence to Beer’s Law

From a 4 mg/L PPIX stock solution (from S-DME) prepared as a representative material, we made a series of dilutions at decreasing 10% concentration intervals, keeping the HCl concentration constant at 1.5 mol/L. From the 10% stock, we made another 10-fold series of dilutions, resulting in a concentration range of 4.0 to 0.04 mg/L (7.12 × 10⁻⁶ to 7.12 × 10⁻⁸ mol/L). We measured the absorbance at the λmax of each solution and calculated me in two ways: for the first method we used a linear regression of absorbance vs concentration in millimoles per liter (where the slope was equal to the me); for the second method, we calculated each concentration individually, from its respective absorbance. To extend the interpretation of this experiment to fluorometry, each of these 19 standards was taken through our acid-extraction procedure (15, 16). We measured fluorescence intensities, using a sensitivity setting of 0.1, and the highest standard was arbitrarily set to read 100 relative fluorescence units. The wavelengths used were 404 nm (excitation) and 658 nm (emission). The concentrations of PPIX in these extracts were estimated to range from 0.031 to 3.08 mg/L (5.48 × 10⁻⁶ to 5.48 × 10⁻⁸ mol/L) after dilution and extraction. Relative fluorescence readings were plotted vs concentration, and linearity was assessed by regression analysis.
Results

Spectrophotometric Comparisons

Table 1 shows the calculated \( m \) and \( \lambda_{	ext{max}} \) for the various porphyrin compounds in 1.5 mol/L HCl. Results for solutions of the same porphyrin compounds prepared in ethyl acetate-saturated HCl are not presented because their spectra, absorbance values, and calculated \( m \) values were essentially identical. The highest \( m \) values were observed for protoporphyrin IX free acid prepared from hydrolyzates of the S-DME and PP-DME. Values for PP-FA and PP-NaP were about 10% lower than for the free acid hydrolyzed from the S-DME and PP-DME. Values for the PP-FTS were 20% lower, except for lot 253, which had substantially lower absorbance values than did the other PP-FTS lots. The manufacturer later confirmed that the variation in lot 253 was ascribable to a production error (personal communications, Drs. Bruce Burnham and Jerry Bommer, Porphyrin Products).

Although PP-NaP appeared to dissolve readily, we observed unacceptable batch-to-batch variability in \( m \) as compared with the other materials (data not shown). We attributed this variation to differences in moisture content among batches, arising from the hygroscopic nature of this sodium salt of PPIX. We also found this material to have poor long-term stability. Therefore, PP-NaP was not included in other comparison studies.

Observed \( \lambda_{	ext{max}} \) for all materials were between 407.0 and 408.0 nm. Spectral scans between 380 and 440 nm revealed no extraneous peaks, and bandwidths at half-maximum were 17.0 to 17.4 nm, indicating that all materials were of the same relative purity.

Fluorescence Comparisons

Fluorescence spectra at the three excitation wavelengths were similar for all the materials, each exhibiting two characteristic emission peaks at 605 and 658 nm. The ratios of these peak heights for all materials at each of the three excitation wavelengths were virtually identical (1.31–1.33), indicating that, by this technique, PPIX solutions prepared from all the starting materials appeared to be of equal purity. However, we observed differences in relative fluorescence intensities similar to the calculated differences in \( m \) shown in Table 1. PPIX solutions prepared from the DMEs had equivalent fluorescence intensities; both were about 10% higher than those of PP-FA and PP-NaP and about 16% higher than those of PP-FTS.

HPLC

The observed retention times (minutes:seconds) for porphyrins in the combined standard solution were: (a) HP (1:30); (b) free acid (3:45); (c) MME (3:29); (d) MP (4:46); and (e) PP-DME (5:46). The PPIX free acid solutions from PP-FTS (lot 260), PP-FA, PP-NaP, and both protoporphyrin IX dimethyl ester hydrolyzates all had a single peak at about 2:45 min. No detectable peaks were seen at the specific retention times corresponding to HP or MP, the most likely contaminants or degradation products of these materials. Also, no extraneous peaks were observed for any of the materials except S-DME, for which an additional peak was detected at 3:20 min. This was the retention time observed for MME, and the peak height was about 10% to 12% as great as that of the DME peak. Upon hydrolysis, however, only one peak eluting at 2:45 min, corresponding to PPIX free acid, was observed for this material. Figure 1 illustrates chromatograms comparing the PP-DME and S-DME before and after hydrolysis. Quantification of the relative concentrations of DME and MME in S-DME was hampered by quenching effects when we tried to use fluorescence detection at the concentrations required in this experiment.

![Figure 1. HPLC chromatograms of PP-DME and S-DME before and after hydrolysis](image)

When chromatographed, S-DME (left) contains an additional small peak identified as MME. After hydrolysis, only PPIX (free acid form) is seen in solutions prepared from both PP-DME and S-DME

TLC

We used qualitative TLC to confirm the identity of the second HPLC peak observed in the S-DME. We thought the additional HPLC peak was MME because it was absent in the chromatogram of S-DME after hydrolysis. Two distinct spots, centered at 5 and 41 mm from the origin on the TLC plate spotted with S-DME, corresponded to pure MME and DME, respectively, and confirmed that some MME was present in S-DME, but none was observed in the TLC of PP-DME.

NMR

Proton NMR spectra taken on three of the standard materials (S-DME, PP-DME, and PP-FA) had chemical shifts relative to trimethylsilane of 6.17, 6.34, and 8.39 ppm from the vinyl protons and 3.59 ppm for the \( R_x \) and \( R_y \) methyl ester protons, which agreed with reported values (27). No other unaccounted peaks were observed in the

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**Table 1. Spectrophotometric Evaluation of Protoporphyrin IX Standards Prepared in 1.5 mol/L HCl**

<table>
<thead>
<tr>
<th>Starting material</th>
<th>Conc, mg/L</th>
<th>n</th>
<th>( \text{Mean } m )</th>
<th>( \text{CV, } % )</th>
<th>( \lambda_{\text{max}} ) nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-DME</td>
<td>1</td>
<td>9</td>
<td>299.1</td>
<td>0.8</td>
<td>406.0</td>
</tr>
<tr>
<td>PP-DME</td>
<td>1</td>
<td>9</td>
<td>300.7</td>
<td>0.6</td>
<td>407.0</td>
</tr>
<tr>
<td>PP-NaP</td>
<td>1</td>
<td>9</td>
<td>272.2</td>
<td>0.6</td>
<td>407.6</td>
</tr>
<tr>
<td>PP-FA</td>
<td>1</td>
<td>9</td>
<td>272.7</td>
<td>1.3</td>
<td>407.0</td>
</tr>
<tr>
<td>PP-FTS</td>
<td>0.5</td>
<td>9</td>
<td>277.1</td>
<td>0.8</td>
<td>407.8</td>
</tr>
<tr>
<td>Lot no. 246</td>
<td>6</td>
<td>6</td>
<td>242.1</td>
<td>0.6</td>
<td>406.0</td>
</tr>
<tr>
<td>Lot no. 248</td>
<td>6</td>
<td>6</td>
<td>242.2</td>
<td>1.2</td>
<td>406.0</td>
</tr>
<tr>
<td>Lot no. 253</td>
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<td>6</td>
<td>171.7</td>
<td>1.4</td>
<td>408.5</td>
</tr>
<tr>
<td>Lot no. 257</td>
<td>6</td>
<td>6</td>
<td>249.0</td>
<td>0.8</td>
<td>408.0</td>
</tr>
<tr>
<td>Lot no. 260</td>
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<td>6</td>
<td>244.3</td>
<td>0.8</td>
<td>406.0</td>
</tr>
</tbody>
</table>
S-DME spectrum, suggesting that the amount of MME present in this material was below the limit of detection by NMR.

Elemental Analyses

Results from the elemental analyses were: (a) for S-DME and PP-DME, respectively, %C found 72.60 and 72.67, calcd 73.20; %H found 6.40 and 6.60, calcd 6.48; %N found 9.44 and 9.39, calcd 9.36; %O found 11.30 and 10.93, calcd 10.83; and (b) for PP-FA, %C found 71.26, calcd 72.57; %H found 6.15, calcd 6.09; %N found 9.80, calcd 9.97; %O found 12.03, calcd 11.37. Results of Karl Fischer water determinations on the materials were S-DME, 0.39%; PP-DME, 0.19%; and PP-FA, 0.87%, indicating negligible water content in these materials as supplied from the vendors.

Effect of HCl Concentration on Millimolar Absorptivity

Table 2 shows the effect on absorption spectra of PPIX (free acid) solutions (prepared from DME) of varying the HCl concentration. We observed two trends with increasing HCl concentration: (a) a shift in \( \lambda_{\text{max}} \) to longer wavelengths and (b) a corresponding decrease in absorbance at \( \lambda_{\text{max}} \) and a concomitant decrease in me. Similar observations have been previously reported (13, 26). Specifically, we noted a 0.5-nm increase in \( \lambda_{\text{max}} \) for each 0.5 mol/L increase in HCl concentration. From linear-regression analysis of me vs HCl concentration data, we estimate that for each 0.5-nm shift in \( \lambda_{\text{max}} \), resulting from increasing HCl concentration, there is a decrease of 2.1 L/mmol cm in me. A constant 1.0-nm difference in \( \lambda_{\text{max}} \) observed in this experiment between solutions of PPIX prepared from S-DME and PP-DME was also observed in all experiments in which these materials were used.

Effect of Temperature on the me for PPIX

Figure 2 shows results of the first experiment on the effect of temperature on me for a 1.0 mg/L PP-FA solution (prepared from S-DME) in 1.5 mol/L HCl. Absorbance at the Soret maximum decreased linearly with increasing temperature. The initial absorbance of the solution at 20 °C was 0.5390 at 407.0 nm, corresponding to an me of 297.8 L · mmol⁻¹ · cm⁻¹. Upon cooling the solution to 2.3 °C, its absorbance increased to 0.5634 at 86.2 °C the absorbance decreased to 0.47399 at 305.3 to 286.2 (−0.6 L · mmol⁻¹ · cm⁻¹/°C). This decrease apparently was attributable to thermal decomposition, because the original absorbance was restored when the solution was recooled to 20 °C.

![Fig. 2. Effect of temperature on me for protoporphyrin IX Absorbance, as well as fluorescence, of PPIX is inversely related to temperature](image)

To demonstrate that these results were not artifactual, due to blanking or to photodegradation of the PPIX, we subjected the 1.5 mol/L HCl blank solution to the same increasing temperature conditions as the PPIX standard. The solution showed a decrease of \(-5.0 \times 10^{-4} \, \text{A} \) over the same temperature range. A second aliquot of PPIX solution, placed in the spectrophotometer and exposed to the tungsten light source for 1 h at ambient temperature, had a decrease of \(-8.0 \times 10^{-4} \, \text{A} \), indicating negligible photodegradation. (Thermal expansion in the quartz cuvette was disregarded as a factor.)

Because absorbance in the first experiment was monitored at a fixed wavelength, 408 nm, we investigated the possibility that the decrease in absorbance we observed was caused by a wavelength shift during heating, possibly similar to the effect previously noted for increasing HCl concentration. Overlaid scans from 320 to 417 nm, recorded at 5 °C intervals, of a third aliquot of the 1 mg/L PPIX solution, heated from 5 to 75 °C, demonstrated that absorbances at \( \lambda_{\text{max}} \) decreased with increasing temperature. Bandwidths at half-maximum for all temperatures were consistently 17.0 nm, and the \( \lambda_{\text{max}} \) of all scans was 408 nm.

Adherence to Beer's Law

Figure 3 shows that Beer's Law appears to have been obeyed over the entire concentration range. The me calculated for individual concentration points showed no trend with concentration.

Figure 4 shows a clear linear relationship between fluo-

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**Table 2. Absorbances at Soret Peak and Greatest me for 1 mg/L Protoporphyrin IX Solutions in Various HCl Concentrations**

<table>
<thead>
<tr>
<th>Protoporphyrin IX prepared from S-DME</th>
<th>HCl concn, mol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance</td>
<td>0.5</td>
</tr>
<tr>
<td>( \lambda_{\text{max}} ), nm</td>
<td>406.5</td>
</tr>
<tr>
<td>me, L · mmol⁻¹ · cm⁻¹</td>
<td>296.5</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Protoporphyrin IX prepared from PP-DME</th>
<th>HCl concn, mol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance</td>
<td>0.5375</td>
</tr>
<tr>
<td>( \lambda_{\text{max}} ), nm</td>
<td>406.5</td>
</tr>
<tr>
<td>me, L · mmol⁻¹ · cm⁻¹</td>
<td>301.4</td>
</tr>
</tbody>
</table>
tions of PPIX having the same apparent concentration.

The variations observed in me between PP-FTS and PP-FA were unexpected, because both are prepared from the same PP-FA material (which is derived from the dimethyl ester). The manufacturer verified our deduction that the amount of PP-FA in the tubes containing PP-FTS standards is not actually 5 μg. Rather, the amount is based on yielding a solution that, when diluted to volume, has an absorbance corresponding to a calculated concentration of 0.5 mg/L if an me of 241 L·mmol⁻¹·cm⁻¹ is assumed. The manufacturer prepares the PP-FTS in this manner to supply a convenient and pure form of PPIX to its customers for EP calibration purposes and to provide a product that conforms with the expectations of those users—that is, in agreement with the consensus me value of 241.

After our analysis of the materials by HPLC, we concluded that, as judged by this technique, there was negligible contamination in any of the free acid standards by other porphyrins or PPIX degradation products. This experiment also substantiated that our 3-h hydrolysis procedure results in complete conversion of DME to the free acid as previously reported (24), without the acid-catalyzed hydration of the vinyl side chains to hydroxyethyl groups as observed by other investigators (29). The dimethyl ester, hydrolyzed for 24 to 48 h, has been used by various investigators for preparing free acid standards (12-14, 18). Solutions of the free acid so prepared may have contained degradation byproducts and their use may have resulted in inaccurate me determinations.

We did not attempt to quantify the amount of MME present in the S-DME beyond that estimated at 10% to 12% from their relative HPLC peak heights. Based on differences in their relative molecular masses, the presence of approximately 10% MME in S-DME would contribute an error of about +0.5% in the calculated me or calculated concentration of PPIX free acid.

NMR is not necessarily a sensitive technique for determining sample purity, but we used it to provide data for comparison of these materials, as is frequently cited and recommended (26).

The results from the elemental and water content analyses indicated the S-DME, PP-DME, and PP-FA to be about 99% pure, substantiating the manufacturer’s stated purities for these compounds. (The amounts of PP-FTS available in preweighed tubes were insufficient for these analyses; we assumed that their elemental composition and water content were similar to those of PP-FA.) PP-NaP was not submitted because of its rehydration potential and the batch-to-batch variability previously mentioned.

Historically, the concentration of HCl in PPIX standard solutions has been specified with published me for PPIX. The 0.5-3.0 mol/L concentration range of HCl selected for this experiment represented most of those found in the literature. The results shown in Table 2 substantiate the need for reporting the HCl concentration at which me was determined and for performing a spectral scan of a PPIX standard material to obtain the absorbance at λmax.

In the temperature-change experiments, the decrease in absorbance with increasing temperature can be explained in part by the thermal expansion of the aqueous solutions resulting in decreasing absorbance values. On the basis of published data for the coefficient of thermal expansion for aqueous solutions (29), we determined that the absorbance of PPIX at 85 °C would be 97% of that measured at 4 °C—but the observed absorbance at 85 °C was 84% of the
absorbance at 4 °C. These observations indicate that PPIX free acid absorbance is dependent on temperature.

The results of these experiments also revealed an inverse relationship between mε and temperature of dilute PPIX solutions in 1.5 mol/L HCl and demonstrate that errors in measuring absorbance can be ascribed in part to the temperature of the solution. The mε of PPIX from 15 to 25 °C in this experiment decreased by about 2%, from 300 L · mmol⁻¹ · cm⁻¹ at 15 °C to 294 at 25 °C. Standard solutions should be equilibrated to ambient temperatures (20 ± 5 °C) before absorbance is measured, especially if the PPIX stock solution has previously been stored at 4 to 8 °C.

PPIX reportedly forms molecular aggregates in dilute aqueous solutions (30). This aggregation is associated with a decrease in the mε and broadening of the Soret band (31; and personal communication, Dr. David Mauzerall). One recommended approach for detecting aggregation is to demonstrate deviation from Beer’s Law (32) by observing the absorbance spectra of porphyrins over a wide concentration range in cells of various pathlengths, keeping the amount of chromophore (i.e., the product of concentration × pathlength) in the lightpath constant (33). We investigated this possibility with cuvettes of five different sizes and solutions with concentrations ranging from 0.1 to 2.0 mg/L, but the results were inconclusive (data not shown).

Aggregation could explain the approximately 10% differences in mεobs for PPIX prepared from the dimethyl-ester samples and PP-FA, an hypothesis further supported by the 10% differences observed in fluorescence intensities between these same solutions, it being assumed that only monomers fluoresce.

If equal purity is assumed, we cannot explain why PP-FA should aggregate more than PPIX prepared from the hydrolysis of the dimethyl ester. Because the final solvent in most of these experiments was 1.5 mol/L HCl, one could speculate that the hydrolysis procedure was in some way contributing to a more dispersed state of PPIX molecules in the final solution by providing the most monomeric form of PPIX among available materials.

In our previous experiments, we measured the absorbance of 1 mg/L (1.78 × 10⁻⁶ mol/L) PPIX solutions in 1.5 mol/L HCl to determine mε. Similar concentrations were used by other investigators (25, 33). Apparently, this concentration of PPIX was originally selected to provide absorbance values of approximately 0.500 A, which is in the range corresponding to minimal spectrophotometric error (34). Because mε appears to be relatively constant over the range 0.4–4.0 mg/L, a 1.0 mg/L PPIX standard would be appropriate for use in routine measurements. Our observations were consistent with reported deviations from Beer’s Law for concentrations greater than 2 mg/L and with the recommendation that mε be determined on porphyrin solutions containing less than 1.5 to 2.0 mg/L (35). This same investigator (35) observed an mε of 310 L · mmol⁻¹ · cm⁻¹ (with a λmax of 408.0 nm) for PPIX in 1.5 mol/L HCl. Apparently, this is the highest value for mε published for PPIX. Unfortunately, the concentration of PPIX used was not reported.

Spectrofluorometry is reported to be 10⁶ more sensitive for PPIX than spectrophotometry and is also useful for detecting aggregation of porphyrins, if it is assumed that only monomers fluoresce (30). Because of the linear relation of fluorescence to concentration in the extracts of these standards, we concluded that there was no molecular aggregation of PPIX in these extremely dilute solutions of PPIX. As a result of the extraction process, the highest standard (400 mg/L) was diluted to give a final concentration less than the starting concentration of the lowest standard (0.04 mg/L). If molecular aggregation had been occurring, fluorescence readings, which are more sensitive than absorbance by a factor of 10³, would have been nonlinear.

On the basis of collective results of the experiments presented, our observations over the past 12 years, and independent confirmation by Porphyrin Products (personal communications, Drs. Bruce Burnham and Jerry Bommer), we believe there is strong evidence that the mε for PPIX free acid is 297, rather than 241, L · mmol⁻¹ · cm⁻¹. We propose that the mε of 297 L · mmol⁻¹ · cm⁻¹ for PPIX free acid be adopted and supersede the value of 241 L · mmol⁻¹ · cm⁻¹ in general use.

The public health impact of this change would be a 19% decrease in the current normal reference interval for EP values, which would change the cutoff value recommended by the Centers for Disease Control for EP screening in childhood lead-monitoring programs from 35 to 28 µg per deciliter of whole blood. United States national normative data for EP (in micrograms per deciliter of erythrocytes) have already been generated and published, this corrected value being used, in the Second National Health and Nutrition Examination Survey (NHANES II, n = 20 000) (18, 36) and the Hispanic Health and Nutrition Examination Survey (HHANES, n = 12 000) (16). EP data generated by other laboratories could be referenced to these existing national databases.

PPIX-DME represents a stable and highly pure standard material that should be used in fluorometric EP procedures rather than a less appropriate secondary standard such as coproporphyrin.

Because of the numerous variables that may affect determination of absorbance of PPIX free acid in acid solutions (e.g., hydrolysis procedure, acid concentration, temperature, etc.), the concentration of standard solutions on the basis of an mε of 297 L · mmol⁻¹ · cm⁻¹ must be determined under well-defined conditions. We can supply a recommended protocol for this procedure.

In addition, we have found that the 10 mg/L intermediate stock solution in 0.35 mol/L HCl may validly be stored in the dark at 4 to 8 °C for as long as a year with no detectable change in absorbance and thus used as an intermediate stock to prepare 1 mg/L and other solutions for the fluorometric EP standard curve. No degradation products were seen by the HPLC, TLC, fluorescence, or absorbance methods previously described. We recommend that the intermediate stock be used only for several months; a new hydrolysate should then be prepared and checked. For long-term studies such as the NHANES, after the purity of a particular lot of DME has been verified, a large quantity of the material may be purchased, divided into 100-mg quantities in glass ampuls, sealed, and stored at −70 °C. Under these conditions, DME is stable for at least five or six years. Alternatively, instead of the PP-FTS, we suggest that the manufacturer could prepare tubes

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3 Data from these surveys for EP by acid-extraction were calculated by using 297 L · mmol⁻¹ · cm⁻¹. Publications and data tapes from these studies may be obtained from the National Center for Health Statistics, Hyattsville, MD (36).
containing 42 mg of PP-DME and supply hydrolysis instructions.

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Use of trade names is for the purpose of identification only and does not constitute endorsement by the Public Health Service or the U.S. Dept. of Health and Human Services.

References