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# DETERMINATION OF PORPHYRINS IN URINE BY DIRECT INJECTION ON A LIQUID CHROMATOGRAPHIC COLUMN COATED WITH TRIBUTYLPHOSPHATE

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SUMMARY

To separate and quantify urinary porphyrins, acidified and centrifuged urine was injected on a reversed-phase high-performance liquid chromatographic column, LiChrosorb RP-18, coated with tributylphosphate. A linear pH gradient from pH 4.40 to pH 6.50 was applied in the eluent using phosphate buffers containing methanol (9:1). The method permits detection of uro- and coproporphyrin down to concentrations normally present in urine and also selective determination of the hepta-, hexa- and pentacarboxylic porphyrins present in urine from patients suffering from porphyria cutanea tarda.

INTRODUCTION

Porphyrias are diseases caused by defects of enzymes in the biosynthesis of haem. Different porphyrins are accumulated depending on the break in the metabolic pathway, and for correct diagnosis it is important to have simple and selective analytical methods to measure the porphyrin content in urine, faeces and erythrocytes. The use of liquid chromatography for the separation and quantitation of porphyrins in biological specimens has been reviewed [1]. Most high-performance liquid chromatographic (HPLC) methods presented used some extraction, concentration and/or esterification step before injection of the sample on the chromatographic column and the porphyrins were detected spectrophotometrically.

The analysis of urinary porphyrin carboxylic acids by direct injection of urine or acidified urine on the liquid chromatographic column [2—6] can easily be done in reversed-phase liquid chromatography in combination with fluoro-

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metric detection. The only specimen pretreatment that is necessary is filtration or centrifugation of the urine to get rid of solid particles.

The separation of porphyrins on columns with hydrophobized silica as the solid phase can be performed in different ways. Englert et al. [2] used a gradient of acetonitrile in phosphate buffer pH 7.5 in the eluent, while Ford et al. [3] used a gradient of methanol in phosphate buffer pH 3.5. Rather high concentrations of acetonitrile and methanol were necessary to elute the porphyrins from the column. The urinary porphyrins have also been separated by reversed-phase ion-pair chromatography with tetrabutylammonium as counter-ion added to the eluent [4—6]. The separation was performed at pH 7.5 in the isocratic mode [4] or by applying a multilinear gradient of methanol in the eluent [5, 6]. A separation on an ion-exchange HPLC column has also been demonstrated using a methanol—acetic acid gradient in the eluent [7].

In the present work the hydrophobic support was coated with tributylphosphate (TBP), to separate the urinary free porphyrin carboxylic acids. TBP is a liquid with hydrophobic properties due to the three butyl groups. It also has strong hydrogen-binding properties due to the phosphate group, and TBP-coated columns thus give high retention of hydrogen-donating compounds like carboxylic acids. TBP has previously been used in extraction chromatography for the separation of metal ions [8], but also in HPLC in the separation of organic substances such as carboxylic acids [9—11], amino acids [11] and catecholamines [12]. The analysis of 5-hydroxyindoleacetic acid by direct injection of urine on a column coated with TBP in combination with fluorometric detection [10] has been running for some time as a routine method in our laboratory. With TBP present, high selectivity for carboxylic acids is obtained with respect to other endogenous compounds in urine. It was our intention to use the same chromatographic system to analyse the urinary porphyrin carboxylic acids. Our method involves direct injection of acidified urine on the column, pH-gradient elution of the porphyrins and fluorometric detection. With this method it is possible to separate and quantify octacarboxylic (uro)porphyrin, hepta-, hexa- and pentacarboxylic porphyrin and tetracarboxylic (copro)porphyrin.

EXPERIMENTAL

*Apparatus*

The liquid chromatographic system consisted of two pumps, Constametric III and Constametric II, combined with a solvent programmer, Gradient Master Model 1601 (Milton Roy Company, Riviera Beach, FL, U.S.A.), a Rheodyne injector with 20-$\mu$l loop and a Schoeffel FS 970 fluorescence detector. The excitation wavelength was set at 405 nm and an emission cut-off filter below 550 nm was used. The isocratic experiments were performed with a Milton Roy Minipump with pulse dampener (LDC, Model 711-26). A Spectro Monitor III (LDC) UV detector with variable wavelength was also used in some of the experiments.

The separation column was of precision bore 316 stainless steel, 100 mm × 3.9 mm I.D., packed with LiChrosorb RP-18, 5 $\mu$m (Merck, Darmstadt,

G.F.R.). A water-bath (Heto Lab Equipment A/S, Denmark) was used for thermostating.

*Chemicals*

Tributylphosphate (TBP) and methanol as well as all the other chemicals were of analytical grade and obtained from Merck.

The buffers were prepared from sodium dihydrogen phosphate and disodium hydrogen phosphate with an ionic strength of 0.1 using distilled water.

Coproporphyrin dihydrochloride type III isomer, uroporphyrin octamethyl ester type III isomer and a synthetic mixture of tetra-, penta-, hexa-, hepta- and octacarboxylic porphyrins type I isomers including mesoporphyrin IX as a dicarboxylic porphyrin were obtained from Porphyrin Products (Logan, UT, U.S.A.). The uroporphyrin III octamethyl ester was hydrolysed overnight in 6 mol/l hydrochloric acid (dark, 4°C) to obtain the uroporphyrin III carboxylic acid.

*Chromatographic conditions*

The separation column was packed at 35 MPa with the support suspended in methanol [13].

The eluents were prepared by first mixing buffer and methanol (9:1) and the solution was then equilibrated with TBP. During the development of the method the eluents were saturated to 90% with TBP and the column and the reservoirs were thermostated at 25.0°C by circulating water [11]. In the final method, however, we found it possible to use eluents saturated to 100% with TBP and ambient temperature. The pH of the eluent was always measured before the addition of methanol. In the gradient studies buffer to eluent A was prepared by dissolving 13.8 g of $NaH_2PO_4 \cdot H_2O$ in 1000 ml of distilled water and buffer to eluent B by dissolving 5.2 g of $NaH_2PO_4 \cdot H_2O$ and 3.6 g of $Na_2HPO_4 \cdot 2H_2O$ in 1000 ml of distilled water.

The columns were coated with TBP by injection of 350 $\mu$l of TBP on the column, followed by recycling of the eluent overnight [11].

The hold-up volume of the column, $V_m$, was determined by injection of coproporphyrin III in an eluent of pH 8, where coproporphyrin III was unretained. The retention volume of the samples, $V_R$, was measured and the capacity ratio, $k'$, was calculated by $(V_R - V_m)/V_m$.

In the isocratic experiments the porphyrins were dissolved in 0.1 mol/l hydrochloric acid and diluted with the eluent.

*Analytical method*

Standards were prepared by dissolving the synthetic mixture of octa-, hepta-, hexa-, penta-, tetra-, tri- and dicarboxylic porphyrins, containing 10 $\mu$mol of each porphyrin, in 4 ml of 1.0 mol/l hydrochloric acid. The standard was further diluted to 0.045–0.28 $\mu$mol/l with 0.1 mol/l hydrochloric acid and injected on the chromatographic column. The peak heights of the standards were measured and standard curves were prepared by plotting the peak height of each porphyrin against the concentration. The standards were protected from light and stored in darkness at 4°C.

Collection of 24-h urine was made in dark plastic bottles with the addition of sodium carbonate (2 g/l of urine). Prior to analysis 5.0 ml of the 24-h urine

were adjusted to about pH 2 by the addition of six droplets of 6 mol/l hydro-chloric acid. The acidified urine was centrifuged before injection. When higher concentrations of porphyrins were found, a fresh aliquot of the 24-h urine was diluted 1:25 with 0.1 mol/l hydrochloric acid and analysed again.

The porphyrin carboxylic acids were eluted from the column by applying a pH gradient in the eluent from pH 4.40 (eluent A) to pH 6.50 (eluent B). The gradient was started immediately after injection of the sample. The Gradient Master was adjusted to give a linear gradient in 10 min, 15-min delay at eluent B and a return to eluent A in 1 min. After 15 min at eluent A a new injection was made. The flow-rate was 0.8 ml/min.

The peaks obtained in urine were identified by comparing the observed retention times with those of the standards, and the concentrations were cal-culated from the peak heights given by the sample and the standard. For copro-porphyrin the concentrations were calculated from the peak area measured by multiplying the peak height by the peak width at half the peak height.

RESULTS AND DISCUSSION

*Retention model*

The chromatographic support, LiChrosorb RP-18, was modified in the study by adsorption of tributylphosphate (TBP). TBP is a strong hydrogen acceptor and is able to form hydrogen bonds with acidic groups.

The capacity ratio of a sample distributed to the adsorbed TBP layer is given by

$$k' = (V_s/V_m) \times D \tag{1}$$

where $C_{HA,org}$ and $C_{HA,aq}$ are the total concentrations of the acid in the sta-tionary and the mobile phase, respectively. The distribution constant of the acid, $K_D$ is given by

$$D = \frac{C_{HA,org}}{C_{HA,aq}} = \frac{[HA]_{org}}{[HA]_{aq} + [A^+]_{aq}} \tag{2}$$

where $C_{HA,org}$ and $C_{HA,aq}$ are the total concentrations of the acid in the sta-tionary and the mobile phase, respectively. The distribution constant of the acid, $K_D$ is given by

$$K_D = \frac{[HA]_{org}}{[HA]_{aq}} \tag{3}$$

When the acid is retained on the column in uncharged form and proteolysis oc-curs in the mobile phase, the equation for the capacity ratio can be trans-formed to

$$k' = (V_s/V_m) \times \frac{K_D}{1 + (K_a^x/a_{H^+})} \tag{4}$$

$$\log k' = \log [(V_s/V_m) \times K_D] - \log (1 + K_a^x/10^{-pH}) \tag{5}$$

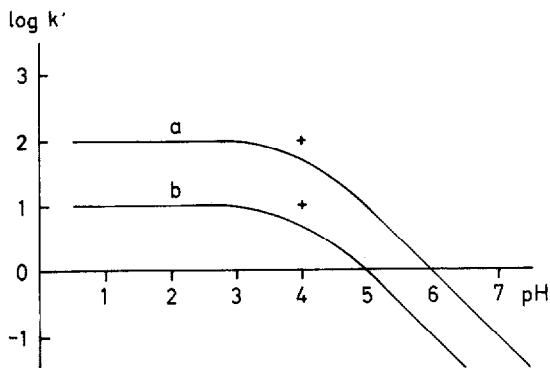where $K_a^x$ is the apparent dissociation constant including the medium factor

Fig. 1. Relation between pH of the eluent and the retention of acids. (a) Acid with $K_D$ = 170 and $pK_a^x$ = 4. (b) Acid with $K_D$ = 17 and $pK_a^x$ = 4. + = intersection point (pH = $pK_a^x$) for the two asymptotes according to eqn. 5. ($V_s/V_m$) is set at 0.6.

(cf. ref. 14) and $a_{H^+}$ is the hydrogen activity in the mobile phase.

The relation between the capacity ratios of two acids containing one carboxylic group each and pH of the eluent according to eqn. 5 is illustrated in Fig. 1. The graphs in Fig. 1 have two asymptotes, one with a slope of 0 and one with a slope of –1. They intersect at a pH equal to $pK_a^x$ for the acids [11,15].
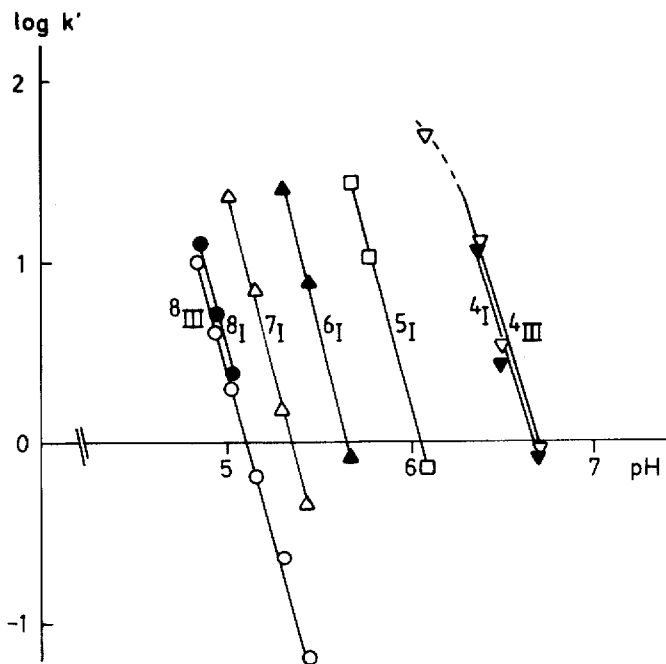


Fig. 2. Change in retention of porphyrin carboxylic acids with pH (isocratic mode). $8_{III}$ (○) = octacarboxylic (uro)porphyrin III, $8_I$ (●) = octacarboxylic (uro)porphyrin I, $7_I$ (△) = heptacarboxylic porphyrin I, $6_I$ (▲) = hexacarboxylic porphyrin I, $5_I$ (□) = pentacarboxylic porphyrin I, $4_I$ (▼) = tetracarboxylic (copro)porphyrin I, $4_{III}$ (▽) = tetracarboxylic (copro)porphyrin III. Solid phase: LiChrosorb RP-18, 5 μm, coated with TBP. Eluent: buffer—methanol (9:1) with 90% relative saturation of TBP.

*Retention of porphyrin carboxylic acids*

The porphyrins studied contain between two and eight carboxylic acid groups. The $pK_a$ values of the acidic groups are not known but the degree of dissociation in the eluent could be related to pH. The capacity ratios of the porphyrins are decreased by increasing pH in the eluent as shown in Fig. 2. The slopes of the lines for the octa-, hepta-, hexa- and pentacarboxylic porphyrins were 3.7—4.1, which indicates that the net charge of the porphyrins in the pH range studied for each porphyrin should be about 4. The slopes of the lines for tetracarboxylic porphyrins were close to 3.

Mesoporphyrin IX could not be eluted from the column even at pH 8. A higher pH should be needed but further increase in pH is limited by the stability of the silica support.

The separation factor between the type I and type III isomers ($\alpha = k'_I / k'_{III}$) was 1.2—1.3 for uroporphyrin and 0.77—0.91 for coproporphyrin. Note the difference in retention order. No complete separation was obtained between the isomers due to rather broad peaks under isocratic conditions.

The porphyrins were eluted from the column by a linear increase of pH in the eluent, from pH 4.40 to pH 6.50. The samples were injected at pH 4.40, where all porphyrins are strongly retained on the column, and the gradient was started. A linear gradient in 10 min at a flow-rate of 0.8 ml/min was found to be suitable to elute the porphyrins one after the other from the column. After 15 min at pH 6.50 a return to pH 4.40 was made in 1 min.
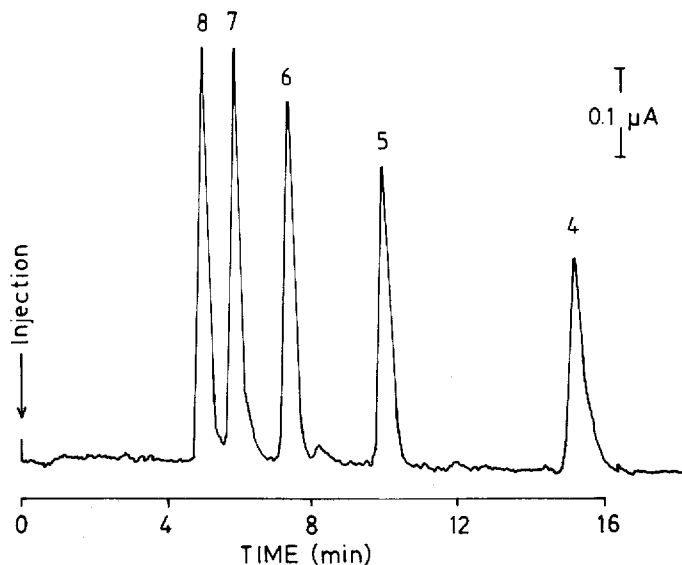


Fig. 3. Separation of a standard mixture of porphyrin carboxylic acids. 8 = octacarboxylic (uro)porphyrin, 7 = heptacarboxylic porphyrin, 6 = hexacarboxylic porphyrin, 5 = penta-carboxylic porphyrin, 4 = tetracarboxylic (copro)porphyrin. Solid phase: LiChrosorb RP-18, 5 $\mu$m coated with TBP. Eluents: A = buffer pH 4.40—methanol (9:1) with 90% saturation of TBP; B = buffer pH 6.50—methanol (9:1) with 90% saturation of TBP; linear gradient from 0 to 100% B in 10 min with 15 min delay. Flow-rate, 0.8 ml/min; pressure, 16.3 MPa; fluorescence detection, excitation at 405 nm, emission cut-off at 550 nm; time constant 6 sec. Sample: 20 $\mu$l porphyrin standard 0.18 $\mu$mol/l (3.6 pmol of each porphyrin).

The concentrations of the buffer ions $H_2PO_4^-$ and $HPO_4^{2-}$ are changed in the eluent during the gradient. The phosphate ions have no fluorescence and only a minor drift in the baseline was observed during the gradient elution. The ions have, however, different weak UV-absorption properties at 405 nm and it was possible to follow the reequilibration of the column to pH 4.40 by connecting
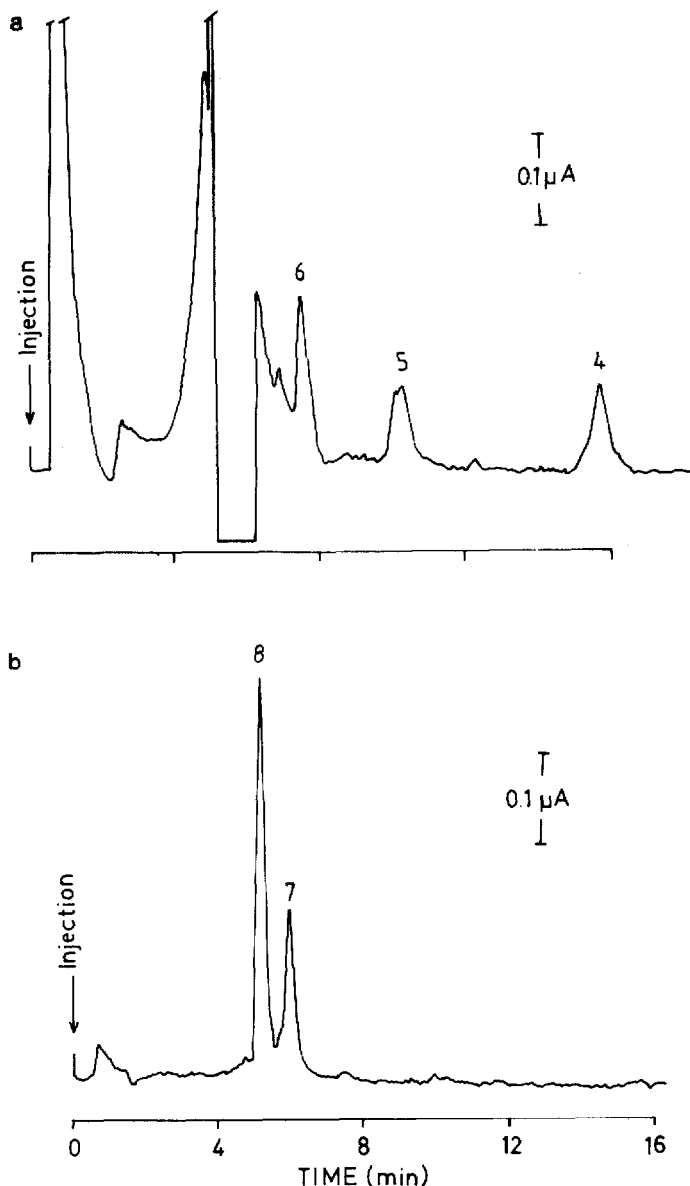


Fig. 4. Chromatogram obtained from a patient suffering from porphyria cutanea tarda (E.A./810924). (a) Sample: 20 $\mu$l of undiluted urine injected. When the uroporphyrin peak appeared there was a break in power due to overloading of the photomultiplier in the detector. Urine concentration of coproporphyrin = 0.09 $\mu$mol/l. (b) Sample: 20 $\mu$l of urine diluted 1:25 with 0.1 mol/l HCl injected. Urine concentration of uroporphyrin = 4.3 $\mu$mol/l and of heptacarboxylporphyrin = 1.8 $\mu$mol/l. Conditions and peaks as in Fig. 3.

TABLE I

URINARY PORPHYRIN EXCRETION IN TWO PATIENTS (H.H. AND E.A.) SUFFERING FROM PORPHYRIA CUTANEA TARDA

Excretion was estimated on three occasions each and compared to the mean excretion in four healthy individuals. Excretion is expressed in $\mu$mol per 24 h.

| Initials/date | Uroporphyrin | Heptacarboxylic porphyrin | Hexacarboxylic porphyrin | Pentacarboxylic porphyrin | Coproporphyrin |
|---|---|---|---|---|---|
| H.H./810811 | 4.9 | 1.8 | 0.10 | 0.20 | 0.25 |
| H.H./811009 | 0.73 | 0.92 | —* | — | 0.19 |
| H.H./820315 | 0.59 | 0.49 | 0.03 | — | 0.04 |
| E.A./810924 | 4.5 | 1.9 | 0.07 | 0.05 | 0.10 |
| E.A./811014 | 5.6 | 2.6 | ★★ | ★★ | ★★ |
| E.A./820311 | 0.78 | 0.41 | — | 0.04 | 0.06 |
| Normals (mean ± S.D., $n$ = 4) | 0.006 ± 0.004 | — | — | — | 0.05 ± 0.02 |

*— = below detection limit.
★★ = not quantified.

a UV detector in series with the fluorescence detector. The column was re-equilibrated to pH 4.40 in 12—13 min and then a new injection could be made. A chromatogram of the porphyrin standard containing 0.18 $\mu$mol/l of each porphyrin is shown in Fig. 3.

*Analysis of porphyrins*

Urine samples have been analysed from patients. Results from two patients suffering from porphyria cutanea tarda are shown in Table I. The chromatograms obtained with undiluted urine and with urine diluted 25 times with 0.1 mol/l HCl are shown in Fig. 4a and b. Coproporphyrin type III isomer, present in human urine to 95% of the total coproporphyrin [6], had a slightly higher retention than coproporphyrin type I isomer used as standard. This was compensated for by calculating the concentration from the peak area instead of the peak height. A chromatogram obtained from a healthy individual is shown in Fig. 5.
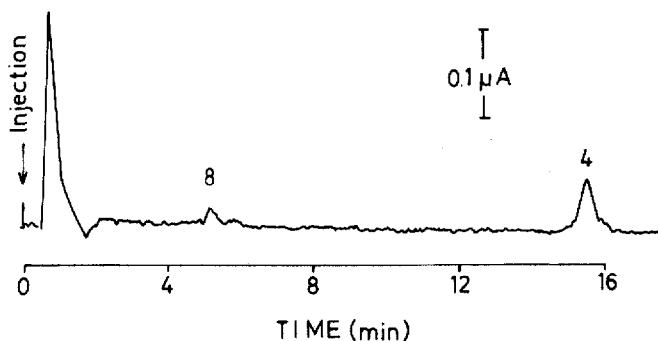


Fig. 5. Chromatogram obtained from a healthy individual. Sample: 20 $\mu$l of urine injected. Urine concentration of uroporphyrin 0.006 $\mu$mol/l and of coproporphyrin 0.04 $\mu$mol/l. Conditions and peaks as in Fig. 3.

TABLE II

IMPRECISION OF THE METHOD AT TWO CONCENTRATIONS

Estimations were made from repeated injections of porphyrin standard ($n=8$) and expressed as coefficient of variation (C.V. %).

| Amount | Uroporphyrin | Heptacarboxylic porphyrin | Hexacarboxylic porphyrin | Pentacarboxylic porphyrin | Coproporphyrin |
|---|---|---|---|---|---|
| 3.6 pmol (0.18 $\mu$mol/l) | 5.9 | 6.8 | 6.3 | 6.9 | 5.4 |
| 0.8 pmol (0.04 $\mu$mol/l) | 5.5 | 4.7 | 5.2 | 6.1 | 9.4 |

The imprecision of the method calculated from eight determinations of 3.6 pmol and 0.8 pmol porphyrin standard is given in Table II.

The detection limit defined as the amount that gave a signal two times the noise, was 0.08 pmol (0.004 $\mu$mol/l) of uroporphyrin and 0.28 pmol (0.014 $\mu$mol/l) of coproporphyrin.

The method presented has now been used as a routine method for more than one year in our laboratory without any deterioration of the column. The

simple specimen handling, the high sensitivity and specificity make it a valuable tool to obtain correct diagnosis and to follow the course of porphyria cutanea tarda.

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