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A novel rapid method for simultaneous determination of three diagnostically important porphyrins in erythrocytes using hyphenated synchronous fluorescence techniques

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ABSTRACT

The species and concentrations of porphyrins in erythrocytes are of great importance in the clinical screening and diagnosis of porphyrias. However, it is difficult to analyze them simultaneously by conventional spectrofluorimetry. In this paper, we proposed a novel, simple and rapid method for the simultaneous determination of three diagnostically important porphyrins in human erythrocytes, protoporphyrin IX (PP), coproporphyrin III (CP) and zinc protoporphyrin IX (ZnPP), using hyphenated techniques based on derivative matrix isopotential synchronous fluorescence spectrometry and nonlinear variable-angle synchronous fluorescence spectrometry (DMI-NLVASFS). The spectral overlapping problems were well resolved and these three components were determined in one scanning without spectral compensation factors and chromatographic separation. The detection limits were 0.58, 0.21, 0.05 nmol L^{-1} for PP, CP and ZnPP, respectively. Only 30 s was needed for a single scanning and the recoveries were from 73% to 105% in erythrocytes. The Bland–Altman analysis indicates no significant difference between the proposed DMI-NLVASFS method and conventional fluorimetry method. The PP level of the erythropoietic protoporphyria (EPP) patients was significantly higher than that of healthy volunteers. This method can determine PP, CP and ZnPP simultaneously in a single scanning, thus providing a potential tool for the clinical analysis of porphyrins in human erythrocytes and the differential diagnosis of porphyrias.

1. Introduction

Biologically, porphyrins are important molecules relating to enzymes in human body, and perform a wide variety of biological functions. The clinical and biochemical significance of porphyrins is that the disorders arising from the defects in enzymes of heme biosynthesis will lead to a group of diseases called porphyria. The variation of the enzymes will cause the excessive accumulation and excretion of certain species of porphyrins in liver, blood and excreta. In the erythrocytes of normal humans, protoporphyrin IX (PP) and zinc protoporphyrin IX (ZnPP) exist in trace level [1]. However, in the case of erythropoietic protoporphyria (EPP), the level of PP in erythrocytes increases dramatically, and in some cases the level of coproporphyrin (CP) may increases as well [2–4]. In contrast, in the case of hepatic erythropoietic protoporphyria (HEP), lead and arsenic poisoning, iron deficiency and sideroblastic anemia, high concentration of ZnPP in erythrocytes occurs [2,4]. In the congenital erythropoietic porphyria (CEP), the concentrations of ZnPP, CP and uroporphyrin (UP) in erythrocytes also increase [2,5]. Besides, the CP and PP levels in erythrocytes from patients with carcinoma of stomach, large intestine and thyroid gland rise markedly [6]. Hence, the species and concentrations of porphyrins in erythrocytes are of great importance in the clinical screening and diagnosis of some porphyrias and serve as biomarkers in the environment monitoring of lead [7] and arsenic pollution [8,9].

Porphyrins exhibit strong absorption in the Soret band and most of them fluoresce after being excited. Many methods have been established for the determination of porphyrins in erythrocytes [10–13]. Mingioli [14] introduced new correction factors to remove interference, and determined UP, CP and PP in erythrocytes by spectrophotometry. The employing of mathematical calculation in this method was inconvenient for routine measurement. In our previous work, we have reported a variable-angle synchronous fluorescence spectrometry method to analyze PP and ZnPP in whole blood [15], and derivative matrix isopotential synchronous fluorescence spectroscopy methods for the simultaneous determination of PP and CP in feces [16], CP and UP in urine [17], respectively. However, they were not suitable for the analysis of ternary mixtures of PP, CP and ZnPP. We have reported a method based on non-linear variable-angle synchronous fluorescence technique (NLVASFS) and partial least squares (PLS) for the simultaneous determination of PP, UP and CP in human whole blood [18]. Nevertheless, the

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spectral overlapping problems cannot be solved by NLVASFS solely without the PLS1 algorithm. Many porphyrins have been quantified simultaneously with high performance liquid chromatography (HPLC) techniques [3,12,19,20]. Gotelli et al. [20] had used it to determine ZnPP, PP and CP in whole blood. Furthermore, high performance liquid chromatography coupled with mass spectrometry (HPLC/MS) method was also used for the detection of porphyrins in blood [21,22]. However, these methods were time-consuming and required complicated pretreatment processes.

Compared with the conventional fluorescence spectrometry, better selectivity and more flexibility can be obtained by variable-angle synchronous fluorescence spectrometry (VASFS), and the spectral resolution of this method can be further improved when coupled with derivative technique [15,23–30]. The first derivative matrix isopotential synchronous fluorescence spectrometry (DMISFS) can eliminate the interference of the matrix and other fluorescent interferent by scanning along their contour lines [16,17,31,32]. This technique can simultaneously determine binary mixtures with seriously overlapped spectra. Multi-component mixtures can be better investigated using these techniques properly.

To the best of our knowledge, the simultaneous determination of PP, CP and ZnPP with a fluorimetric technique that could avoid chromatographic separation has not yet been reported. In this paper, we proposed a novel, simple and rapid method for the simultaneous determination of three diagnostically important porphyrins in human erythrocytes, PP, CP and ZnPP, using hyphenated synchronous fluorescence techniques, derivative matrix isopotential synchronous fluorescence spectrometry coupled with nonlinear variable-angle synchronous fluorescence spectrometry (DMI-NLVASFS).

2. Materials and methods

2.1. Reagents and solutions

Protoporphyrin IX disodium salt was purchased from Sigma–Aldrich Corporation, coproporphyrin III dihydrochloride and zinc protoporphyrin IX were from Strem Chemicals Incorporation (Newburyport, USA). *N*,*N*-dimethylformamide (DMF), ethyl acetate, glacial acetic acid and hydrochloric acid were from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and all were analytical grade reagents. Methanol was of HPLC grade.

The standard stock solutions of PP, CP and ZnPP were prepared using the similar procedures. About 1 mg of each porphyrin was dissolved in DMF. These porphyrin solutions were transferred into 50 mL volumetric flasks and brought to the mark with DMF as individual standard stock solutions. The volumetric flasks were protected from light by wrapping them with aluminum foil and stored at −20 °C. The exact concentrations of them were determined spectrophotometrically. An aliquot of 0.1 mL standard stock solutions of PP, CP and ZnPP were individually diluted to 3.00 mL with 1.5 mol L^{-1}, 0.1 mol L^{-1} and 1.5 mol L^{-1} HCl, respectively. The absorption was measured on Beckman DU-7400 spectrophotometer ($\varepsilon_{408\,nm} = 3.1 \times 10^5$ dm^3 mol^{-1} cm^{-1} for PP and ZnPP, $\varepsilon_{399.5\,nm} = 4.89 \times 10^5$ dm^3 mol^{-1} cm^{-1} for CP) [33]. The standard working solutions of PP, CP and ZnPP were prepared from corresponding standard stock solutions by serial dilutions in DMF/methanol (v/v = 1/5).

2.2. Instrumentation

All the fluorescence spectra were obtained on a laboratory-constructed, computer-controlled MYF spectrofluorimeter which was similar to that described previously [15–17]. It was equipped with 150 W xenon arc lamp (OSRAM GmbH, Steinerne Furt 6286167 Augsburg, Germany) and tunable slit band passes. The excitation and emission monochromators were controlled by a personal computer through a software package written in Turbo C 2.0. Experimental data were collected by the computer through the software package. A program written in Visual Basic for Excel was used for further data processing. The excitation–emission matrices calculated from the excitation and emission spectral data were used to acquire a suitable scanning route by means of this program. The matrix isopotential-nonlinear variable-angle synchronous fluorescence spectra were recorded with the selected scanning route. The derivative spectra were obtained by the electronic derivative system. Laser scanning confocal fluorescence microscopy (TCS·SP5-AOBS-MP, Leica, Germany) was used for imaging erythrocytes after washed by physiological saline. A microquartz cuvette (5 mm × 5 mm × 35 mm) was used for fluorescence measurements throughout the study. The desktop centrifuge was purchased from Wuxi Ruijiang Analytical Instruments Co., Ltd. (Wuxi, Jiangsu Province, China). The vortex-mixer was from Kylin-Bell Lab Instruments Co., Ltd. (Haimen, Jiangsu Province, China).

2.3. Sample preparation

Blood samples from 20 healthy volunteers were collected into glass tubes containing Na_2EDTA as anticoagulant by co-workers from the hospital of Xiamen university (Xiamen, China). Blood samples from two erythropoietic protoporphyria (EPP) patients were provided by Doctor You-Sheng Yan from Maternal and Child-care hospital of Gansu province (Gansu, China). After mixing, the blood samples were centrifuged at 1000 rpm for 1 min to remove the plasma, and the residual parts were washed with physiological saline for three times to get intact erythrocytes [34]. An aliquot of 8.0 μL erythrocytes was taken into a disposable plastic stoppered centrifugal tube, and 0.4 mL DMF/methanol (v/v = 1/5) was added and vortex-mixed for about 10 s to extract the porphyrins, then the mixture was centrifuged at 8000 rpm for 3 min. The supernatant was collected for measurement. After extraction and centrifugation, the sample was clear and most of the matrix of the erythrocytes potentially interfering in the determination of porphyrins was eliminated.

3. Results and discussion

PP and ZnPP can be determined simultaneously by derivative variable-angle synchronous fluorescence spectrometry (DVASFS) [15], while PP and CP, whose spectra were almost superposed, can be determined by derivative matrix isopotential synchronous fluorescence spectrometry (DMISFS) [16]. However, it was still difficult to determine these three diagnostically important porphyrins simultaneously by either DVASFS or DMISFS technique only. The secondary peak (λ_{em} = 640 nm) of ZnPP overlapped with the primary peaks of PP (λ_{em} = 632.7 nm) and CP (λ_{em} = 623 nm). Furthermore, the spectra of PP and CP were almost superposed on each other (Fig. 1). Nevertheless, the interference between PP and CP can be eliminated by derivative matrix isopotential synchronous fluorescence spectrometric (DMISFS) technique [16], and if proper contour lines of PP and CP were chosen, the interference from the secondary peak of ZnPP can also be eliminated. Fig. 1b shows the contour maps of PP, CP and ZnPP and it also indicates that it is difficult to analyze these three porphyrins simultaneously by either DMISFS or DVASFS. Thus, a rapid and selective method based on hyphenated synchronous fluorescence techniques, derivative matrix isopotential synchronous fluorescence spectrometry coupled with nonlinear variable-angle synchronous fluorescence spectrometry (DMI-NLVASFS), was investigated to

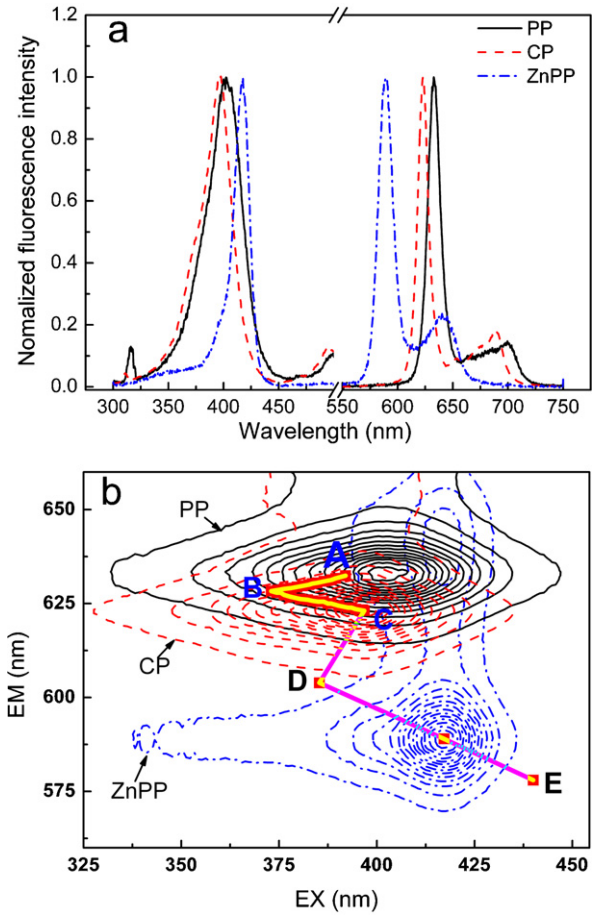

Fig. 1. (a) Normalized excitation and emission spectra and (b) theoretical contour maps of PP (solid line), CP (dashed line) and ZnPP (dash-dotted line) in DMF/methanol (v/v = 1/5). (a) The λ_{em} of their excitation spectra are 632.7, 623 and 589.6 nm, respectively. The λ_{ex} of their emission spectra are 402.4, 397 and 416.5 nm, respectively. (b) ABCDE was the whole scanning route (thick solid line).

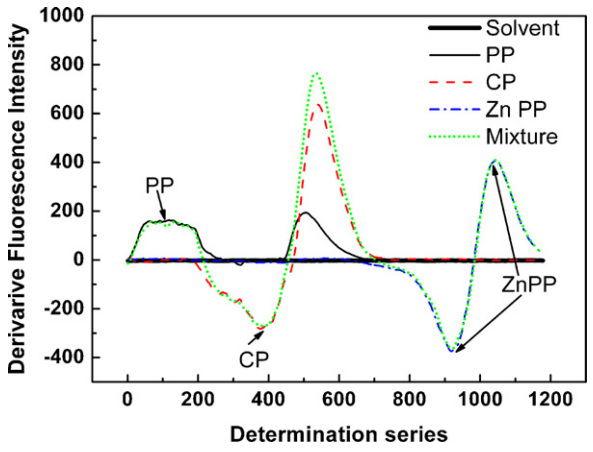

Fig. 2. DMI-NLVASF spectra of PP (33 nmol L^{-1}, solid line), CP (27 nmol L^{-1}, dashed line), ZnPP (16 nmol L^{-1}, dash-dotted line) in DMF/methanol (v/v = 1/5), solvent (thick solid line) and their mixture with the same concentrations as above (short dashed line).

determine the ternary components of PP, CP and ZnPP simultaneously.

3.1. Selection of the optimal DMI-NLVASFS scanning route

In order to establish a suitable DMI-NLVASFS method for the simultaneous determination of PP, CP and ZnPP, the selection of an optimal scanning route is critically important since it can lead to the optimal DMI-NLVASFS fluorescence spectra with the highest fluorescent signal and without interference among them [12–14].

The first step was to obtain the three-dimensional spectra of PP, CP and ZnPP. In this work, they were derived from pairs of excitation and emission spectra of these three porphyrins as mentioned in Section 2. In order to provide favorable guidance for choosing an optimal scanning route, the excitation and emission spectra were normalized before inputting them into the homemade program for gaining the theoretical three-dimensional fluorescence spectra. The second step was to select the detection point of each component, where each component exhibits maximum signal and minimum interference. As shown in Fig. 1b, CP was first supposed as interfering component, point (λ_{ex} = 392 nm, λ_{em} = 632.4 nm) was selected as the starting point considering the secondary peak of ZnPP overlapped with the primary peaks of PP and CP. Then we searched for the fluorescent intensity of CP at this point and obtained a set of points with the same intensity, and linked these points together to form the determination route of PP (indicated by "AB" in Fig. 1b). In a similar way, we obtained the determination route for CP (indicated by "BC" in Fig. 1b). The two parts were united together as a matrix isopotential synchronous fluorescence scanning route for the simultaneous determination of PP and CP. Since the interference of PP and CP on the primary peak of ZnPP can be negligible, the fluorescence maximum point of ZnPP (λ_{ex} = 416.5 nm, λ_{em} = 589.6 nm) was chosen as the detection point for ZnPP. Other points should be chosen to form a nonlinear variable-angle synchronous scanning route for ZnPP (indicated by "DE" in Fig. 1b). Finally, the two routes ABC and DE were connected with each other to obtain a whole scanning route. When combined with the derivative technique, the interference signal of CP and PP were eliminated in the first (AB) and second (BC) segments, respectively, which then showed the net derivative signal of PP and CP. Therefore, PP, CP and ZnPP could be determined simultaneously in only a single scanning within 30 s.

3.2. Spectral characteristics

Using the selected trajectory, the DMI-NLVASF spectra of porphyrins in standard solutions were recorded, and they were well resolved with almost no mutual interference (Fig. 2). The derivative fluorescence peaks of PP, CP and ZnPP appeared in turn with no overlap; in the spectral region of PP, the derivative signal of CP and ZnPP tended to zero. Similarly, the contribution of other two components was nearly zero in the spectral region of CP and ZnPP. According to Fig. 2, peak-to-peak measurement was used for the determination of ZnPP, while the peak-to-zero measurement was used for PP and CP. The peaks in the spectrum of the ternary components mixture were consistent with that of single-component solutions completely (Fig. 2). The time for a whole single scanning was only 30 s.

3.3. Linearity and detection limits

Individual calibrators were prepared by diluting the standard stock solutions of PP, CP and ZnPP in DMF/methanol (v/v = 1/5). The linear equation of PP from 1 to 82 nmol L^{-1} without interference was $y = 0.447 + 6.66\,x$ ($r = 0.999$). The linear equation of CP from 0.4 to 69 nmol L^{-1} without interference was $y = 0.945 + 11.57\,x$ ($r = 0.999$). The linear equation of ZnPP from 0.25 to 64 nmol L^{-1} without interference was $y = 4.1 + 72.5\,x$ ($r = 0.999$).

According to the definition of the detection limit (LOD), $LOD = 3S_{bl}/k$ (where S_{bl} is the standard deviation of the blank solvent and k is the slope of the calibration curve). Thirteen blank solvent samples were measured by the DMI-NLVASFS method, and

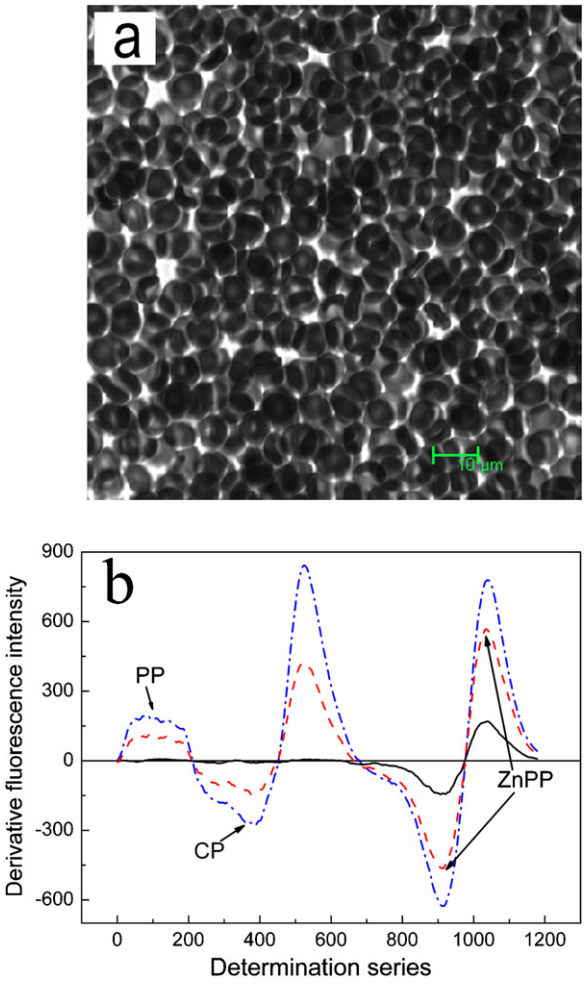

Fig. 3. (a) Bright field image of erythrocytes by laser scanning confocal fluorescence microscopy. (b) DMI-NLVASF spectra of the extract of erythrocytes (solid line); spiked erythrocytes (dashed line), spiked with 6.6 nmol L^{-1} PP, 5.5 nmol L^{-1} CP, and 6.4 nmol L^{-1} ZnPP; and the standard mixture of 13 nmol L^{-1} PP, 11 nmol L^{-1} CP and 12.8 nmol L^{-1} ZnPP (dash-dotted line).

the standard deviation was calculated. The detection limits of PP, CP and ZnPP were 0.58, 0.21 and 0.05 nmol L^{-1}, respectively.

3.4. Real samples analysis

In order to check the effect of washing procedure (the erythrocytes should be intact and the remaining should contain the cells only), laser scanning confocal fluorescence microscopy was used to investigate the washed erythrocytes in glass bottom cell culture dishes. The image (Fig. 3a) was collected using 20× objective lens (numerical aperture: 0.7, pinhole: 60.61 μm; zoom: 6). Erythrocytes remained intact with centre-concave and edge-thick round-pie shape.

The extract of erythrocytes, spiked erythrocytes, and the mixed standard solution of porphyrins were measured by the proposed DMI-NLVASFS method. The spectra are shown in Fig. 3b. We can see that the peak positions of each porphyrin remain the same as their individuals in these three spectra. The background matrix of the extract of erythrocytes and spiked erythrocytes could be eliminated by the derivative technique. Moreover, the signal-to-noise ratio of ZnPP increased markedly.

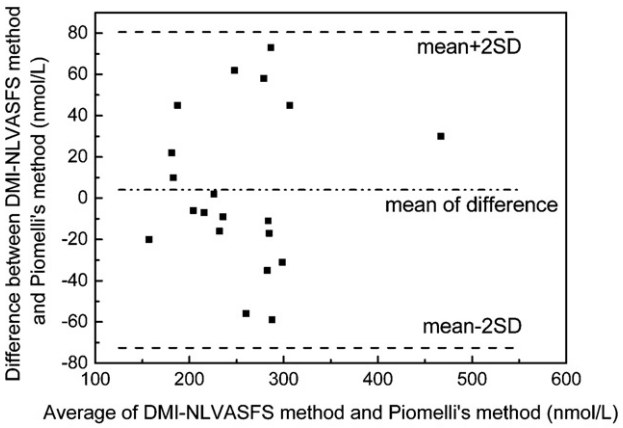

Fig. 4. Bland–Altman plot of the proposed DMI-NLVASFS method and Piomelli's method (erythrocytes samples of 20 healthy volunteers).

3.5. Within-run precision and recoveries

To assess the within-run precision of the method, we measured five aliquots of erythrocytes from the same spiked sample. The obtained relative standard deviations of PP, CP and ZnPP in erythrocytes were 9.8%, 4.4% and 7.5%, respectively. The results met the requirement of quantitative analysis [35].

Five aliquots of erythrocytes from five different sources were spiked with different concentrations of standard solutions. The concentrations of these three porphyrins components under investigation were determined by the proposed DMI-NLVASFS method. The recoveries were all between 73% and 105%, and also met the requirement of quantitative analysis (Table 1) [35].

3.6. Method comparison

In order to validate this DMI-NLVASFS method, we compared it with the Piomelli's method [11]. Because the acid extraction solvent was used in the Piomelli's method, the total porphyrins were determined. The porphyrins in erythrocytes of 20 healthy volunteers and 2 erythropoietic protoporphyria (EPP) patients were measured by these two methods and the results were listed in Table 2. The CP level of healthy volunteers and the two EPP patients was not detectable, thus not listed in Table 2. It was similar to that reported previously [20]. Due to the high levels of porphyrins in patients, the supernatant of their erythrocytes were diluted by several times before measuring.

The sum amounts of PP and ZnPP obtained by the proposed DMI-NLVASFS method were used for comparison. Fig. 4 shows the Bland–Altman plot of the 20 samples of healthy volunteers. The mean of difference was 4 nmol L^{-1}, and the standard deviation of the difference was 38 nmol L^{-1}. The limits of agreement were between −72 and 80 nmol L^{-1}. The 95% confidence intervals for mean, upper limit and lower limit of differences were 4 ± 18, 80 ± 31, and -72 ± 31 nmol L^{-1}, respectively. The narrow range for the limits of agreement (between −72 and 80 nmol L^{-1}) in the Bland–Altman analysis indicates that the results of total porphyrins obtained by these two methods are consistent with each other, without significant difference between them.

The results shows that the level of PP in erythrocytes of erythropoietic protoporphyria (EPP) patients is about several hundred times higher than that of healthy volunteers, which agrees with the results reported by Hindmarsh et al. [4]. The proposed DMI-NLVASFS method showed coincident results of total porphyrins with Piomelli's method, indicating that the proposed DMI-NLVASFS

Table 1
Recoveries of PP, CP and ZnPP in 8 μL spiked erythrocytes by the proposed DMI-NLVASFS method.

Sample	Added (nmol L^{-1})			Found[a] (nmol L^{-1})			Recovery (%)		
	PP[b]	CP[b]	ZnPP[b]	PP[b]	CP[b]	ZnPP[b]	PP[b]	CP[b]	ZnPP[b]
1	6.6	2.7	6.4	4.8	2.2	6.7	73	81	105
2	13.2	5.5	12.8	11.5	4.9	10.9	87	89	85
3	16.5	2.7	25.6	15.3	2.1	24.0	93	78	94
4	31.3	3.6	9.2	23.6	3.2	6.8	75	89	74
5	53.7	8.4	15.8	52.0	7.7	11.6	97	92	73

[a] The amount found refers to the net found that has been subtracted the amount in the erythrocytes.
[b] PP, protoporphyrin; CP, coproporphyrin; ZnPP, zinc protoporphyrin.

Table 2
Concentrations of total porphyrins determined by Piomelli's method and the proposed DMI-NLVASFS method for 8 μL erythrocytes from 20 healthy volunteers and 2 EPP patients.[a]

Sample source	Sample number	DMI-NLAVSFS[b] (μmol L^{-1})			Piomelli (μmol L^{-1})
		PP[c]	ZnPP[c]	Total	Total
Healthy volunteers	1	ND[d]	0.258	0.258	0.317
	2	ND	0.231	0.231	0.240
	3	0.063	0.419	0.482	0.452
	4	0.053	0.179	0.232	0.288
	5	0.081	0.248	0.329	0.284
	6	ND	0.276	0.276	0.293
	7	ND	0.323	0.323	0.250
	8	0.095	0.213	0.308	0.250
	9	ND	0.210	0.210	0.165
	10	ND	0.188	0.188	0.178
	11	ND	0.227	0.227	0.225
	12	ND	0.201	0.201	0.207
	13	ND	0.265	0.265	0.300
	14	ND	0.192	0.192	0.170
	15	ND	0.224	0.224	0.240
	16	ND	0.283	0.283	0.314
	17	ND	0.212	0.212	0.219
	18	ND	0.147	0.147	0.167
	19	ND	0.279	0.279	0.217
	20	ND	0.278	0.278	0.289
EPP[e] patients	1	26.0	0.842	26.8	24.2
	2	39.6	0.491	40.1	38.5

[a] The CP level of both healthy volunteers and EPP patients was not detectable, thus not listed in the table.
[b] DMI-NLVASFS: derivative matrix isopotential-nonlinear variable-angle synchronous fluorescence spectrometry.
[c] PP, protoporphyrin; ZnPP, zinc protoporphyrin.
[d] Not detectable.
[e] Erythropoietic protoporphyria.

method can be used to determine PP, CP and ZnPP simultaneously in erythrocytes of patients.

4. Conclusion

A novel, simple, rapid and selective method based on hyphenated synchronous fluorescence techniques, DMI-NLVASFS, was developed for the simultaneous determination of three diagnostically important porphyrins, protoporphyrin IX (PP), coproporphyrin III (CP) and zinc protoporphyrin IX (ZnPP), in human erythrocytes. Compared with conventional synchronous fluorescence spectrometry, this method exhibits more flexibility and better selectivity. PP, CP and ZnPP were determined simultaneously and rapidly by the proposed DMI-NLVASFS method in a single scanning within 30 s. This method was used to determine these porphyrins in erythrocytes of patient and the PP level of the two patients was significantly higher than that of healthy volunteers. The coincident results between the proposed DMI-NLVASFS method and Piomelli's method indicate that the proposed DMI-NLVASFS method is applicable to determine PP, CP and ZnPP simultaneously. This method provides a potential tool for the clinical analysis of diagnostically important porphyrins in human erythrocytes and the differential diagnosis of porphyrias, especially for the rapid routine screening of large number of samples. The experimental results also indicate that the successful combination of different synchronous fluorescence techniques could provide powerful and flexible approaches for the analysis of complex samples.

Acknowledgements

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