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Biochemical Differentiation of the Porphyrias

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Objectives: To differentiate the porphyrias by clinical and biochemical methods.
Design and methods: We describe levels of blood, urine, and fecal porphyrins and their precursors in the porphyrias and present an algorithm for their biochemical differentiation. Diagnoses were established using clinical and biochemical data. Porphyrin analyses were performed by high performance liquid chromatography.
Results and conclusions: Plasma and urine porphyrin patterns were useful for diagnosis of porphyria cutanea tarda, but not the acute porphyrias. Erythropoietic protoporphyria was confirmed by erythrocyte protoporphyrin assay and erythrocyte fluorescence. Acute intermittent porphyria was diagnosed by increases in urine delta-aminolevulinic acid and porphobilinogen and confirmed by reduced erythrocyte porphobilinogen deaminase activity and normal or near-normal stool porphyrins. Variegate porphyria and hereditary coproporphyria were diagnosed by their characteristic stool porphyrin patterns. This appears to be the most convenient diagnostic approach until molecular abnormalities become more extensively defined and more widely available. Copyright © 1999 The Canadian Society of Clinical Chemists

KEY WORDS: Porphyria; porphyrins; feces; urine; blood; plasma; erythrocytes; renal failure; abdominal pain; neuropathy; erythema; urticaria; photodermatitis.

Introduction

The biochemical differentiation of the porphyrias resides, at present, with the measurement of porphyrins and their metabolites in urine, feces and blood (1). Enzyme tests are often technically difficult and require tissues such as cultured fibroblasts, lymphocytes, or liver biopsy material and therefore, except for the use of erythrocyte porphobilinogen deaminase in acute intermittent porphyria, they are rarely used (2). Genetic analysis produces a precise diagnosis in some of the porphyrias provided the patient has one of the previously described point mutations, but this technique is currently confined to a few research laboratories (3). As a consequence of our role as a major reference laboratory for porphyrin analysis performing approximately 2500 tests per year, we have assembled reference intervals for urine, fecal, and blood porphyrins and their precursors in the various porphyrias and in normal subjects and have devised an algorithm for investigation of these diseases. Except for Porphyria Cutanea Tarda (PCT), our numbers of patients in each category of porphyria are small and therefore our reference ranges for these should be considered approximate.

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Materials and methods

Reagents and chemicals

Porphyrin standards were obtained from Porphyrin Products Inc. (Logan, UT). All solvents were of high performance liquid chromatography (HPLC) grade.

Equipment

HPLC was performed using a Varian 5500 (Varian Canada Inc., Mississauga, ON) equipped with a Shimadzu RF-1501 spectrofluorometer (Shimadzu Scientific Instruments, Inc., Columbia, MD). The column was a Perkin-Elmer (Norwalk, CT) reversed phase Percospher 3 C18 Brownlee, 33 × 4.6 mm. Data analysis was performed using Dionex A1-450 chromatography software (Dionex Canada Ltd., Oakville, ON).

Plasma porphyrins

Plasma porphyrin analysis was performed according to the method of Hindmarsh *et al.* (4). Heparinized plasma was used, samples being centrifuged immediately after collection and frozen at −20° C. Stock standards were prepared using porphyrin markers in porphyrin-free plasma obtained from our hospital blood transfusion service; 2-vinyl 4-hydroxymethyl-deuteroporphyrin IX was used as an internal standard. The recovery of individual porphyrins added to porphyrin-free plasma varied from 89% to 114% except for coproporphyrin-III, whose recovery varied from 71% to 99%. Recoveries of protoporphyrin were low, therefore, no attempt was made to quantify this fraction. Between-run preci-

sion varied from 5.4% to 13.2%, coproporphyrin-III being the least precise.

Urine porphyrins

Urine porphyrins were measured by a reversed phase HPLC method based upon that of Johnson *et al.* (5). To achieve separation of the I and III isomers of uroporphyrin and coproporphyrin, we substituted their mobile phase with a gradient of a 1 mol/L ammonium acetate solution (pH 5.16) and methanol, varying from 27% (starting) to 90% (ending) of methanol V/V. Direct standardization was used with uroporphyrin-I, uroporphyrin-III, heptacarboxyl-I, hexacarboxyl-I, pentacarboxyl-I, coproporphyrin-I, and coproporphyrin-III. Recoveries of standard porphyrins added to urine samples varied from 92% to 115%. Between-run precision for the various fractions varied between 7% and 10%. Samples were preserved at 4° C with 5-g sodium carbonate per daily collection in brown glass bottles.

Fecal porphyrins

Our fecal porphyrin method that was based upon the reversed phase HPLC method of Pudek *et al.* (6), but using direct standardization with uroporphyrin-I, uroporphyrin-III, heptacarboxyl-I, hexacarboxyl-I, pentacarboxyl-I, coproporphyrin-I and coproporphyrin-III, deuteroporphyrin, mesoporphyrin, and protoporphyrin. We used a 3×0.43 cm (ID) cartridge analytical column containing 3-μm octadecylsilyl particles. The solvent system was a gradient of a 1 mol/L solution of ammonium acetate and methanol varying from 27% (starting) to 90% (ending) of methanol V/V. Recovery of standards added to samples varied from 75% to 102%. Random samples of feces were preserved at −20° C in opaque containers. Between-run CVs varied from 10% to 15%.

Erythrocyte protoporphyrin

Erythrocyte protoporphyrin was measured by the method of Piomelli (7). Blood samples were collected in EDTA tubes (Becton Dickinson Canada, Inc., Mississauga, ON) wrapped in aluminum foil and preserved at 4° C. Our between-run CV for this method was 10%. When defining our normal reference interval, all results from samples with hematocrit ≤ 0.35 were excluded.

Erythrocyte fluorescence

Erythrocyte fluorescence was demonstrated by briefly viewing an erythrocyte smear (diluted with 154 mmol/L saline, if necessary) using an Olympus fluorescence microscope with a BG3 (405 nm) excitation filter.

Erythrocyte porphobilinogen deaminase activity was measured in duplicate using delta-aminolevulinic acid (ALA) as substrate (8). Between run precision was 4.7%.

Urine ALA and porphobilinogen (PBG) were measured by the method of Mauzerall and Granick (9) using a BioRad kit (BioRad Laboratories, Mississauga, Ontario, Canada).

Screening tests

Urine was screened for increased porphyrin content by spectrophotometric scanning of an acidified sample between 350 and 415 nm and measuring the peak absorption at 405 nm. The molar absorptivity was then used to obtain the total porphyrin content (10) and a "cut off" of 150 nmol/d was used to ensure a maximal detection of normal subjects. Currently in our service laboratory we use a "cut off" of 110 nmol/d to ensure maximal detection of abnormal subjects (11). Fecal screening was performed by extracting interfering substances from an acidified sample with diethylether and scanning the remaining aqueous fraction in a spectrophotometer. Peak absorption was measured and a "cut off" of 35 nmol/g wet feces was used (12). We prefer spectrophotometric absorption screening techniques rather than fluorometric screening methods because we have encountered too many false positive results with the latter.

Results

Tables 1, 2, and 3 report plasma, urine, and fecal porphyrin and precursor results in a variety of porphyrias and include our normal reference intervals. The data on Table 1 have been published previously (4). All data were derived from samples we received in our role as a major porphyrin reference laboratory and were checked for proper preservation and transportation (acid pH for urine ALA and PBG measurements, alkaline pH for urine porphyrin samples, samples frozen and transported in dry-ice for plasma porphyrins, samples frozen and wrapped in aluminum foil for fecal porphyrins). All abnormal data include the complete range of results encountered by our laboratory for a particular disease classification. Disease stratification was made by an experienced clinician and porphyrin chemist (J.T.H.) after discussion with the referring physician and perusal of porphyrin and precursor patterns in urine and feces and erythrocyte porphobilinogen deaminase activities.

Normal reference intervals

"Normal" samples were chosen by visual inspection of the results of all specimens processed. In the case of urine and fecal samples, those designated "normal" had all tested negative by our screening tests (10,12). The reference intervals were determined as a central 95% interval of ranked data using a nonparametric method (13). Modest elevations above the apparently normal range were occasionally seen for urine coproporphyrin (total coproporphyrin up to two times normal) without obvious

TABLE 1
Reference Intervals for Plasma Porphyrin, Erythrocyte Protoporphyrin, and Erythrocyte Porphobilinogen Deaminase Quantitations in Normal Subjects and Patients with Various Porphyrias [see (4)]

Porphyrin Fraction	Normal Subjects $n = 245$	Porphyria Cutanea Tarda $n = 32$	Acute Intermittent Porphyria $n = 3$	Porphyria Variegata $n = 5$	Hereditary Coproporphyria $n = 5$	Erythropoietic Protoporphyria $n = 4$
Uroporphyrin I, nmol/L	0–11	5–549	6.9–16	0.5–7.2	0.4–4.9	0
Uroporphyrin III, nmol/L	0–3	5–220	3.4–9.6	0.2–8.8	0.3–4.4	0
Heptacarboxyl III, nmol/L	0–5	10–402	0.4–0.9	0–3.8	0–1.7	0–0.2
Hexacarboxyl III, nmol/L	0–2	4–90	0	0–2.9	0–0.5	0
Pentacarboxyl III, nmol/L	0–2	2–41	0.4–1.7	0–3.7	0–1.0	0
Coproporphyrin I, nmol/L	0–10	0–16	1.4–36	0–5.9	0–3.5	0.8–174
Coproporphyrin III, nmol/L	0–12	0–26	3–24	0.2–8.7	0–9.3	2.2–203
Erythrocyte protoporphyrin, μmol/L erythrocytes	0.4–1.0 $n = 224$	—	—	0.7–1.3 $n = 4$	—	2.3–63 $n = 10$
Erythrocyte porphobilinogen deaminase, μmol/L erythrocytes/h	20–43 $n = 174$	—	12–18 $n = 7$	29–36 $n = 1$	27	—

n = number of subjects; — = not measured.

clinical explanations and these were excluded from our normal range study. Some of these cases could perhaps have been due to occult liver disease.

PLASMA PORPHYRINS

There is very little present in normal subjects. The values in PCT present an abnormal and distinctive pattern, but the ranges in the other porphyrias overlap the normal reference interval (except for uroporphyrin-III in acute intermittent porphyria [AIP], but our sample is small).

URINE PORPHYRINS

In normal subjects, uroporphyrins and coproporphyrins predominate. Only small amounts of hepta-, hexa-, and pentacarboxyl porphyrins were present, with the III-isomer predominating. Also reported are the urine porphyrin:creatinine ratios on all our

TABLE 2
Reference Intervals for Urine Porphyrin and Precursor Quantitations in Normal Subjects and Patients with Various Porphyrias

Porphyrin or Precursor Fraction	Normal Subjects		Porphyria Cutanea Tarda		Acute Intermittent Porphyria ($n = 4$) nmol/d	Porphyria Variegata ($n = 4$) nmol/d	Hereditary Coproporphyria ($n = 3$) nmol/d	Erythropoietic Protoporphyria ($n = 4$) nmol/d
	nmol/d ($n = 96$)	μmol/mol creatinine ($n = 96$)	nmol/d ($n = 141$)	μmol/mol creatinine ($n = 100$)				
Uroporphyrin I	0–44	0.4–3.9	77–12159	7.7–375	360–3587	16–1473	2–43	21–51
Uroporphyrin III	0–20	0–2	25–5283	3.7–264	296–3439	0–1312	0–14	0–42
Heptacarboxyl III	0–16	0–1.3	25–8526	2.2–414	16–205	3–34	0–29	19–91
Hexacarboxyl III	0–2	0–0.7	0–1944	0.5–44	0–16	0–16	0	0–13
Pentacarboxyl III	0–2	0–1	0–1379	0.6–56	40–267	0–78	0–24	0–21
Coproporphyrin I	5–90	0.3–8.5	3–2109	1.8–27	102–445	44–118	7–98	14–1243
Coproporphyrin III	15–242	1.7–26	0–963	4–125	313–1227	134–720	54–1735	35–534
Coproporphyrin III / Coproporphyrin I ratio, range and (median)	2.6–5.3 (4.3)		(2.6)					
Delta-aminolevulinic acid	0–50 μmol/d ($n = 118$)	1–5 mmol/mol creatinine ($n = 118$)	—	—	30–818 ($n = 6$)	26–226 ($n = 5$)	25–88 ($n = 2$)	—
Porphobilinogen	0–9 μmol/d	0.1–0.8 mmol/mol creatinine	—	—	25–732	2–101	—	—

n = number of subjects; — = not measured.

TABLE 3
Reference Intervals for Fecal Porphyrins in Normal Subjects and Patients with Various Porphyrias (nmol/g dry weight)

Porphyrin Fraction	Normal Subjects $n = 100$	Porphyria Cutanea Tarda $n = 47$	Acute Intermittent Porphyria $n = 2$	Porphyria Variegata $n = 4$	Hereditary Coproporphyria $n = 5$	Erythropoietic Protoporphyria $n = 4$
Uroporphyrin I	0–5	0.1–13	0.6–9.2	0–1.2	0–1.6	0.1–5.2
Uroporphyrin III	0–1	0–9	0–5.5	0–0.8	0–0.6	0–0.4
Heptacarboxyl III	0–1	1.6–151	0–0.6	0	0–0.7	0
Hexacarboxyl III	0–1	0–69	0–0.4	0	0–0.3	0
Pentacarboxyl III	0–1	0.5–57	0–0.7	0–3.2	0–1.8	0
Coproporphyrin I	0–13	4–70	0.3–7.4	6.8–28	12–48	0–18
Coproporphyrin III	0–12	1–96	0.4–5	3.5–114	37–983	0–5.2
Median Coproporphyrin III / Coproporphyrin I ratio	0.63	0.99				
Protoporphyrin	0–38	0–61	0–16	109–257	11–85	24–695

n = number of subjects

samples, which were all 24-h collections. Urine porphyrins were clearly abnormal in PCT and AIP, but overlapped with the normal reference interval in the other porphyrias.

FECAL PORPHYRINS

Fecal porphyrins were frequently abnormal in the various porphyrias, except in AIP in which they were usually normal or near normal. In all porphyrias, however, the abnormal range overlapped the normal, with the exceptions of protoporphyrin in variegate porphyria (VP) and coproporphyrin-III in hereditary coproporphyria (HCP).

URINE ALA AND PBG

Urine ALA and PBG were elevated in all cases of AIP, but were usually normal in VP and HCP. Table 4 presents the data from a suspected case of mixed PCT and VP.

Discussion

We have previously reported our method for plasma porphyrin measurement (4) and have shown that it is useful in the diagnosis and management of PCT. It can also differentiate porphyria cutanea tarda of renal failure from pseudoporphyria of renal failure. It was not useful in the diagnosis of the acute porphyrias because plasma levels were often normal. It cannot reliably quantify protoporphyrin, thereby limiting its utility in VP.

Values for urine and fecal total uroporphyrin and coproporphyrin have often been determined using solvent fractionation techniques, which are known to be inaccurate (14,15). There have been fewer studies using HPLC. Also, there have been few reports that included the I- and III-isomers of uro- and coproporphyrin and the intermediates between uro- and coproporphyrin (hepta-, hexa-, and pentacarboxyl-III) (16–19). Our normal reference intervals for urine porphyrins are similar to those of others (1,16,20). Uroporphyrins and coproporphyrins predominate, with the I-isomer exceeding the III-isomer for uroporphyrin and the III-isomer of coproporphyrin exceeding the I. Only small amounts of hepta-, hexa-, and pentacarboxyl porphyrins are present in normals, with the III-isomer predominating. Also reported are the urine porphyrin:creatinine ratios on all our samples, which were all 24-h collections. Others (21,22) have demonstrated that this is a reliable estimate of daily porphyrin excretion and it has the advantage that random samples

TABLE 4
Porphyrin Values in Patients with Suspected Mixed Porphyria (PCT and VP)

	Uro-porphyrin I	Uro-porphyrin III	Hepta-carboxyl III	Hexa-carboxyl III	Penta-carboxyl III	Copro-porphyrin I	Copro-porphyrin III	Proto-porphyrin
Urine porphyrin nmol/d	1093	927	1485	228	295	140	1213	—
Fecal porphyrin nmol/g dry weight	2.8	3.5	39	24	37	61	43	101

can be used. We do not use this ratio if the urine creatinine is < 4 mmol/L (1).

When assessing fecal porphyrin patterns, some authors prefer to measure fecal total porphyrins and then make an assessment of the individual porphyrins using a visual interpretation of an HPLC fractionation (6). We have elected to measure them using direct standardization. Our results are substantially lower than those reported by Elder *et al.* (1) and Logan *et al.* (23), but similar to those of Beukeveld *et al.* (18), when we use a conversion factor of $\times 3.3$ (24) to convert their fecal wet weight data to dry weight that we use. The recovery of dicarboxylic porphyrins in HPLC assays can be incomplete, but our recovery experiments indicate that our method did not suffer this problem. Antibiotic therapy can reduce fecal porphyrin levels (18), especially the secondary porphyrins (mesoporphyrin and deuteroporphyrin), but as most of our subjects were outpatients being investigated for skin lesions, it is unlikely that many of them were taking antibiotics.

Porphyria cutanea tarda

In this disease, plasma, urine, and fecal porphyrins demonstrated an easily recognizable pattern with moderate to large elevation of both uroporphyrin isomers, a pronounced elevation of heptacarboxyl-III (in plasma, the highest elevation of all fractions measured) usually followed by lesser elevations of hexacarboxyl-III, pentacarboxyl-III, and the coproporphyrin isomers. Heptacarboxyl-III was always elevated in plasma and urine, even when all other fractions were normal (usually in patients undergoing therapeutic phlebotomy). In mild disease, plasma and urine heptacarboxyl levels demonstrated equal sensitivity in detecting disease. Kalb *et al.* (19) have also shown that serum and urine total porphyrin levels have equal sensitivity in detecting PCT. Conversely, fecal porphyrins were sometimes completely normal in mild disease when the urine and plasma patterns were abnormal; therefore, fecal porphyrin quantitation would not be useful in diagnosis or management. However, our technique does not identify fecal isocoproporphyrin, and therefore we cannot comment about its sensitivity in this disease. Isocoproporphyrin is usually identified by thin-layer chromatography but we have abandoned that tedious procedure. There are often substantial elevations of plasma, urine and fecal coproporphyrin levels in PCT, even though this metabolite is produced distal to the enzyme deficiency. While some of this must be derived from nonenzymatic decarboxylation of the preceding porphyrins in the pathway, there is also, probably, in-vivo overproduction of coproporphyrin, due to an over-correction of the mechanism controlling heme synthesis, a phenomenon Jacob and Doss (25) have called "counter-regulatory compensatory enhancement." We believe that the modest increase in stool protoporphyrin which we encountered in some cases of PCT was due to bacterial action on the increased porphyrins present.

The median urine coproporphyrin-III:coproporphyrin-I ratio in PCT was 2.6, contrasting with 4.3 in our normal urines. Coproporphyrin isomer excretion in PCT is certain to be complex. Those patients with hepatic alcoholic liver damage may excrete in the urine, a predominant excess of the I-isomer (some of our patients fit this pattern), whereas others excreted excess III-isomer, presumably derived from the accumulated heptacarboxyl-III, which predominates in this disease. Our patients' median fecal coproporphyrin-III:I ratio in PCT was 0.99, our normal being 0.63. This is contrary to the findings of Badcock *et al.* (26), who reported the ratio to be lower than normal in sporadic PCT, the category in which most of our patients would belong. We cannot explain this difference. In contrast, Lim and Peters (17) found patterns similar to ours in the urine and feces of patients with PCT.

We have also reported urine porphyrin excretion per mole of creatinine on all our samples (they were all 24-h collections) and it can be seen that, in PCT, the pattern again clearly differs from that of normal urine. This interpretation would allow investigators to use random samples of urine which would avoid the tedium and inaccuracy of 24-h collections. Plasma porphyrin fractionation is also a reliable diagnostic test for PCT (4), and plasma is often easier to obtain than urine, the latter also requiring a preservative. However, our plasma HPLC assay is technically more demanding than our urine assay.

We have encountered five patients with florid PCT, which we have attributed to taking antiepileptic medications (phenytoin, carbamazepine). We have not been able to prove this hypothesis. It was impractical to stop their medications, therefore, the criterion of the disease being cured by discontinuing the drug could not be tested. Nevertheless, it is likely that the medications were related to the etiology. Our patients did not have any other risk factors related to PCT, such as chronic alcoholism or estrogen therapy (we did not check for hepatitis C status or hemochromatosis). Antiepileptic medications are potent inducers of cytachrome P450 isoenzymes in the liver and this is known to induce porphyrin synthesis. We are surprised that an association between antiepileptic therapy and PCT has, to our knowledge, only once been previously described as a single case study (27). It must be remembered, however, that many cases of sporadic PCT do not have any obvious precipitating cause. Yeung-Laiwah *et al.* (28) have described an association between antiepileptic therapy and an acute porphyric syndrome; however, our patients had normal levels of urine ALA and PBG thereby ruling out active acute porphyria.

The acute porphyrias

Our plasma porphyrin assay was not helpful in the diagnosis of the acute porphyrias (AIP, VP, and

HCP) as results were commonly normal (4) (Table 1). Unfortunately, we were unable quantitatively to recover protoporphyrin in our plasma assay, which seriously limits its utility in VP. In any event, plasma fluorescence emission scanning for the characteristic peak present in VP is easier to perform and this is apparently the most sensitive routine test to date for this disease (29).

Acute intermittent porphyria

Plasma porphyrin measurement in this disease varied from normal to a slight elevation of uroporphyrins-I and -III and coproporphyrins-I and -III. Urine porphyrins demonstrated a pattern of marked elevation of uroporphyrins-I and -III with less pronounced elevations of coproporphyrins-III and -I. Compared with PCT, the intermediates (hepta-, hexa-, and pentacarboxyl porphyrin) were only modestly increased, thus the urine porphyrin patterns of the two diseases are quite different. Urine porphyrins were performed on only four patients and they were all experiencing acute symptoms when the samples were taken. The I- and III-isomers of uroporphyrin were increased about equally (I slightly exceeding III in all cases) whereas the III-isomer of coproporphyrin was clearly increased more than its I counterpart. These excess urine porphyrins are most likely derived from nonenzymatic condensation of the excess porphobilinogen which occurs in acid urine. Urine porphyrins can, of course, be normal in AIP (30). Fecal porphyrins in AIP varied from normal to a slight elevation of uroporphyrin (Table 3).

The patterns of urine and fecal porphyrins that we observed agree with previous reports (1,14,17,20,25, 30–32); thus, plasma, urine, and fecal porphyrins offer little diagnostic information in AIP. In all our cases of AIP, the diagnosis was confirmed by demonstrating reduced activity of erythrocyte porphobilinogen deaminase (Table 1). All our cases of AIP had elevated urine PBG and ALA concentrations, although some of our cases were not symptomatic at the time of sampling. Urine PBG and ALA are usually, but not invariably elevated between clinical attacks in AIP (1,20,33).

Variegate porphyria

Plasma porphyrin concentrations, by our method, were normal in all but one patient. We have been unable to quantitatively recover protoporphyrin in our extraction (4), probably because of covalent bonding of protoporphyrin to plasma proteins in this disease (34). Urine ALA, PBG, and porphyrins ranged from normal to abnormal, depending upon clinical symptoms, which is typical of this disease (31). Two patients (who had active skin lesions) showed a marked elevation of urine uroporphyrins I and III in approximately equal amounts. Coproporphyrins-I and -III were also markedly elevated in these patients with the III-isomer predominating. They also had a moderate elevation of the intermediates between uroporphyrin and coproporphyrin but the pattern (modest elevation of intermediates compared with uroporphyrins) was quite different from that of PCT. Two patients also demonstrated a slight increase in erythrocyte protoporphyrin (1.2 and 1.3 μmol/L erythrocytes).

Fecal protoporphyrin concentrations were consistently increased, varying from three to seven times normal. The patient with the threefold elevation was in an inactive (subclinical) state, but she certainly has VP because she had previously experienced two acute attacks (weakness and abdominal pain) associated with elevated urine ALA and PBG. Also, her brother has florid VP with skin lesions and the typical biochemical changes, including a plasma fluorescence peak at 625 nm (29).

Two patients demonstrated fecal coproporphyrin-III increases of about ninefold. One might argue that they could have HCP and the differentiation of this from VP may be in doubt without definitive tests such as enzyme assays or perhaps biliary porphyrins (23). However, both probably had VP, because one demonstrated the presence of the typical plasma fluorescence emission peak at 625 nm (her fecal porphyrin, coproporphyrin-III, and -I were 109, 114, and 20 nmol/g dry weight, respectively). Plasma peak fluorescence was not measured on the other patient, but he had a substantial elevation of fecal protoporphyrin (257 nmol/g dry weight). His fecal coproporphyrin-III and -I levels were 112 and 28 nmol/g dry weight, respectively. Rarely, fecal porphyrins can be normal in VP (29), particularly in long dormant cases.

Hereditary coproporphyria

Plasma porphyrins were normal in this disease by our method, even though at least one patient had active skin lesions when the specimen was taken (Table 1).

Urine porphyrins varied from normal to a marked elevation of coproporphyrin-III, consistent with previous reports that urine porphyrins can be completely normal when these patients are asymptomatic (1,35). A modest elevation of heptacarboxyl porphyrin was seen in one case and of pentacarboxyl porphyrin in another but the pattern was quite different from that of PCT. In patients with HCP there appears to be some preferential excretion of uroporphyrin-III by the kidney, as its renal clearance can exceed that of creatinine (36).

Fecal porphyrins remain the better test for this disease, demonstrating a moderate to marked elevation of the I and III coproporphyrin isomers with a marked preponderance of the latter. Two of our five cases demonstrated a moderate elevation of protoporphyin, but their relative increases in fecal coproporphyrins always exceeded those of protoporphyrin. Some clinicians prefer to see at least a fivefold elevation in fecal coproporphyrins to make the diagnosis of HCP, but all our cases with lesser elevations

had strongly suggestive clinical histories, although most were asymptomatic when testing was performed. Therefore, we felt that they probably had the disease. Occasionally, fecal porphyrins can be normal in this disease (35).

It should be noted that patients with VP and HCP have a predominance of the III-isomer of coproporphyrin in their urine and feces, in keeping with their metabolic defects, whereas, as we will show, patients with erythropoietic protoporphyria (EPP) demonstrate, if coproporphyrin is increased, an excess of the I-isomer in urine and feces. These findings are in general agreement with those of others (1,17,18,31,37,38).

Blake's group (31) has observed that in symptomatic HCP the fecal coproporphyrin-III:I ratio is greater than in symptomatic VP, and our results support their findings except that Blake's ratios in symptomatic HCP were > 10, whereas ours were only > 8. Urine ALA and PBG varied from normal to abnormal in HCP, as is characteristic of this disease (1).

Erythropoietic protoporphyria

Plasma porphyrin assays, by our technique, were not helpful in the diagnosis of this disease (4); protoporphyrin, which is the most elevated plasma fraction, is poorly recovered in our method. Our two unusually florid cases with abnormal patterns showed marked elevations of coproporphyrin-I and -III, the latter exceeding the former.

Urine porphyrins are usually normal in EPP unless there is liver disease, secondary to hepatic porphyrin deposition in longstanding cases. Thus, our mild cases demonstrated normal urine porphyrins, whereas our two florid cases exhibited elevated levels particularly of coproporphyrin-I, as described by Doss and Frank (39). Hepta-, hexa-, and pentacarboxyl porphyrins were also moderately elevated in our florid cases, particularly heptacarboxyl, but the pattern was quite different from that in PCT.

It should be noted that the fecal and urine porphyrin patterns in VP and florid EPP may, at first glance, be similar: elevations of protoporphyrin in feces and coproporphyrin in urine, but in VP it is the urine coproporphyrin-III that is more elevated whereas in EPP, if urine coproporphyrin is elevated, it is predominantly the I-isomer.

Fecal porphyrins in this disease varied from normal to marked elevations of protoporphyrin, this latter finding being pronounced in our florid cases, one of whom also had a slight elevation of coproporphyrin-I.

Fecal deuteroporphyrin and mesoporphyrin (secondary porphyrins) were sometimes elevated in VP, HCP, and EPP (data not shown) but did not add any diagnostic information. This is presumably the consequence of bacterial action on the elevated (primary) fecal porphyrins in these diseases.

We have been able to define the reference intervals for erythrocyte protoporphyrin in normal subjects and in several conditions in which it is elevated. Levels in anemia were usually less than 2.2 μmol/L erythrocytes) except for one severe case (hematocrit 0.27) when it was 3.04. In our single case of lead poisoning (blood lead 2.87 μmol/L) erythrocyte protoporphyrin was 3.5 μmol/L erythrocytes. Our method does not distinguish zinc protoporphyrin from free protoporphyrin. Levels in erythropoietic protoporphyria were usually > 4, but two of our mild cases were as low as 2.3 and 2.6 μmol/L erythrocytes. Our florid cases had levels of 34 and 63. These results are in general agreement with those of Piomelli (7) who reported that in iron-deficiency anemia erythrocyte protoporphyrin was often elevated up to three times the upper limit of the normal reference interval, but in EPP it was usually higher. Because of the occasional overlap between the levels of erythrocyte protoporphyrin in anemia and EPP (and also to exclude lead poisoning), we always confirm the diagnosis of EPP by demonstrating erythrocyte fluorescence. Very transient fluorescence is also seen in lead poisoning but the fluorescence in erythropoietic protoporphyria is more persistent.

Mixed porphyria

One of our patients probably has dual porphyria (Table 4). He has a urine and fecal porphyrin pattern of PCT except that the urine coproporphyrin is higher than our other PCT cases and the III-isomer predominates. Also, his fecal protoporphyrin is elevated. Thus, he probably has PCT superimposed on VP although enzyme studies or genetic analysis would be needed to confirm the diagnosis. This syndrome has been reported previously (40,41).

Procedure for investigating a suspected case of porphyria

Many authors have published schemes for the investigation of porphyrias (1,20,42). Elder's (1) is comprehensive, but relies upon thin-layer chromatography that we find difficult to standardize in our reference laboratory; therefore, we prefer HPLC.

Specimens can be screened for increased porphyrin metabolites at the referring laboratory but detailed porphyrin testing is most effectively performed by a porphyrin reference laboratory. The referring laboratory should provide a stat screening test for porphobilinogen for the urgent evaluation of suspected acute neurovisceral porphyric attacks and, although the classical Watson-Schwartz technique will be positive in most acute attacks, it is better to employ one of the screening procedures that provides improved sensitivity over the classical technique (43,44). Also, to avoid sending normal samples to the reference laboratory, the referring laboratory should perform screening tests for urine porphyrins (10) and fecal porphyrins (12).

The pathway for heme synthesis is shown in Figure 1 and a classification for the porphyrias,

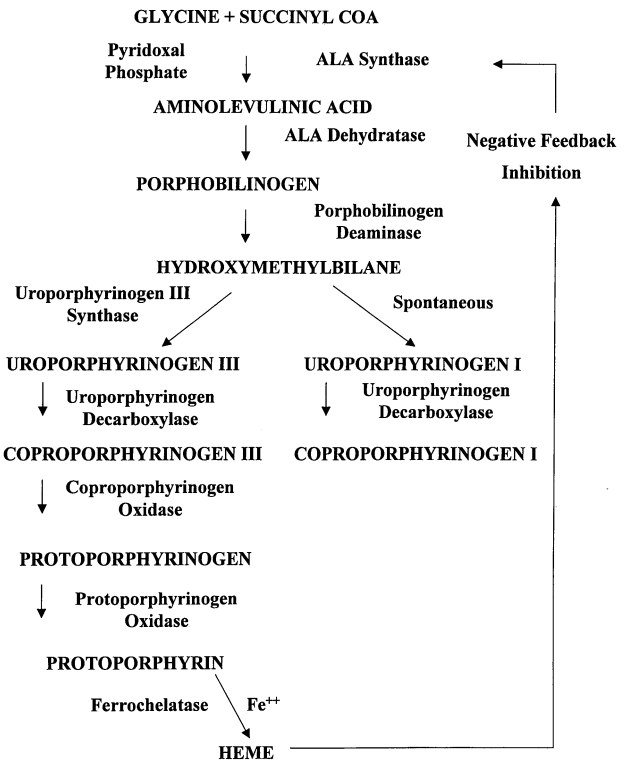

Figure 1 — Heme synthesis pathway.

which includes identification of the related enzyme defects, in Table 5. Figure 2 presents an algorithm for their investigation. Often, when we see a patient, we collect all specimens that may be necessary, but only analyze those that are required to achieve a diagnosis. This algorithm we will deal only with identification of the more common porphyrias: thus congenital erythropoietic porphyria, toxic porphyria, hepatoerythropoietic porphyria, harderoporphyria, and ALA dehydratase deficiency porphyria will not be discussed.

If a patient presents with acute neurovisceral manifestations, quantify urine PGB and ALA. If these are normal, the physician can be reassured that the patient is not having an acute attack of porphyria. This, of course, does not rule out that the patient has porphyria, it simply tells us that they are not having an acute attack at this time and that their symptoms and signs have another cause. Patients with AIP usually (but not invariably) have elevated urine ALA and PBG even between attacks but patients with VP or HCP often have normal urine ALA and PBG between attacks. Therefore, if the previous clinical history is compelling but the urine ALA and PBG normal, then AIP should be sought by assay of erythrocyte porphobilinogen deaminase, and VP and HCP by fecal porphyrin quantitation.

If the urine ALA and PBG are increased then the patient probably has an acute porphyria and these should be differentiated by performing erythrocyte porphobilinogen deaminase and fecal porphyrin assays. It should be remembered that erythrocyte porphobilinogen deaminase may not be completely discriminatory as it may fall in the normal/abnormal overlap range (20) or be completely normal if the patient has the rare variant where the enzymatic defect is not present in erythrocytes (45). If the porphobilinogen deaminase assay is not available, or is equivocal, then AIP can be differentiated from VP and HCP during an acute attack by demonstrating a normal or near-normal fecal porphyrin level, characteristic of AIP (1).

We prefer to use ALA rather than PBG as the substrate for our erythrocyte porphobilinogen deaminase assay. It has the disadvantage that whole-blood controls are somewhat unstable, keeping for only about 3 weeks at 4° C, whereas when using PBG as substrate, whole-blood controls are apparently more stable. This phenomenon is probably the consequence of deterioration of the enzyme

TABLE 5
Clinical Classification of Porphyria

Acute porphyrias			
	Neurovisceral Manifestations Only		
1) Acute intermittent	Autosomal dominant	Porphobilinogen deaminase	Liver
2) ALA dehydratase deficiency	Autosomal recessive	ALA dehydratase	Liver
	Neurovisceral and/or Cutaneous Manifestations		
3) Variegate porphyria	Autosomal dominant	Protoporphyrinogen oxidase	Liver
4) Hereditary coproporphyria	Autosomal dominant	Coproporphyrinogen oxidase	Liver
Nonacute porphyrias			
	Cutaneous Manifestations Only		
1) Congenital erythropoietic	Autosomal recessive	Uroporphyrinogen-III synthase	Erythroid cells
2) Porphyria cutanea tarda	Sporadic or autosomal dominant	Uroporphyrinogen decarboxylase	Liver
3) Toxic porphyria	Acquired	Uroporphyrinogen decarboxylase (usually)	Liver
4) Hepato-erythropoietic porphyria	Autosomal recessive	Uroporphyrinogen decarboxylase	Erythroid cells & liver
5) Harderoporphyria	Autosomal recessive	Coproporphyrinogen oxidase	Liver
6) Erythropoietic protoporphyria	Autosomal dominant	Ferrochelatase	Erythroid cells & liver

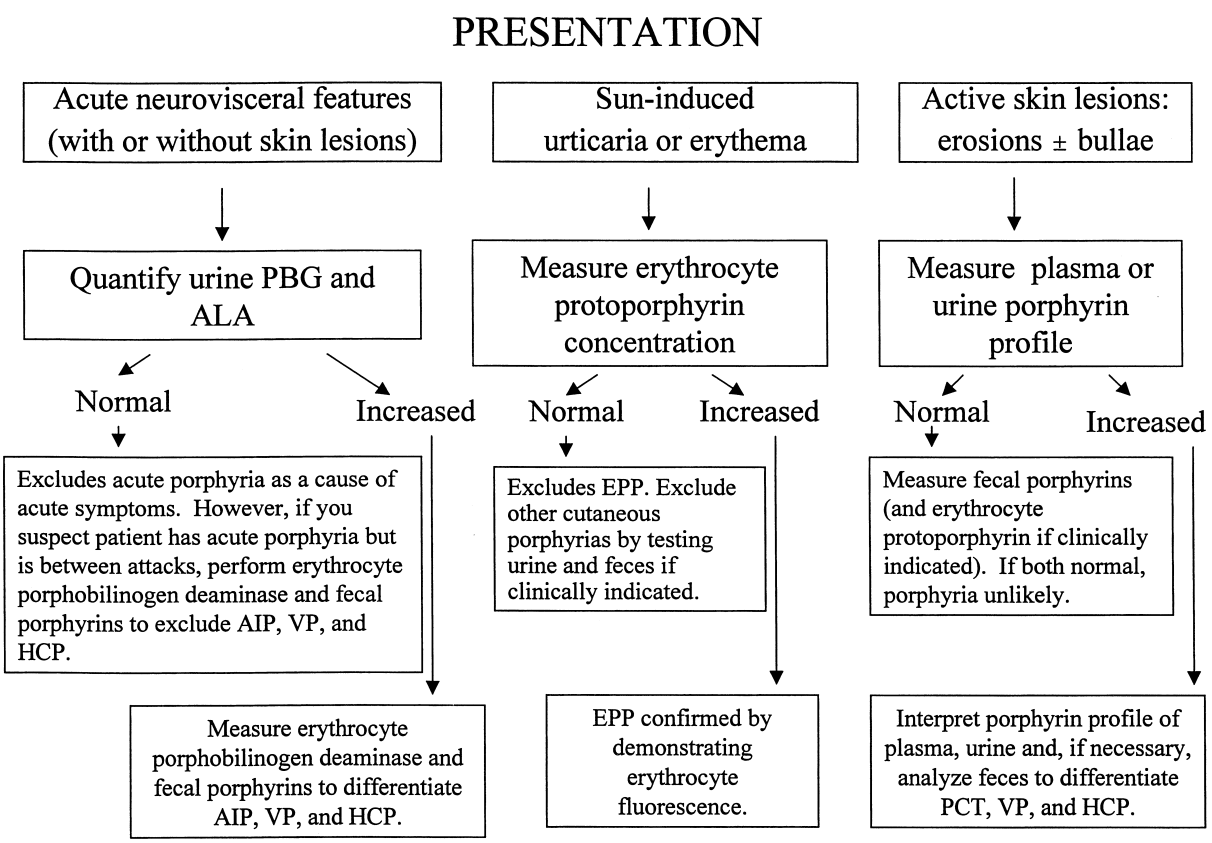

Figure 2 — Algorithm for the differential diagnosis of the porphyrias. Abbreviations: AIP = acute intermittent porphyria; VP = variegate porphyria; HCP = hereditary coproporphyria; PCT = porphyria cutanea tarda; EPP = erythropoietic protoporphyria.

ALA dehydratase on storage. The advantages of ALA over PBG as substrate are: it is more stable and less expensive; also, a low level of porphobilinogen deaminase would be encountered in ALA Dehydratase Deficiency Porphyria, thereby adding an additional disease to our diagnostic armamentarium. We have addressed the problem of unstable whole blood controls by using samples from two laboratory staff, collecting specimens every three weeks. The intrapersonal variation in erythrocyte porphobilinogen deaminase activity is quite small, the overall between run CV of the method being $< 5\%$ (80 consecutive assays from 30 samples).

The clinical features of EPP are fairly specific: sun-induced urticaria or erythema, often chronic and with skin induration, and usually presenting in childhood. Thus, the investigator can usually proceed directly to the specific test: erythrocyte protoporphyrin quantitation, and if this proves to be normal, then EPP is excluded. If the clinical history is vague, it is often advisable to test urine and feces to detect PCT, VP or HCP.

If erythrocyte protoporphyrin concentration is increased then one must determine if this is due to EPP, lead poisoning or anemia. Most laboratories, including our own, do not have facilities to differentiate free protoporphyrin from zinc protoporphyrin (free protoporphyrin is elevated in EPP whereas zinc protoporphyrin is elevated in anemia and lead poisoning). Thus, we confirm the diagnosis of EPP by demonstrating erythrocyte fluorescence in a blood smear. A clue can be obtained from the fact that erythrocyte protoporphyrin levels are often higher in EPP than in anemia (not so for lead poisoning however). Nevertheless, we had two patients with mild EPP whose erythrocyte protoporphyrin was within our "anemia" range.

If the patient has the characteristic active skin lesions of trauma-induced bullae and erosions in light-exposed areas, perhaps associated with hirsutism and pigmentation, and they have a history of exposure to one of the risk factors for PCT such as alcoholism or estrogen therapy, then we proceed directly to plasma porphyrin fractionation and quantitation. Those laboratories that cannot offer plasma porphyrin fractionation, including the isomers of uroporphyrin and coproporphyrin and their intermediates hepta-, hexa- and pentacarboxyl-III, should use the urine HPLC assay, provided it offers the same fractionation, as it has equal sensitivity to the plasma assay. We use plasma porphyrin fractionation as our primary test for PCT because our clinicians prefer to send us plasma rather than urine. If the patient does not have a history of exposure to PCT risk factors, then we collect and store a sample of feces for future analysis should this prove necessary.

If the plasma or urine assay is normal then fecal

porphyrin fractionation should be performed to determine if the patient has VP or HCP. Urine porphyrins can be completely normal between attacks in VP and HCP but fecal porphyrins are usually elevated in these diseases, except in dormant cases when all of the above tests may be normal. The dermatologic features of EPP are not often confused with those of PCT, VP, or HCP. However, if the clinical history is vague, it may be advisable to perform erythrocyte protoporphyrin quantitation also.

If the plasma or urine porphyrin fractionation is increased then the profile should be studied and if the characteristic pattern of PCT is present then the diagnosis is made and the feces need not be analyzed. If the pattern is not that of PCT then fecal fractionation should be performed and studied to differentiate VP from HCP. The diagnosis of VP can be confirmed by plasma fluorescence scanning (29), but we have limited experience with this test. VP exhibits a characteristic fluorescence emission peak between 621 and 627 nm. Patients with EPP may have a peak at 636 nm but we have found that this is not always present (4). A peak between 618 and 622 may be present in AIP, HCP, congenital erythropoietic porphyria, PCT, renal failure, and cholestasis, but may also be present in normal subjects (29,46), so this test is only of value in VP.

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