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High-Performance Thin-Layer Chromatography of Free Porphyrins for Diagnosis of Porphyria

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In this simple high-performance thin-layer chromatographic technique for evaluating porphyrin excretion, porphyrins rapidly extracted from urine or feces are separated on reversed-phase octadecylsilyl (C_{18})-bonded silica thin-layer chromatography plates. We observed >12 distinct bands of different porphyrins by viewing the plates under long-wave ultraviolet light. Positive screening tests can readily be characterized by the relative fluorescence of various free porphyrins. The two patients presented in this paper are possibly the first two cases of porphyria cutanea tarda reported in Hong Kong Chinese.

Indexing Terms: *porphyria cutanea tarda/chromatography, reversed-phase/solid-phase extraction/uroporphyrin/coproporphyrin/urine/feces*

Investigation of suspected porphyria has recently been reviewed by Elder et al. (*1*). Although solvent extraction methods remain the most popular follow-up tests for patients with porphyrin excess, they lack specificity. According to the review, thin-layer chromatography (TLC) or HPLC methods should now replace solvent extraction techniques for the fractionation of mixtures of porphyrins.[2]

Quantitative methods for porphyrins involving HPLC with fluorometric detectors are preferable for those laboratories with this facility and expertise. The ammonium acetate buffer–organic modifier reversed-phase (RP) HPLC systems (*2, 3*) are robust and provide complete resolution of urinary and fecal porphyrin mixtures, including separation of type I and type III isomers. However, such systems require expensive, dedicated equipment and highly skillful expertise.

For most laboratories in general hospital settings, a less complicated separation technique such as TLC is desirable. Previously, the analysis of porphyrins as their methyl esters in normal-phase TLC or HPTLC was recommended (*1*). The methyl esters are more stable than the free porphyrins, but their preparation is labor-intensive and time-consuming. A very simple TLC procedure (*4*) allows qualitative evaluation of urinary and fecal porphyrin patterns to provide tentative differential diagnoses, thereby saving time and decreasing the rate of referral of samples to more specialized laboratories. We initially used this silica-gel TLC method for investigating our first porphyria cutanea tarda (PCT) patient; however, we failed to demonstrate a discrete isocoproporphyrin band in the fecal sample, which is considere diagnostic for PCT (*5*). Thus, we explored the potentia of using a novel RP-HPTLC method for urine and feca porphyrin separation, which provides better resolutio and more informative porphyrin patterns. We believ this new method is suitable for routine laboratory use

Materials and Methods

Subjects

Patient 1. A 64-year-old male Chinese intravenou drug abuser and chronic alcoholic with liver cirrhosi was admitted to hospital because of septic arthritis. Th patient had increased pigmentation over the body an hypertrichosis of the face, particularly on the tempora regions. There was no history of photosensitivity an fragility of the skin and no bullae were found. Cutane ous porphyria was suspected, and urine and feces wer sent for porphyrin analysis.

Patient 2. A 41-year-old male Chinese laborer com plained of increased susceptibility to minor trauma c the skin over the past 1.5 years. He was a heav drinker, having consumed 4–20 pints of beer per day fo the past 20 years. Physical examination showed hyper pigmentation with multiple small superficial erosion over the backs of both hands. Hypertrichosis was note over the temporal regions. The patient is married an has a 19-year-old son and a 4-year-old daughter, bu there is no familial history of similar illness. The pro visional diagnosis was porphyria cutanea tarda. Urin and feces were collected and analyzed for porphyrins.

Apparatus

We used a Vac-Elut reduced pressure manifold fo solid-phase extraction from Analytichem International Harbor City, CA; an ultraviolet (UV) cabinet with long-wavelength UV lamp from Luckham, Sussex, UK and a Pentax Zoom 90 camera from Asahi Optical Co. Tokyo, Japan.

Reagents

All reagents were analytical or chromatographi grade: Acetonitrile, dioxane, ethanol, *N*-cetyl-*N,N,N* trimethyl ammonium bromide, paraffin (viscous, DAI 8), and RP-18 HPTLC precoated plates were fron Merck, Darmstadt, Germany; uroporphyrin III, copro porphyrin III, protoporphyrin IX, and mesoporphyrin D calibrators were from Sigma, St. Louis, MO; mixed cal ibrators of free porphyrins (uroporphyrin I, heptacar boxylic porphyrin I, hexacarboxylic porphyrin I, penta carboxylic porphyrin I, coproporphyrin I, mesoporphyri IX) and deuteroporphyrin were purchased from Porphy rin Products, Logan, UT.

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[1] Author for correspondence. Fax 852 370-0969.
[2] Nonstandard abbreviations: TLC, thin-layer chromatography; HPTLC, high-performance TLC; RP, reversed-phase; PCT, porphyria cutanea tarda; QC, quality control; and UV, ultraviolet.
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The commercial urine control, Lyphochek Quantitative Urine Control, Level 2, was purchased from Bio-Rad ECS Division, Anaheim, CA.

The external quality-control (QC) samples taken from PCT patients were obtained from an external QC program run by the Australian Association of Clinical Biochemists Working Party on porphyria.

Solid-phase extraction minicolumns (C_{18}, 0.5 g) were obtained from Syva, Palo Alto, CA, as part of the Emit digoxin kit.

The photographic film used was Kodak 200 negative from Eastman Kodak, Rochester, NY.

Sample Preparation

Calibrators. Porphyrin calibrators were dissolved in 3 mol/L HCl to a concentration of 10 μmol/L. For solid-phase extraction, 2 mL was used.

Urine. Fresh urine (3 mL) was acidified with 300 μL of glacial acetic acid for solid-phase extraction.

Feces. About 500 mg of fresh feces was weighed in a screw-cap tube. The mass of feces was suspended and homogenized in 5 mL of 4 mol/L HCl by vigorous vortex-mixing for 5 min. After centrifugation (1000*g*, 15 min), 1 mL of the acid extract was withdrawn and neutralized with 1.2 mL of 10 mol/L NaOH. Then 300 μL of glacial acetic acid was added to adjust the pH of the extract to 3.0 for solid-phase extraction.

Solid-phase extraction. C-18 minicolumns were preconditioned with 1 mL of methanol and then 1 mL of 0.2 mol/L phosphate buffer, pH 3.0. Calibrators, patients' samples, or control specimens were passed through columns under negative pressure. The columns were then washed twice with 1 mL of 0.2 mol/L phosphate buffer, pH 3.0. Porphyrins were eluted and collected with 0.5 mL of 900 mL/L methanol in 0.2 mol/L phosphate buffer, pH 7.5. The final extract of the samples (10–20 μL) were applied as fine lines (1-cm wide) onto silica-gel TLC or RP-HPTLC plates.

Silica-Gel TLC

Normal-phase TLC was based on the method of Henderson (*4*). However, the chromatogram was developed on a silica-gel TLC plate (Merck no. 5567) with a solution of chloroform, methanol, concentrated ammonia, and water (28:28:6:4 by vol).

RP-HPTLC

The method is based on a solvent system suggested by Junker-Buchheit and Jork (*6*) for porphyrin standard solutions. The chromatographic conditions were modified and optimized for the separation of major porphyrins in urine and feces by using ascending one-dimensional development in a saturated chamber (ambient temperature, absence of light) and HPTLC precoated plates RP-18 WF_{254s}.

Developing solutions were 20 mL of ethanol and 20 mL of dioxane (solvent 1) and 40 mL of acetonitrile, 20 mL of ammonium acetate buffer (0.1 mol/L, pH 4.1), and 0 mL of *N*-cetyl-*N,N,N*-trimethylammonium bromide (0.01 mol/L) (solvent 2).

Migration time and distance were 90 s, 13 mm (solvent 1) and 25 min, 80 mm (solvent 2).

For fluorometric viewing, the porphyrin bands were observed by illuminating the plates with long-wave UV light in a dark cabinet. These bands are best viewed when the plates are still damp from the solvent. The fluorescence fades within ~10 min, but can be revived by dipping the solvent-free plates into a paraffin:*n*-hexane solution (35:65 by vol). Porphyrin bands in post-treated plates stored in the dark were stable for at least 24 h without loss of fluorescence.

For a permanent record, the plates could easily be photographed over a window of the UV cabinet with Kodak 200 film with a Pentax Zoom 90 on macro mode exposed for 15 s, with autofocusing and autoaperture.

Results

The patterns of the normal-phase TLC based on the method of Henderson (*4*) are shown in Fig. 1. The migration of the porphyrins on the TLC plates was inversely proportional to the number of carboxyl side chains around the tetrapyrrolic porphyrin macrocycle. Both patients had distinctive urine and fecal porphyrin patterns. The urinary patterns were characterized by prominent bands of 8-carboxyl uroporphyrin and 7-carboxyl porphyrin. The fecal samples showed prominent bands of 7-carboxyl porphyrin and an extra cluster of fluorescence between the 5-carboxyl porphyrin and coproporphyrin. We suspect that the extra fluorescence cluster is caused by isocoproporphyrin, which is overlapped by some 5-carboxyl porphyrin isomers. A very similar pattern was obtained with the external QC sample from a PCT patient with a total porphyrin concentration of 820 μmol/kg feces.

The RP-HPTLC chromatograms of urine and fecal porphyrins of patient 2 and of feces of patient 1 are shown in Fig. 2. The enhanced resolving power of the HPTLC system is demonstrated by the discrete separa-

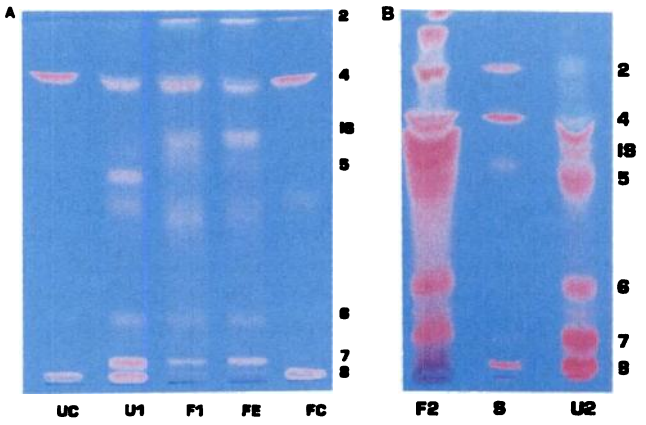

Fig. 1. Normal-phase TLC of patients 1 and 2 and an external QC sample of PCT.

(*Left*) UC, urine control; U1, urine of patient 1; F1, feces of patient 1; FE, feces of the external QC sample of PCT; FC, normal feces control. (*Right*) F2, feces of patient 2; S, calibrators containing uroporphyrin, coproporphyrin, and protoporphyrin; U2, urine of patient 2. 8, uroporphyrin; 7, heptacarboxylic porphyrin; 6, hexacarboxylic porphyrin; 5, pentacarboxylic porphyrin; 4, coproporphyrin; 2, protoporphyrin; Is, isocoproporphyrin.

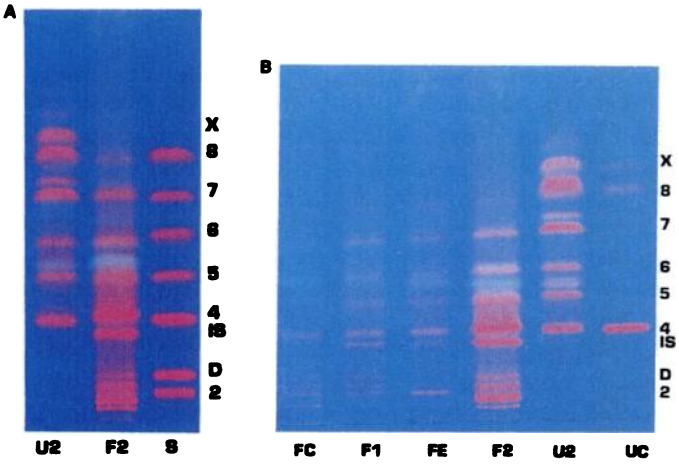

Fig. 2. HPTLC of patients 1 and 2 and an external QC sample of PCT.

S, mixed calibrators of free porphyrin and deuteroporphyrin; D, deuteroporphyrin; X, peptide-conjugated porphyrins; other symbols as in Fig. 1.

tion of minor bands in addition to the major bands of 2-, 4-, 5-, 6-, 7-, and 8-carboxyl porphyrins. Thus, the fecal sample of patient 2 shows an intense distinct band below the coproporphyrin band. This band is not found in the patient's urine nor in the normal feces control. The relative mobility of this band in the chromatogram is consistent with that of isocoproporphyrin (*8*).

The better resolution in our HPTLC method is also evident in the separation of various dicarboxylic porphyrins. As shown in the fecal sample, sharp minor bands migrated to positions other than those of the protoporphyrin and deuteroporphyrin calibrators. In the external QC sample (Fig. 2), deuteroporphyrin is absent, possibly due to antibiotic usage by the patient before collection of the feces. In normal feces, the dicarboxylic porphyrin fraction is approximately one-third protoporphyrin, the other two thirds being deuteroporphyrin and pemptoporphyrin. During complete suppression of bacterial flora by antibiotic therapy for prolonged periods, the production of proto- and coproporphyrins was decreased and deutero-, pempto-, and mesoporphyrins were absent (*7*).

Two additional bands above the uroporphyrin band were also detected in the urine sample of patient 2 (Fig. 2); the lower one was also found in the commercial urine control, albeit with a lower intensity. These two bands were not detected by the normal-phase TLC (Fig. 1). The polarity of these two porphyrins would be higher than that of uroporphyrin in our RP-HPTLC system. On the basis of the relative polarity of porphyrins, these two bands could possibly be the peptide-conjugated porphyrins or porphyrin X (*8*).

Discussion

The enhanced separation of free porphyrins in our method is attributable to the high capacity of sample cleanup by solid-phase C_{18} minicolumn and the high efficiency of chromatography by RP-HPTLC. The chromatographic mechanism of this method is analogous to the RP-HPLC method of Lim and Peters (*2*). RP techniques allow separation of free porphyrins near the isoelectric points and thus allow maximum resolution of various porphyrins according to the polarity of their side chains.

The mechanism of HPTLC separation has been described by Junker-Buchheit and Jork (*6*): Porphyrin free acids are separated on an RP-HPTLC plate coated with octadecylsilyl-bonded silica. After concentration of the chromatogram zones with the polar mixture of ethanol and dioxane, a buffered ternary solvent system is used as the mobile phase. This system contains the ion-pairing reagent *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide to improve zone formation of ionic species. A pH of 4.1 buffered by the ammonium acetate system is required to obtain reproducible separation of various porphyrin acid isomers.

Although PCT is the most common porphyria diagnosed in some Caucasian populations (*1*), it is not common in Chinese (*9*). These two PCT patients are possibly the first cases reported in Hong Kong Chinese. Urinary porphyrins of these two patients were increased and consisted almost entirely of uroporphyrin and 7-carboxyl porphyrin. The most important features of the fecal porphyrin profile of PCT are the presence of 7-carboxyl porphyrin and isocoproporphyrin, both of which are believed to be diagnostic for PCT (*5*). Penta- and hexacarboxylic porphyrins were also observed in both the urine and fecal profiles. The dicarboxylic porphyrins found in normal feces were also present in the fecal profile.

This simple HPTLC procedure for the qualitative evaluation of porphyrins in urine and feces is a useful supplement to any routine clinical laboratory that currently relies solely on simple screening tests to detect porphyria. Within 30 min, the most diagnostically important porphyrins in urine or feces are well resolved for fluorescence viewing. The TLC technique described by Henderson (*4*), which involves alkali conditions and normal-phase separation, does not provide enough resolving power, particularly for fecal protoporphyrin and its derivatives pempto-, deutero-, and mesoporphyrins. The significance of detecting these dicarboxylic porphyrins has been discussed by Beukeveld et al. (*7*) and Ros et al. (*10*). Also, peptide-conjugated porphyrins were not identified in Henderson's method (*4*). Our method produces a clear separation of isocoproporphyrin from coproporphyrin and resolves fecal dicarboxylic porphyrins into discrete bands. In addition, the direct fractionation of free porphyrins with this method obviates the problems of methyl ester analysis, including the time-consuming prechromatographic derivatization and the risk of incomplete porphyrin methyl ester formation. This would be a problem for quantitative measurements of porphyrin methyl esters from TLC plates by elution or fluorescence scanning. Such measurements may not accurately reflect the composition of the starting material because of differential losses during esterification, protoporphyrin and other porphyrins with unsaturated β-substituents being particularly unstable during these procedures (*11*).

By using the optimized procedure described here, one an readily identify abnormal band patterns in urinary nd fecal samples. These patterns are characteristic for ach of the porphyrias and permit tentative diagnoses to e made.

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