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DEVELOPMENT AND UTILIZATION OF A PROCEDURE FOR MEASURING URINARY PORPHYRINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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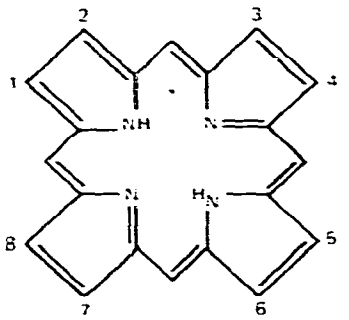
SUMMARY

We describe a procedure for determining the five principal urinary porphyrins — uroporphyrin, heptacarboxylporphyrin, hexacarboxylporphyrin, pentacarboxylporphyrin, and coproporphyrin — by using high-performance liquid chromatography (HPLC). The method involves a 12- to 15-min isocratic separation on a Bondapak® Phenyl column. Studies have indicated that urine samples must be preserved to maintain porphyrin stability for extended periods. We have found that the pH of preserved samples must be adjusted into the acidic range before the samples can be accurately analyzed by this HPLC procedure. Our studies demonstrate that good reproducibility and recovery are achieved with this method. Urinary porphyrin values for normal and porphyric individuals are reported.

INTRODUCTION

The recognition of characteristic urinary porphyrin patterns is important in the diagnosis of porphyria, a disease characterized by excessive porphyrins in body fluids and excreta. Five porphyrins may be found in urine (Fig. 1). Type I porphyrins are unused byproducts of the heme biosynthetic pathway and are usually the principal porphyrin constituents in normal urine. Type III porphyrins are precursors to heme and are not observed in normal urine except in trace amounts. Most normal urine samples contain only coproporphyrin and perhaps a small amount of uroporphyrin. The heptacarboxyl, hexacarboxyl, and pentacarboxyl porphyrins are found in normal urine only in trace amounts, and their detection depends upon the sensitivity of the analytical procedure. The individual measurement of these five porphyrins provides a relative distribution pattern which, in the case of chronic hepatic porphyria, is so characteristic that a diagnosis can be made on the basis of a single examination of a small urine sample [1].

Porphyria is either hereditary (of genetic origin) or acquired from exposure



	Type I	Type III
uroporphyrin (octacarboxylic)	1,3,5,7=A 2,4,6,8=P	1,3,5,8=A 2,4,6,7=P
heptacarboxylic acid	1=M 3,5,7=A 2,4,6,8=P	1=M 3,5,8=A* 2,4,6,7=P
hexacarboxylic acid	1,3=M 5,7=A* 2,4,6,8=P	1,3=M 5,8=A* 2,4,6,7=P
pentacarboxylic acid	1,3,5=M 7=A 2,4,6,8=P	1,5,8=M 3=A* 2,4,6,7=P
coproporphyrin (tetracarboxylic)	1,3,5,7=M 2,4,6,8=P	1,3,5,8=M 2,4,6,7=P
$A=CH_2CO_2H$	$P=CH_2CH_2CO_2H$	$M=CH_3$

* = more than this isomer possible

Fig. 1. Porphyrin structures.

to chemicals. Our particular interest is in chemically induced porphyria. Among the chemicals which cause or precipitate porphyria are halogenated hydrocarbons [2–6], steroid hormones [7], ethanol [8], lead [9], barbiturates, and other drugs [10]. Many of our case studies involve low-level or chronic poisoning or exposure to environmental chemicals, and we are interested in analyses which identify chemically induced disease or injury at early stages before clinical symptoms occur. Doss [11] reported that some definitive early (subclinical) stages of chronic hepatic prophyria can be readily recognized by analyzing the urinary porphyrin pattern.

Classical clinical laboratory methodology for porphyrin analysis involves the separation and spectrometric or fluorescent measurement of porphyrins as two fractions — the uroporphyrins and coproporphyrins — which, for non-porphyric patients, contain principally uroporphyrin and coproporphyrin, respectively. However, for porphyric patients, each fraction actually contains several different porphyrins so that, for example, the uroporphyrin fraction consists of uroporphyrin, heptacarboxylporphyrin, and hexacarboxylporphyrin, and similarly the coproporphyrin fraction contains coproporphyrin, pentacarboxylporphyrin, and hexacarboxylporphyrin. The classical methodology, therefore, is unsuitable for diagnosing porphyria on the basis of porphyrin pattern. Procedures involving thin-layer chromatography have been used for porphyrin separations but, although specific, these analyses are time-consuming and tedious. Recent reports on the use of high-performance liquid chromatography (HPLC) for the analysis of free urinary porphyrin acids have been promising [12–17]. Many of these procedures involve gradient separations [13–15, 17], which are more time-consuming and require more elaborate equipment than isocratic separations [12, 16]. Bonnett [12] reported an isocratic separation for the porphyrin acids, but difficulties with this separation have been reported [14–17]. In 1980, we published a preliminary report of a rapid isocratic HPLC procedure for determining urinary porphyrins [16]. In this present paper, we provide supporting data and experimental details in the development and use of this procedure.

MATERIALS AND METHODS

Porphyrins

Uroporphyrin I, heptacarboxylporphyrin I, hexacarboxylporphyrin I, pentacarboxylporphyrin I, and coproporphyrin I were purchased from Porphyrin Products, Inc., Logan, UT, U.S.A., as separate standards or in mixed standards, known as porphyrin acid-marker kits. These marker kits, which contained 10 nmol of each of the five Type I porphyrins, were used to prepare standard solutions. A stock solution was prepared from a marker kit vial by dissolving the vial contents in 1 ml of 60% methanol—water solution containing 10 mmol/l tetrasodium EDTA (Sigma, St. Louis, MO, U.S.A.). This was either allowed to stand overnight or was put into an ultrasonic bath to effect complete solution of the porphyrin acids. Standards were prepared by diluting various volumes (50—400 μl) of tetrasodium EDTA (0.4—1.5 ml). All porphyrin standards were made by using this solvent system. Concentrations ranged from 4 μg/ml to 0.2 μg/ml. Aliquots of 5 μl each were injected into the HPLC system to produce standard curves. The heptacarboxyl, hexacarboxyl, and pentacarboxylporphyrins were available individually only as the methyl ester and had to be hydrolyzed to free acid forms. The esters (1 mg) were hydrolyzed with 0.5 ml of Ultrex® hydrochloric acid (J.T. Baker Chemical Co., Phillipsburg, NJ, U.S.A.) and 0.5 ml of water for at least 2 h in a water bath at 60—65°C. The solution was evaporated to dryness at 40—45°C, and was appropriately diluted in 60% methanol—water containing 10 mmol/l tetrasodium EDTA.

Reagents

UV-grade methanol was purchased from Burdick and Jackson Labs., Muskegon, MI, U.S.A., or from Fisher Scientific, Fairlawn, NJ, U.S.A. Water was doubly deionized with a Milli-Q-Water Purification System from Millipore, Bedford, MA, U.S.A. The HPLC mobile phase consisted of 15.6 mmol/l sodium 1-pentanesulfonate (Eastman Kodak, Rochester, NY, U.S.A.) and 0.10 mmol/l tetrasodium EDTA in 60—64% methanol in water solution adjusted to pH 2.1 with sulfuric acid (J.T. Baker). The exact concentration of methanol depends upon the retention characteristics of the particular column being used. The 60—64% methanol composition was generally the range used for several columns. The preservative for collected urine specimens was prepared by mixing 14.4 g of sodium carbonate with 19.3 g of tetrasodium EDTA. A 0.3-g portion of this mixture was added to each 250-ml collection bottle.

Equipment

The chromatographic system consisted of a Waters U6K injector (Waters Assoc., Milford, MA, U.S.A.), a Waters M6000A pump, and a Schoeffel FS970 L.C. Fluorometer (Kratos/Schoeffel Instruments, Westwood, NJ, U.S.A.). The detector was set at an excitation wavelength of 403 nm with a 7-59 transmission prefilter and a 600-nm emission cutoff filter. A Waters 10-μm Bondapak® Phenyl column (300 × 3.9 mm) was used with a MPLC® RP-18 guard column (Brownlee Labs., Santa Clara, CA, U.S.A.).

PROCEDURE

Allow collected urine to stand at room temperature for 24 h before analysis to allow oxidation of porphyrinogens to porphyrins. Five to 10 min before analysis, adjust the pH of the urine sample to between 2 and 6 with 6 *N* sulfuric acid. Set the solvent flow-rate at 1.5 ml/min. Inject a 50-μl aliquot of the pH-adjusted urine. Smaller aliquots or dilution of urine may be necessary if peak heights are used for measurement. Compare peak heights or peak areas with a standard curve or linear regression line generated from external standard solutions. Urinary porphyrin levels are reported in micrograms per liter (μg/l) unless the urine analyzed was a 24-h collection, in which case the level may be reported in milligrams per 24-h period.

RESULTS AND DISCUSSION

Our efforts were initially directed toward the use of a previously reported isocratic procedure [12]; however, problems related to reproducibility and sample carryover required a new approach. We obtained good separation for standards with all five urinary porphyrins by using 73% methanol—water with 5.2 mmol/l pentasulfonic acid acidified with phosphoric acid to pH 2.5, with a C_{18} reversed-phase column. When we analyzed the urine samples, however, we found that an unidentified compound had coeluted with the uroporphyrin. This interference was largest in specimens from persons taking vitamin supplements, and we soon recognized the interference as vitamin B_2 or riboflavin — a common, almost ubiquitous component of urine. With a C_{18} column we could resolve the riboflavin and uroporphyrin peaks only by gradient elution, an unsatisfactory method for our purposes since the time of analysis and regeneration to original conditions was at least 30 min. Our goal was to develop a rapid isocratic procedure (a 15-min separation) so that one analyst could analyze at least 15—20 samples with standards and controls in an 8-h work period using simple HPLC equipment (injector, pump, detector, and recorder/integrator).

We found that an isocratic separation could be achieved by using a Bondapak Phenyl® column which resolved riboflavin and uroporphyrin and still allowed elution of the coproporphyrin within 12—15 min (Fig. 2). Optimum conditions for separation on this column were achieved by adjusting the pH and the counter-ion concentration in the mobile phase. Decreasing the pH produced sharper peaks, particularly the coproporphyrin peak. Pentanesulfonic acid and octanesulfonic acid were evaluated as ion pairing agents. The octanesulfonic acid extended the retention time by 20—30% more than the pentanesulfonic acid, but since it did not improve the separation, pentanesulfonic acid was selected as the ion pair reagent. Both sulfuric acid and phosphoric acid were tested as sources of acidification for the mobile phase. Both gave almost identical separations, but the mobile phase with sulfuric acid produced a slightly faster eluting coproporphyrin peak; therefore, we adopted sulfuric acid for acidification.

Tetrasodium EDTA was added to prevent the formation of trace metal complexes and the precipitation of calcium salts. In early experiments, we found

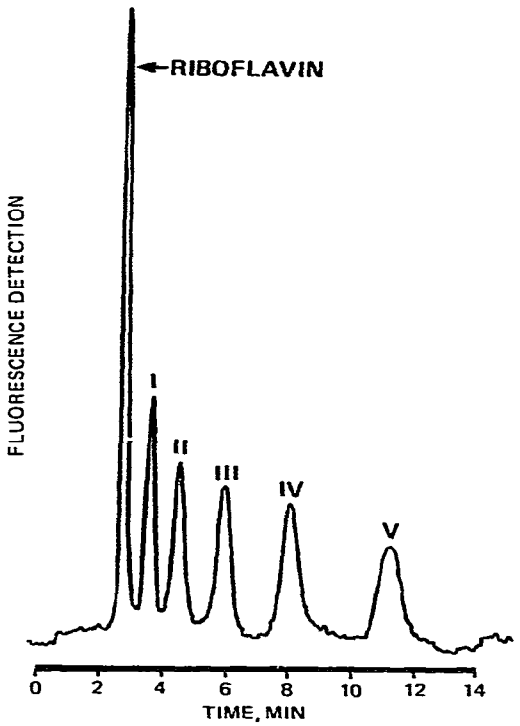


Fig. 2. Chromatogram of porphyrin standard and riboflavin. I = Uroporphyrin (26 ng), II = heptacarboxylporphyrin (25 ng), III = hexacarboxylporphyrin (23 ng), IV = pentacarboxylporphyrin (22 ng), V = coproporphyrin (20 ng).

that porphyrin standards prepared by hydrolysis with ordinary hydrochloric acid apparently formed trace metal complexes with the porphyrins, and these complexes had different retention times and reduced fluorescence. Esters hydrolyzed with Ultrex® hydrochloric acid and diluted with methanol—water containing EDTA were found to be free of these trace metal porphyrin complexes. Whether EDTA is a necessity in the mobile phase is not clear; however, we routinely use EDTA in the mobile phase and have always obtained good results.

We found that the addition of Na_2CO_3 and EDTA as a preservative in the urine samples was necessary to maintain stable samples for extended periods. Unpreserved refrigerated samples showed discernible losses of uroporphyrin and heptacarboxylporphyrin after one week and demonstrated significant losses in the second week (Fig. 3). Although most clinical laboratories would rarely keep samples this long, we must store hundreds of samples for extended periods before analysis. Our usual procedure is to add preservative and then freeze the specimens. Freezing specimens without preservative may also maintain samples for extended periods, but this has not been investigated.

The preservative often changes the pH of the urine sample into the basic range (pH > 7), and we found that urine samples must be adjusted into an acidic range (between pH 2 and 6) just before analysis. In Fig. 4A is shown the chromatogram of a urine specimen with preservative in which the pH

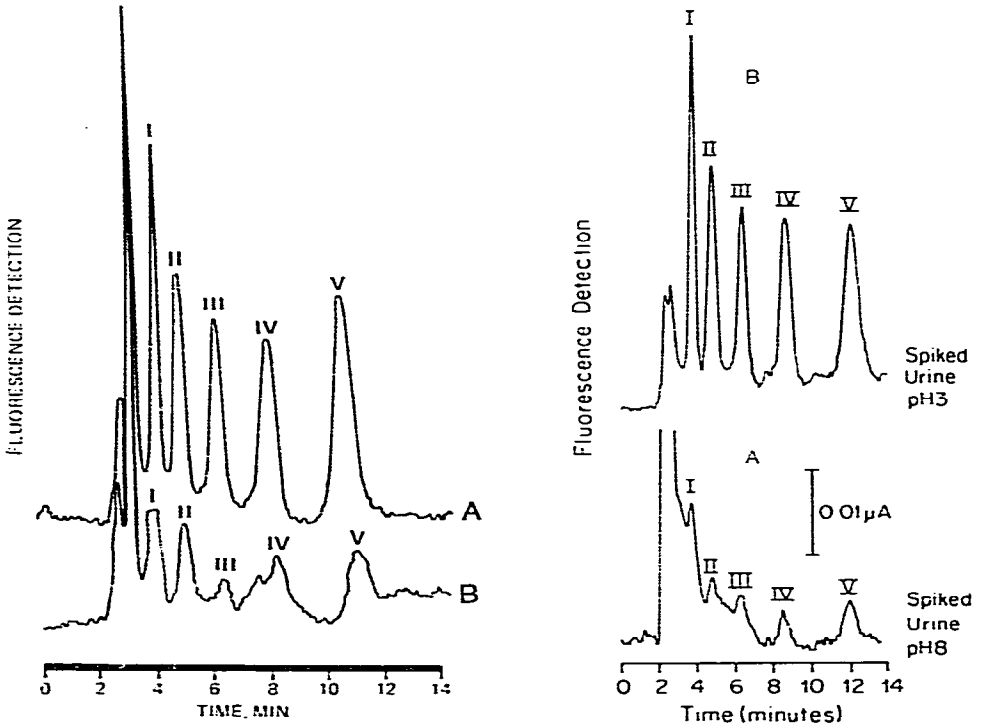


Fig. 3. Chromatograms of spiked urine samples after 2 weeks of refrigerated storage (A) with preservative, (B) without preservative. Peaks as in Fig. 2.

Fig. 4. Chromatogram of spiked urine sample with preservative (A) at pH 8 without acidification, (B) at pH 3 after acidification.

(pH 8) was not adjusted into the acidic range; the chromatogram in Fig. 4B is the same specimen with the pH adjusted to 3.

We attempted unsuccessfully to find a compound which could be used as an internal standard. Our requirements were rather strict, i.e. a compound whose retention time fell just beyond the coproporphyrin peak, was fluorescent under our conditions, and showed similar behavior at differing pH values. All 30 compounds examined either coeluted with the five porphyrins or eluted too late to be considered.

The recovery and reproducibility of the procedure was determined by preparing urine pools spiked at various levels with uroporphyrin, heptacarboxyl, and coproporphyrin. Three aliquots were taken from each of these pools on each of six analytical days covering an 18-day period. Each aliquot was analyzed for the three analytes, and the results are shown in Table I.

The detection limits for each of the porphyrins were estimated to be approximately 5—10 μg/l, or 0.2—0.5 ng per injection. Standards over the range of 5—104 ng/injection produced linear responses with a pooled correlation coefficient for all five porphyrins of 0.9954 ± 0.0065 ($\bar{X}$±S.D.) for six successive days of analysis. When refrigerated, standards were found to maintain their stability for at least four weeks.

TABLE I

REPRODUCIBILITY AND RECOVERY IN PORPHYRIN ANALYSIS

Three samples were analyzed for each analyte on each of six days over an 18-day period.

Porphyrin	Overall mean concentration (μg/l)	Relative standard deviation (C.V., %)		Mean recovery (%)
		Within-run	Total*	
Uro-	75	5.7	9.0	104
	150	4.4	11.6	107
	560	6.9	12.7	90
Hepta-	65	5.0	12.1	90
	230	3.8	12.5	99
	500	6.9	12.3	89
Copro-	36	17.6	24.3	110
	180	5.0	6.2	106
	290	6.3	11.7	91

*Combines the within-run and the between-run components of variance.

To establish normal values for this HPLC procedure, we obtained random, fist-morning, and 24-h urine samples from volunteers for analysis. Although we usually collect random urine specimens in our field studies, we have also reported limited data on first-morning and 24-h urine samples (Table II). All samples contained coproporphyrin, and most also had detectable levels of uroporphyrin. We have also analyzed several cases of clinically diagnosed porphyria, and values for these are summarized in Table III. Typical chromatograms of a normal urine sample and some porphyric samples are shown in Fig. 5. Our analytical results confirm earlier reports of characteristic patterns

TABLE II

URINARY PORPHYRIN VALUES FOR NORMAL VOLUNTEERS

	Porphyrin concentration (μg/l)			
	Uro-	Hepta-	Copro-	Total
Random samples (n*=59)				
Mean	6	1	52	58
S.D.	6	3	27	33
Range	0—24	0—9	12—130	12—150
Percentage detectable values	71	10	100	100
1st morning samples (n=36)				
Mean	11	0.4	56	68
S.D.	10	2	35	39
Range	0—37	0—8	5—160	10—170
Percentage detectable values	86	8	100	100
24-h samples (n=10)				
Mean	10 (12)**	0	60 (81)**	70 (92)**
S.D.	11 (12)	0	31 (23)	39 (29)
Range	0—31 (2—44)	0	26—120 (32—120)	33—150 (40—130)
Percentage detectable values	80	0	100	100

*n = number of individuals.

**In parentheses, mg of porphyrin per 24-h period.

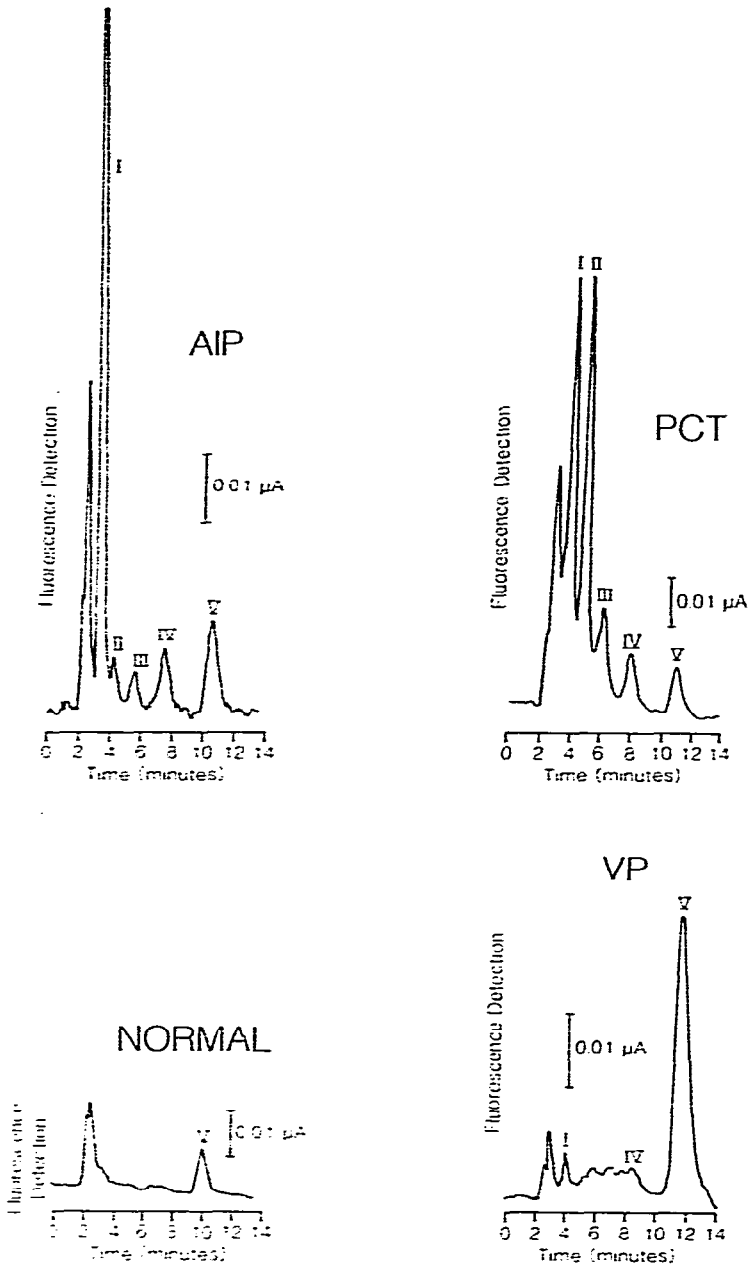


Fig. 5. Chromatograms of urine samples showing patterns for normal individual, acute intermittent porphyria (AIP), porphyria cutanea tarda (PCT), and varigate porphyria (VP).

for various porphyrias. Porphyria cutanea tarda (PCT) is characterized by high levels of uroporphyrin and heptacarboxylporphyrin, the latter being particularly specific for this porphyria [1, 3, 13—15, 17—23]. In PCT, the uroporphyrin/coproporphyrin and heptacarboxylporphyrin/coproporphyrin ratios are greater than one, and — according to some authors — these ratios

TABLE III

URINARY PORPHYRINS IN CLINICALLY CONFIRMED PORPHYRIA

Patient	*n*	Concentrations (μg/l)					Porphyria diagnosis*
		Uro-	Hepta-	Hexa-	Penta-	Copro-	
A	1	1600	400	61	120	95	PCT
B	1	1400	680	29	120	130	PCT
C	5	530 ± 240	140 ± 120	24 ± 30	80 ± 41	53 ± 28	PCT
D	1	670	280	35	83	44	PCT
E	1	1300	1100	45	78	97	PCT
F	1	360	390	78	94	80	PCT
G	4	820 ± 260	540 ± 130	36 ± 28	93 ± 18	80 ± 12	PCT
H	2	1900 ± 410	1200 ± 130	1200 ± 220	670 ± 120	320 ± 36	PCT
I	1	260	290	20	33	86	PCT
J	1	2700	890	110	340	360	PCT
K	1	500	140	23	180	98	PCT
L	1	1000	26	30	54	56	AIP
M	1	1100	110	20	110	2200	VP
N	1	170	20	ND	130	2100	VP

*PCT = porphyria cutanea tarda; VP = varigate porphyria; AIP = acute intermittent porphyria.

can be used to recognize various subclinical states of hepatic porphyria [1, 2]. The single urine specimen that was from a patient with acute intermittent porphyria also conformed to previously reported patterns, showing highly elevated uroporphyrin, but not heptacarboxylporphyrin [9, 15, 18, 23—25]. Specimens from two patients with varigate porphyria (VP) both contained highly elevated coproporphyrin as the dominant component in the porphyrin pattern. Whether this latter pattern is characteristic for VP is less clear. These patterns appear similar to those of patients with cutaneous VP [21]. As better methodology is used for porphyrin analysis, clearer characteristic patterns may emerge.

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

REFERENCES

1 M. Doss, in M.C. Curtins and M. Roth (Editors), Clinical Biochemistry, Principles and Methods, Vol. II, Walter de Gruyter, Berlin, 1974, p. 1365.
2 J.J.T.W.A. Strik, F.M.H. Debets and G. Koss, in R.D. Kimbrough (Editor), Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins and Related Products, Elsevier/North-Holland Biomedical Press, New York, 1980, p. 191.
3 D.J. Cripps, A. Gocmen and H.A. Peters, Arch. Dermatol., 116 (1980) 46.

4 K. Chang, F. Lu, T. Tung and T. Lee, Res. Commun. Chem. Pathol. Pharmacol., 30 (1980) 547.
5 J.N.M. Chalmers, A.E. Gillem and J.E. Kench, Lancet, ii (1940) 806.
6 M. Doss, C.E. Lange and G. Veltman, in J.J.T.W.A. Strik and J.H. Koeman (Editors), Chemical Porphyria in Man, Elsevier/North-Holland Biomedical Press, New York, 1979, p. 107.
7 J.S. Taylor and H.H. Roenigk, Jr., in M. Doss (Editor), Porphyrins in Human Diseases, S. Karger, New York, 1975, p. 328.
8 G. Romeo, Prog. Med. Genet., 4 (1980) 169.
9 M. Doss and R.V. Tieperman, J. Clin. Chem. Clin. Biochem., 16 (1978) 57.
10 P.B. Beeson, W. McDermott and J.B. Wyngaarden (Editors), Cecil Textbook of Medicine, Vol. 2, W.B. Saunders, New York, 15 ed., 1979, p. 2046.
11 M. Doss, Klin. Wochenschr., 49 (1971) 939.
12 R. Bonnett, A.A. Charalambides, K. Jones, I.A. Magnus and R.J. Ridge, Biochem. J., 173 (1978) 693.
13 E. Englert, Jr., A.W. Wayne, E.E. Wales, Jr. and R.C. Straight, J. High Resolut. Chromatogr. Chromatogr. Commun., 2 (1979) 570.
14 H.D. Meyer, K. Jacob and W. Vogt, J. High Resolut. Chromatogr. Chromatogr. Commun., 3 (1980) 85.
15 H.D. Meyer, K. Jacob, W. Vogt and M. Knedel, J. Chromatogr., 199 (1980) 339.
16 R.H. Hill, Jr., S.L. Sirmans and L.L. Needham, J. High Resolut. Chromatogr. Chromatogr. Commun., 3 (1980) 588.
17 R.E. Ford, C. Ou and R.D. Ellefson, Clin. Chem., 27 (1981) 397.
18 C.H. Gray, C.K. Lim and D.C. Nicholson, Clin. Chim. Acta, 77 (1977) 167.
19 A.V. Benedetto, J.R. Kushner and J.S. Taylor, N. Engl. J. Med., 298 (1978) 358—362.
20 R.E. De Salamanca, R. Rico, M.L. Pena, F. Romers, A. Olmos and J. Jimanez, Int. J. Biochem., 12 (1980) 861.
21 L. Eales, R.S. Day, and G.H. Blekkenhorst, Int. J. Biochem., 12 (1980) 837.
22 P.H. Magnin, E.A.W. De Xifra, M. Lenczner, A.M. Stella and A.M. del C. Batille, Int. J. Biochem., 12 (1980) 873.
23 Z.J. Petrylca and C.A. Pierach, in G. Hawk (Editor), Biological/Biomedical Applications of Liquid Chromatography, Marcel Dekker, New York, 1979, p. 103.
24 M. Doss and E. Schermaly, in M. Doss (Editor), Porphyrins and Human Diseases, S. Karger, New York, 1976, p. 189.
25 M. Doss, P. Nawrocki and E. Schermaly, in M. Doss and P. Nawrocki (Editors), Porphyrins in Human Diseases, Report of the Discussion, S. Karger, New York, 1976, p. 163.