*Journal of Chromatography*, 433 (1988) 73–79
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4386

# DETERMINATION OF ERYTHROCYTE PORPHYRINS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING CAPSULE-TYPE SILICA GELS COATED WITH SILICONE POLYMER

TADASHI SAKAI*, YUKIKO TAKEUCHI, TAKAHARU ARAKI and KOICHI USHIO

*Center of Occupational Medicine, Tokyo Labor Accident Hospital, 13-21, Omoriminami-4, Ota-Ku, Tokyo 143 (Japan)*

(First received March 28th, 1988, revised manuscript received July 13th, 1988)

## SUMMARY

A simple, rapid and reliable method is described for determining erythrocyte porphyrins using a new type of reversed-phase silica whose surface is coated with silicone polymer. After a single-step extraction of blood with N,N-dimethylformamide, zinc protoporphyrin IX and protoporphyrin IX were separately quantified in a single run in 5 min. The column life was so long that no alteration in elution profile or retention time was apparent after 1500 injections of samples. The method described would be useful for the screening of lead exposure or iron-deficiency anaemia, and also for the diagnosis of erythropoietic protoporphyria.

## INTRODUCTION

Accumulation of protoporphyrin in erythrocytes provides an index of disturbance of the final step in haem synthesis [1]. Erythrocyte protoporphyrin (EP) occurs as a zinc-chelated protoporphyrin (ZP) in human lead poisoning and iron-deficiency anaemia [2,3]. Unchelated free protoporphyrin (PP), on the other hand, accumulates in erythropoietic protoporphyria [2,3].

Several methods have been developed for determining EP concentrations; they include acid extraction [4], detergent dilution [5], neutral solvent extraction [6], haematofluorimetry [7] and high-performance liquid chromatographic (HPLC) methods [8–10]. The method most widely used is the acid extraction method, although a two-step extraction is complicated and the second extraction with dilute hydrochloric acid cleaves zinc from ZP molecules to form free PP. ZP concentrations are therefore measured as free PP fluorescence by the acid extraction method. The detergent dilution method is subject to serious haemoglobin interference [6]. The disadvantages of neutral solvent extraction are low extrac-

0378-4347/88/$03.50 

tion efficiency, variable recovery and interference from PP [6,10]. The limitation of haematofluorimetry which is a simple and rapid technique for ZP detection, lies in weak quality control of standardization and in interference from bilirubin and PP [10,11]. In these three methods the fluorescence of ZP is usually measured.

The significant advantage of HPLC methods over these methods is that ZP and PP can be separately quantitated in a single run, eliminating the optical interference from co-extractable substances. However, two difficulties with the HPLC method for determining blood porphyrins have been reported [8–10]. One of the requirements for HPLC analysis of fragile ZP is an efficient extraction under mild conditions that leave the ZP molecules intact. Other problems are the stability of both packing materials and samples (ZP and PP); ZP is labile in acid and only stable under neutral or weakly alkaline conditions. However, under these conditions silica gel dissolves into solutions and the column life is short.

Recently, a new type of reversed-phase silica whose surface is coated with silicone polymer and which shows strong resistance to alkali has been developed, allowing separations in alkaline mobile phases [12]. In the present study we successfully applied these packing materials for the separation of blood porphyrins (ZP and PP) with a mobile phase containing ion pairs at a weakly alkaline pH after a single extraction of blood with N,N-dimethylformamide (DMF), which we also first introduced in porphyrin extraction.

## EXPERIMENTAL

### *Automated HPLC*

A Shimadzu liquid chromatograph (Shimadzu, Kyoto, Japan) consisting of an LC-3A pump, a SIL-2A automatic sample injector, a CTO-2A column oven, an RF-530 spectrofluorimeter with an R-928 photomultiplier and a C-R3A automatic integrator was used. The Capcell Pak $C_{18}$ column (150 mm×40 mm I.D., Shiseido, Tokyo, Japan) used was packed with capsule-type $C_{18}$ (5 $\mu$m) silica gel, which is silicone polymer-coated silica gel modified with octadecyl groups. The silicone coating layer provides excellent stability against alkaline and organic solvents. The mobile phase was a mixture of 50 m*M* tetrabutylammonium hydroxide (pH 7.5 adjusted with phosphoric acid)–acetonitrile (34:66, v/v) [8]. The column temperature and flow-rate were set at 40°C and 1.0 ml/min, respectively. The excitation and emission wavelengths were set at 420 and 630 nm, respectively.

### *Chemicals*

PP IX and ZP IX were obtained from Porphyrin Products (Logan, UT, U.S.A.). Acetonitrile, tetrabutylammonium hydroxide (TBA), DMF and sodium diethyldithiocarbamate trihydrate (DDTC) were purchased from Wako (Osaka, Japan). 4-Methyl-2-pentanone (MIBK) was from Kanto Kagaku (Tokyo, Japan). Acetonitrile was of HPLC grade, and DDTC and MIBK were of AAS (atomic absorption spectrophotometry) grade. Other chemicals were of analytical grade.

*Standard solutions*

PP IX and ZP IX were dissolved in DMF (stock solution, 25 mg per 500 ml). The solution was stable spectrophotometrically and spectrofluorimetrically for over two years at 4 °C in the dark. The millimolar absorptivities (at 408 nm) of the free and zinc protoporphyrin standards used were 276 and 274, respectively, in 1.5 *M* hydrochloric acid. For use, the stock solution was further diluted with DMF to give a concentration of 40 μg/l. When 20 μl of the solution were injected as described below, the concentration was equivalent to that of 204 μg/dl of blood on the chromatogram.

*Procedure*

Without any dilution or haemolysis, 50 μl of blood were simply extracted with 2.5 ml of DMF. After agitation and centrifugation, a slightly yellowish and transparent supernatant was obtained, of which 20 μl were automatically injected at 5-min intervals.

*Blood samples and other analyses*

Samples used were the heparinized venous blood from 64 lead workers and 68 normal subjects. Free EP (FEP) and erythrocyte ZP were also determined by the acid extraction method [4] and haematofluorimetry [7], respectively. For the determination of blood lead (Pb-B) concentrations, blood was mineralized with sulphuric acid, nitric acid and perchloric acid, and chelated with DDTC. The lead–DDTC complex was extracted with MIBK, and then the extract was used for the determination of Pb-B by AAS.

## RESULTS AND DISCUSSION

Fig. 1 shows the effect of the injection volume on the chromatographic separations of ZP and PP standard. The elution profile of ZP and PP varies with increases in the injection volume from 20 to 50 μl. The retention of porphyrin is altered by the injection of large amounts of DMF (more than 40 μl). Decreasing TBA concentrations in the mobile phase resulted in changes in the elution profiles similar to those seen in Fig. 1b and c (data not shown). Without addition of TBA to the mobile phase, ZP and PP were not separated and eluted at 1.75 min. Therefore the appearance of an early-eluting peak at 1.75 min with increased

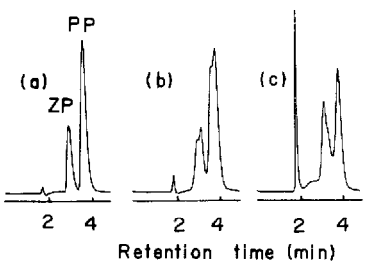


Fig. 1. Effect of the injection volume on the chromatographic separations. The injection volumes of standard solutions containing ZP and PP (40 μg/l each) were (a) 20 μl, (b) 40 μl, and (c) 50 μl.

injection volume or decreased TBA concentration may be caused by inhibitory (competitive) action of DMF on the formation of ion pairs between TBA and porphyrins. The interaction of sample molecules with DMF or TBA on the stationary phase should be further examined for an explanation of the retention mechanismus in the present HPLC system. However, the peak-shapes are unchanged if the volume does not exceed 20 $\mu$l, which therefore seems to be the optimum volume.

Fig. 2 shows a typical chromatogram of actual samples from a normal subject and from lead workers. Various amounts of ZP and PP in the extract are completely separated in less than 5 min. A linear relationship was found between peak height and blood volume used (5–200 $\mu$l), when the total volume of the extraction mixture (DMF plus blood) was not altered. In the present method 50 $\mu$l of blood was used for the extraction. Standard curves were linear over a wide range of concentrations of each porphyrin (more than 2000 $\mu$g/l of red blood cells).

Recoveries of increasing amounts of ZP and PP added simultaneously to blood from a normal subject are 88 and 96%, respectively (Table I). Similar results were obtained when ZP or PP was separately added to blood, indicating that zinc

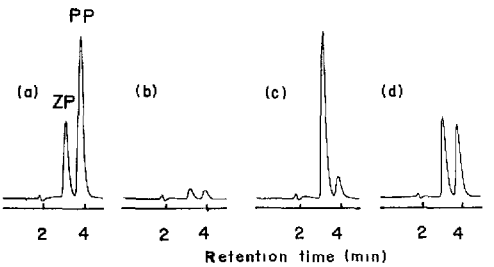


Fig. 2. Chromatographic separation of ZP and PP. The mixture of ZP and PP standards (a) and extracts from a control subject (b) and from lead workers (c and d) were analysed by HPLC. A 50-$\mu$l volume of blood was extracted with 2.5 ml of DMF, and 20 $\mu$l of the extract were injected. The levels of ZP and PP standards were equivalent to a concentration of 476 $\mu$g/dl of red blood cells (haematocrit = 42%). The concentrations of Pb-B ($\mu$g per 100 g), ZP and PP ($\mu$g/dl of red blood cells) were 4.9, 31.6 and 4.1 in (b), 103.4, 972.0 and 54.5 in (c) and 73.0, 295.1 and 146.6 in (d), respectively.

TABLE I

RECOVERY OF ZP AND PP ADDED TO BLOOD

| Concentration added ($\mu$g/dl) | ZP recovered | | PP recovered | |
|---|---|---|---|---|
| | $\mu$g/dl | % | $\mu$g/dl | % |
| Blood alone | 28.5 | | 18.5 | |
| 100 | 113.4 | 84.9 | 101.3 | 82.8 |
| 200 | 199.3 | 85.4 | 218.9 | 100.2 |
| 500 | 479.5 | 90.1 | 515.0 | 99.3 |
| 1000 | 937.5 | 90.9 | 1041.5 | 102.3 |
| Mean | | 87.8 | | 96.2 |

was not cleaved from ZP throughout the extraction and HPLC procedure. Within-assay coefficients of variation (C.V.) are listed in Table II. The data indicate that the method has good precision. Detection limits for ZP and PP at a signal-to-noise ratio of 3 were ca. 10 and 5 μg/dl of red blood cells, respectively, under the standard conditions. The method is sufficiently accurate to detect the lowest levels of both ZP and PP in normal subjects, which are described below. For the more precise determination of blood porphyrins, detection limits can be further lowered to a quarter of the above values, if the blood volume used for the extraction is increased to 200 μl, as mentioned above. Another way of lowering the limits is to change the excitation and emission wavelengths to be specific to ZP or PP [5].

To assess the suitability of the present method, we compared it with the extraction method [4] and haematofluorimetry [7] (Fig. 3). Thirty samples from lead workers were analysed by HPLC for ZP, PP and total protoporphyrin (TP = 0.9ZP + PP). In the method of acid extraction ZP is converted into PP, and both porphyrins are measured as PP, which is commonly called FEP. Therefore we compared TP by HPLC with FEP by acid extraction, and ZP by HPLC with that by haematofluorimetry. Consistently good agreement is found between the HPLC method and each of the two established methods.

TABLE II

PRECISION OF THE METHOD

| Compound | Concentration (μg/dl) | Coefficient of variation (%) |
|---|---|---|
| ZP | 54.2 | 2.6 |
| | 991.7 | 0.8 |
| PP | 20.7 | 2.5 |
| | 51.4 | 2.5 |

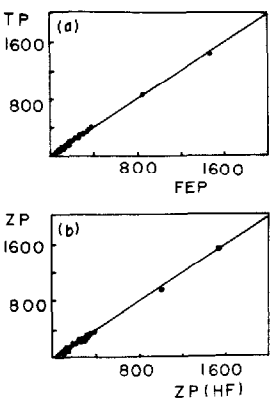


Fig. 3. Linear regression for TP or ZP concentrations by HPLC and FEP by the acid extraction method or ZP by haematofluorimetry (HF). Lines indicate the regression: (a) $y = 0.991x + 14.03$ ($r = 0.997$); (b) $y = 0.989x - 13.9$ ($r = 0.998$).

Fig. 4 shows the correlations of protoporphyrin concentrations with Pb-B levels in 64 lead workers. The correlation coefficients between 0.9ZP or TP and Pb-B are significantly higher than that between PP and Pb-B. These results indicate that the concentrations of ZP or TP determined by the present method are useful indicators for the evaluation of occupational exposure to lead. The ratio of 0.9ZP to TP in the workers ranged from 0.53 to 0.99 (mean $\pm$ S.D., $0.86 \pm 0.085$) and the regression equation between 0.9ZP ($y$) and TP ($x$) was $y=0.940x-9.53$ ($r=0.996$). No correlation was found between the ratio and Pb-B concentration. Reference values for protoporphyrins by the present method were determined in blood from 34 male and 34 female subjects not exposed to lead. The mean $\pm$ S.D. concentrations of ZP, PP and TP were $57.4 \pm 14.7$, $10.6 \pm 8.6$ and $62.2 \pm 20.0$ $\mu$g/dl of red blood cells in male and $69.5 \pm 22.5$, $10.0 \pm 8.4$ and $72.6 \pm 26.4$ $\mu$g/dl red blood cells in female, respectively. These TP values obtained by the present method are compatible with FEP values obtained by the acid extraction method [13].

ZP shows unique solubility characteristics and it is fragile. Neutral and organic solvents such as acetone and ethanol did not extract all the porphyrin and the extraction efficiency was low [6]. If the supernatant was injected onto the HPLC column after lysis and dilution of whole blood without extraction, the column life reportedly decreased to ca. 150 sample injections per column [8]. When the lysing solution contained acid (formic or acetic acid), zinc was removed from the ZP standard [10] or large amounts of haemin were co-extracted from blood [9]. The retention time of haemin was very sensitive to the column acidity, requiring deactivation of the column by washing with strong acid [9]. The present HPLC system has considerable advantages over methods reported so far [8–10], the most notable being that the extraction procedure is simple and rapid, that the recovery is high and that the column life is very long, even in the weak alkaline

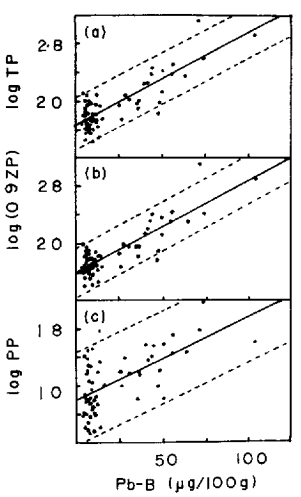


Fig. 4. Relationship between EP by HPLC and Pb-B. Solid and dashed lines show the regression and 95% predictive intervals, respectively: (a) $y=0.0129x+1.67$ ($r=0.834$); (b) $y=0.0130x+1.60$ ($r=0.852$); (c) $y=0.118x+0.79$ ($r=0.605$). In each case, $n=64$.

solvent used. Haemin is not co-extracted by DMF and zinc is not cleaved from ZP during the extraction and HPLC separation. No alteration of the elution profile or retention time was observed even after 1500 sample injections, and weak peak tailings were observed in chromatograms only after 2000 injections. Under the same conditions, we confirmed that conventional reversed-phase silica deteriorated after 100–200 injections, as reported by Gotelli et al. [8]. In addition to its great resistance to alkali, the column is highly stable to organic solvents, apparently because of the formation of chemical bonds between the silanol groups and the polymer [12]. Hence the method described would be useful for the screening of lead exposure or iron deficiency and also for the diagnosis of erythropoietic protoporhyria.

REFERENCES


1 J.T. Hindmarsh, Clin. Chem., 32 (1986) 1255.
2 A.A. Lamola and T. Yamane, Science, 186 (1974) 936.
3 A.A. Lamola, S. Piomelli, M.B. Poh-Fitzpatrick, T. Yamane and L.C. Harber, J. Clin. Invest., 56 (1975) 1528.
4 S. Piomelli, J. Lab. Clin. Med., 81 (1973) 932.
5 A.A. Lamola, M. Joselow and T. Yamane, Clin. Chem., 21 (1975) 93.
6 T.L. Hanna, D.N. Dietzler, C.H. Smith, S. Guputa and S. Zarkowsky, Clin. Chem., 22 (1976) 161.
7 W.E Blumberg, J. Eisinger, A.A. Lamola and D.M. Zuckerman, Clin. Chem., 23 (1977) 270
8 G.R. Gotelli, J.H. Wall, P.M. Kabra and L.J. Marton, Clin. Chem., 26 (1980) 205.
9 R.M. Smith, D. Doran, M. Mazur and B Bush, J. Chromatogr., 181 (1980) 319.
10 G.G. Bailey and L.L. Needham, Clin. Chem., 32 (1986) 2137.
11 E. Buhrman, W.C. Mentzer and B.H. Lubin, J. Lab. Clin. Med., 91 (1978) 710.
12 Y. Ohtsu, H. Fukui, T. Kanda, K. Nakamura, M. Nakano, O. Nakata and Y. Fujiyama, Chromatographia, 24 (1987) 380.
13 W.L. Marsh, D.P. Nelson and H.M. Koenig, Am. J. Clin. Pathol., 79 (1983) 655.