Thin-Layer Chromatography of Free Porphyrins for Diagnosis of Porphyria

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In this simple thin-layer chromatographic (TLC) technique for evaluating porphyrin excretion, porphyrins are extracted from urine or feces, then separated on silica-gel TLC plates. The distinct porphyrin bands are observed by viewing the plates under long-wave fluorescent light. Positive screening tests can readily be confirmed or rejected, and a more comprehensive investigation confidently undertaken.

In most general clinical chemistry laboratories, porphyria investigations consist only of the use of simple screening tests and the referral of samples to specialist centers. Consequently, there is often little interest or understanding of the porphyrins within a laboratory, so that the performance of even these screening tests may be inadequate (1). Yet this is a group of diseases in which the laboratory can play a vital and leading role in the diagnostic process.

The single most important of these tests is that for a porphyrin precursor, porphobilinogen. Detection of porphobilinogen is vital for the rapid diagnosis of an acute porphyrinic attack. Analyses for porphyrins are of less immediate importance, although the demonstration of porphyrinuria may well provide important corroborative evidence. This is true even for acute attacks resulting from acute intermittent porphyria, in which uroporphyrin is formed from porphobilinogen in the urine. However, such assays are necessary for the diagnosis of photosensitive porphyrias, for the differential diagnosis of acute porphyrias, and for family studies.

Screening tests for porphyrins usually consist of an extraction procedure involving use of organic solvents (2) or t alc (3), followed by examination of the extract under an ultraviolet light or by spectrophotometric scan. Reported methods for qualitative and quantitative porphyrin analyses by thin-layer chromatography (TLC) (4) or "high-pressure" liquid chromatography (2) invariably involve forming and purifying porphyrin methyl esters. The methyl esters are more stable and easier to handle than are the free porphyrins, but their preparation is time consuming and necessitates use of a lyophilizer. Methods involving solvent extraction and separation by solubility in hydrochloric acid solutions (6) are similarly laborious, and the final porphyrin fractions are far from pure.

Thus many laboratories are deterred from pursuing diagnoses other than at a very elementary level. By using the technique described here, any laboratory could, without great difficulty, make a greater contribution to porphyria diagnosis and thereby stimulate interest in and awareness of this group of diseases.

**Materials and Methods**

All solvents were of analytical-reagent grade (BDH Ltd., Poole, U.K.). The method is based on a solvent system suggested by Labbé (6). The samples, 5 mL of urine or a "pea-sized" portion of feces, were extracted with 3 mL of ethyl acetate/glacial acetic acid (4/1 by vol). An aliquot of the organic phase (10 μL for urine, 8 μL for feces) was applied as a 10-mm strip on the baseline 15 mm from the lower edge of a 200-mm-wide silica-gel TLC plate (Merck, Darmstadt, F.D.R.; Art. no. 5553). Mixed standards of free porphyrins (Porphyrin Products, Logan, UT; available from Pierce & Warriner, Poole, U.K.) were reconstituted to give a final concentration of 10 nmol/mL by adding 1 mL of extraction solvent and 100 μL of concentrated HCl per vial. Aliquots of this solution were placed in smaller glass bottles and dried under nitrogen. The dried standards were stored desiccated, in the dark. Before analysis they were reconstituted in 100 μL of the same reagents and 5-μL aliquots were applied to the TLC plates.

The plates were developed in chloroform/methanol/ammonia/water (24/25/6/4 by vol). The porphyrin bands could then be observed by illuminating the plates with long-wave ultraviolet light in a dark room. These bands were best visible when the plates were still damp from the solvent. The fluorescence faded within about 10 min, but could be revived by overspraying the plates with the chromatographic solvent. For a permanent record, the plates could easily be photographed with a yellow filter over the camera lens.

**Results**

The migration of the porphyrins on the TLC plates was inversely proportional to the number of carboxyl groups around the ring. Uroporphyrin, with eight carboxyl groups, remains at the origin; protoporphyrin, with only two, travels the farthest (Rf about 0.6).

Fresh urine samples from nonporphyric subjects ordinarily contain only a single band for coproporphyrin. Fecal samples usually show bands for coproporphyrin and protoporphyrin and orange-fluorescent bands, probably from dietary chlorophyll pigment (6), that migrate with the solvent front.

Figure 1 illustrates some of the characteristic porphyrin patterns seen in various porphyrias. Table 1 offers interpretations of observed patterns. These are based on observations I made by using this technique, together with previously reported accounts of porphyrin excretion in porphyria (2, 7).

**Discussion**

This simple TLC procedure for the qualitative evaluation of porphyrin excretion in urine and feces is a useful supplement for any laboratory that currently relies solely on simple screening tests to detect porphyria.

Screening tests for fecal porphyrins are invariably positive, owing to the presence of bacterial and dietary chlorophylls. By using the TLC procedure described here, one can remove these interfering substances before evaluating the porphyrin pattern and, because the readily identified abnormal patterns are characteristic for each of the porphyrias, reliable tentative diagnoses can be made.
Fig. 1. Thin-layer chromatogram, illuminated by ultraviolet light and photographed through a yellow filter.

Lanes: a,b,l and u, standards; b,d,m, and o, control urine; c,e,n, and p, control feces; f and g, urine and feces from variegate porphyria; h and i, urine from acute intermittent porphyria during attack; j, feces from hereditary coproporphyria; q and r, urine and feces from porphyria cutanea tarda (unfortunately these were old, stored samples; bands are typically heavier than this); a and t, feces from erythropoietic protoporphyria. The solvent front (SF) is at the top. The lowest band (for uroporphyrin) has not migrated from the baseline (9). The numbers at the right indicate the number of carboxyl groups for each standard.

Nevertheless, confirmation of new diagnoses should always be sought from an experienced reference center. This is particularly important for patients who have presented with photosensitive skin lesions but who may be at risk of life-threatening acute attacks if they have variegate porphyria or hereditary coproporphyria. Once a diagnosis has been established, the TLC procedure can be used to screen other family members.

The two disorders that may be difficult to diagnose are acute intermittent porphyria and erythropoietic protoporphyria. It is important to be aware of the likelihood of missing the diagnosis of acute intermittent porphyria in patients who are in clinical remission. In such cases, a diagnosis can be made only by analysis of erythrocytes for porphobilinogen deaminase (EC 4.3.1.8).

Fecal excretion of protoporphyrin is not always clearly increased in erythropoietic protoporphyria. Therefore the TLC technique should not be relied upon to exclude this disorder. Patients presenting with typical photosensitive lesions or a positive family history should be investigated by quantitative measurements of erythrocyte protoporphyrin or plasma porphyrin (8).

Weakly positive results of screening tests for urinary porphyrins are relatively common, and are generally related to a modest increase in urinary coproporphyrin excretion caused by cholestatic liver disease or alcohol consumption. The problem that many laboratories face is how to deal with such results: whether to disregard them, having considered the clinical history, or to refer the samples to another laboratory. Probably the major advantage of introducing this TLC technique to a laboratory is that the laboratory can then evaluate such samples, thus saving time and decreasing the rate of referral of samples to more-specialized laboratories.

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References