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Rapid simultaneous determination of protoporphyrin IX, uroporphyrin III and coproporphyrin III in human whole blood by non-linear variable-angle synchronous fluorescence technique coupled with partial least squares

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ABSTRACT

Fluorescence spectroscopy provides high sensitivity in quantitative analysis. However, due to spectral interference, it is difficult to determine the individual components of fluorescent multi-component mixtures in such complicated and important body matrices as blood, urine and feces without any pre-separation. In this study, a simple and rapid approach based on non-linear variable-angle synchronous fluorescence spectrometry coupled with partial least squares analysis (NLVASF/PLS) was developed for the simultaneous determination of protoporphyrin IX (PP), uroporphyrin III (UP) and coproporphyrin III (CP). The detection limits were 0.18, 0.29 and 0.24 nmol L^{-1} for protoporphyrin IX (PP), uroporphyrin III (UP) and coproporphyrin III (CP), respectively. The individual components of blood porphyrins were quantified, by this method, simultaneously in one scan with only about 30 s. The recoveries of this method were above 80% in human whole blood samples. This method provided a potential tool for the determination of porphyrins in whole blood and the differential diagnosis of porphyria, especially for rapid routine screening of large number of samples.

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1. Introduction

Biologically, porphyrins are a class of important molecules. Protoporphyrin IX (PP) and zinc protoporphyrin IX (ZnPP) are trace in the blood of normal human. The deficiency of the enzyme that is required in any step of heme biosynthetic pathway leads to the excess excretion and accumulation of certain porphyrins in the blood, liver and excreta. In the case of erythropoietic protoporphyria (EPP), the level of PP increases greatly [1]; in porphyria cutanea tarda (PCT), uroporphyrin (UP) is produced excessively [1,2]; similarly, in hereditary coproporphyria (HCP), the blood level of coproporphyrin (CP) increases as well [1]. Hence, the species and concentrations of porphyrins in blood as biomarkers are important in the clinical screening and diagnosis of porphyrias.

Porphyrins have strong absorption in the Soret band, and most of them fluoresce after being excited. Many methods have been established for determining the porphyrins in the whole blood, erythrocyte and plasma [3–10]. Mingioli [3] introduced new correction factors to emend the interference that are associated with the spectrophotometric determination of porphyrins in blood. The employing of mathematical calculation in this method is inconvenient for routine measurement. Adjarov and Kerimova [4] had developed a fluorimetric method to measure UP in plasma treated with trichloroacetic acid. However, it is not suitable for the determination of other porphyrins existed in plasma. Many porphyrins have been quantified simultaneously with high performance liquid chromatographic (HPLC) techniques [5–8]. Marton and co-workers [5] used it to quantify zinc protoporphyrin IX, protoporphyrin IX, and coproporphyrin in whole blood. Besides, HPLC coupled with mass spectrometric method (HPLC/MS) was also used for the detection of porphyrins in blood [9,10]. However, these methods were time-consuming and required complicated pretreatment procedures.

Variable-angle synchronous fluorescence spectrometry (VASFS) has better selectivity and more flexibility than conventional fluorescence spectroscopy and constant-wavelength synchronous fluorescence spectrometry (CWSFS) [11]. Multi-component analysis can be better investigated by using this technique combining with a multivariate calibration method. Partial least squares (PLS) was one of the most useful calibration methods with the characteristics of resolving multicollinearity problems well [12–18]. The combination of PLS with CWSFS has been proved successful in multi-component analysis [14–17]. However, the application of the coupled technique of PLS with variable-angle synchronous fluorescence approach [12,13], especially non-linear variable-angle synchronous fluorescence (NLVASF) approach [18] was rare. Berzas Nevado et al. [18] combined partial least squares with non-linear variable-angle synchronous fluorimetry firstly for the simultaneous

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determination of spectrally overlapped pyridoxal, pyridoxamine and pyridoxic acid. It is an attractive method for multi-component analysis, but not yet been widely used.

To our knowledge, the simultaneous quantitative measurement of PP, UP and CP with fluorimetric technique avoiding chromatographic separation has not yet been reported [6,19]. In this study, we developed a new and rapid method using the non-linear variable-angle synchronous fluorescence technique combined with partial least squares for the simultaneous analysis of PP, UP and CP in human whole blood samples.

2. Experimental

2.1. Instrumentation

All the fluorescence spectra were scanned on a laboratory-constructed, computer-controlled MYF spectrofluorimeter which is similar to that described previously [20]. It is equipped with 150 W xenon arc lamp (OSRAM GmbH, Steinerne Furt 6286167 Augsburg, Germany) and tunable slit band passes. The excitation and emission monochromators are 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 path by means of this program. The NLVASF spectra were recorded with the selected scanning route. Data manipulation and statistical treatment were performed with Unscrambler software Version 9.7 (www.camo.com). Spectrophotometer was from Beckman Coulter, Inc. (DU-7400, Beckman, USA). A 1 cm × 1 cm quartz cuvette was used for measurements throughout the study. The desktop centrifuge was purchased from Wuxi Ruijiang Analysis Instruments Co., Ltd. (Wuxi, Jiangsu Province, China). The vortex mixer was from Kylin-Bell Lab Instruments Co., Ltd. (Haimen, Jiangsu Province, China).

2.2. Reagents

Protoporphyrin IX disodium salt was purchased from Aldrich Chem. Co., uroprophyrin III octaethyl ester and coproporphyrin III were from Strem Chemicals. Ethyl acetate, acetic acid and hydrochloric acid were from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and all were analytical grade reagents.

The standard stock solutions of PP and CP were prepared using the similar procedures. About 1 mg of PP or CP was dissolved in a small volume of HCl (about 0.5 mL, 2.0 mol L^{-1}). The resulted porphyrin solutions were transferred into a 50 mL volumetric flask, brought to the mark with 2.0 mol L^{-1} HCl and mixed well. The concentrations of PP and CP standard stock solutions were determined spectrophotometrically: 0.050 mL and 0.100 mL stock solutions of PP and CP were diluted to 3.00 mL with 1.5 and 0.1 mol L^{-1} HCl, respectively. The absorption was measured on Beckman DU-7400 spectrophotometer (For PP, $\varepsilon_{408\,nm} = 3.1 \times 10^5\ dm^3\ mol^{-1}\ cm^{-1}$, for CP, $\varepsilon_{399.5\,nm} = 4.89 \times 10^5\ dm^3\ mol^{-1}\ cm^{-1}$) [21]. Uroprophyrin octaethyl ester was hydrolyzed in 6.8 mol L^{-1} HCl in the dark for 24 h, and was prepared to standard stock solution with 2.0 mol L^{-1} HCl as PP and CP. The concentration of the standard stock solution of UP were also determined spectrophotometrically: an aliquot of 0.100 mL stock solution was diluted to 3.00 mL with 0.5 mol L^{-1} HCl ($\varepsilon_{405.5\,nm} = 5.28 \times 10^5\ dm^3\ mol^{-1}\ cm^{-1}$) [21]. The standard solution containers were protected from light by wrapping them with aluminum foil and stored at −20 °C.

2.3. Sample preparation

Human blood samples were collected into glass tubes containing Na_2EDTA as anticoagulant by coworkers from the hospital of Xiamen University (Xiamen, China). These samples were stable for at least 20 days at 4 °C. After mixed well, 100 μL of blood was pipetted into a disposable plastic stoppered centrifugal tube, then 2.0 mL of ethyl acetate/acetic acid (v/v = 4:1) mixed solvent was added [22], and then vortex-mixed for about 10 s. After that, 1.5 mL 1.0 mol L^{-1} HCl was added. The mixture was centrifuged at 8000 rpm for 1 min. Then the supernatant was collected for measurement. After extraction and centrifugation, the sample was cleared and got rid of most of the matrix of the whole blood probably interferring the detection of porphyrins.

2.4. Determination procedure

In order to determine porphyrins simultaneously, it is important to establish suitable PLS1 models for NLVASF spectra. The first step is to choose a proper scanning path for non-linear variable-angle synchronous fluorescence spectroscopy. Owing to the flexibility of NLVASF, it is possible to choose a scanning path with highest signal as well as good selection. The second step is to choose a proper training set and get their NLVASF spectra. The concentration data of the training set and their NLVASF spectra were directly imported to Unscrambler software 9.7 to establish PLS1 models for PP, UP and CP, respectively. It can offer parameters to evaluate and adjust the models, such as factors, root mean square difference (RMSD), the first weight loading vector (w1) and the regression coefficients (b1). Then, the PLS1 models for them can be established by adjusting the concentration range, factors and wavelength range. Finally, the models were tested by the determination of the synthetic samples to evaluate the prediction ability.

Once the models for PP, UP and CP were established, the blood samples could be analyzed easily. Prepare the blood samples as described in Section 2.3 and get their NLVASF spectra in the same experimental condition with the training set. Next, the spectra were imported into the PLS1 models. The concentrations of each porphyrins in blood sample were then predicted by the models.

3. Results and discussion

PP, UP and CP are structurally similar and their spectra are severely overlapped. Fig. 1 shows their excitation and emission spectra, which are almost superposed on each others, so the simultaneous determination of the individual components of their ternary mixture is impossible while using conventional fluorimetric method or synchronous fluorimetry. Thus, a non-linear variable-angle synchronous technique combined with partial least squares was investigated.

3.1. Selection of the optimal non-linear variable-angle synchronous scanning path

In order to establish a suitable NLVASF method for the simultaneous determination of PP, UP and CP, it is important to select an optimal scanning path. The goal is to acquire the optimum non-linear variable-angle synchronous scanning spectrum with highest possible signal values [23]. Under the experimental conditions, the analytes exhibit their highest fluorescence intensities at the following wavelength pairs: PP, $\lambda_{ex} = 409$ and $\lambda_{em} = 606$ nm; UP, $\lambda_{ex} = 406.1$ and $\lambda_{em} = 598.3$ nm; CP, $\lambda_{ex} = 401.3$ and $\lambda_{em} = 595.8$ nm. Besides, other two points ($\lambda_{ex} = 370$ and $\lambda_{em} = 595.7$ nm; $\lambda_{ex} = 430$ and $\lambda_{em} = 609.8$ nm) were selected to form a complete scanning path. A trajectory through these wavelengths was projected with

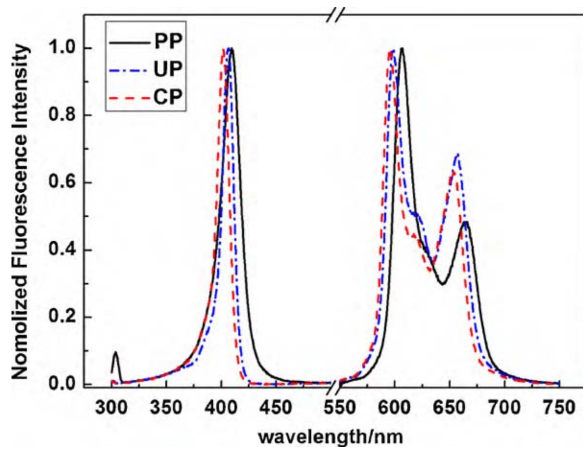

Fig. 1. Normalized excitation spectra (left) and emission spectra (right) of PP (solid line), UP (dash dot line) and CP (dash line). The maximum emission wavelengths of their excitation spectra are 606, 598.3, and 595.8 nm, respectively, and the maximum excitation wavelengths of their emission spectra are 409, 406.1 and 401.3 nm, respectively.

the aid of a sigmoidal fitting in the Origin 7.0 software, according to the follow algebraic equation.

$$\lambda_{em} = A_1 + \frac{A_2 - A_1}{1 + 10^{(\log \lambda_{ex0} - \log \lambda_{ex})P}} \quad (1)$$

After fitting, the mathematical function was obtained.

$$\lambda_{em} = 595.7 + \frac{14.08}{1 + 10^{0.373*(407.8 - \lambda_{ex})}} \quad (2)$$

This trajectory was the scanning path used in this study and was represented in the excitation–emission wavelengths plane in Fig. 2. The maximal point of each porphyrin was included in the path.

Using the above selected trajectory, NLVASF spectra of porphyrins was recorded (Fig. 3). The three compounds were located at intermediate points along this trajectory. On the one hand, the highest peak of each porphyrin could be acquired in only one scan with best sensitivity. On the other hand, the spectrum was simplified. Nevertheless, the spectra of the individual components are still overlapped. Thus, the multivariate algorithm was applied to resolve the data. Due to the distinct advantage of figuring out multicollinearity, PLS1 was used to solve the spectral overlapping problem [24].

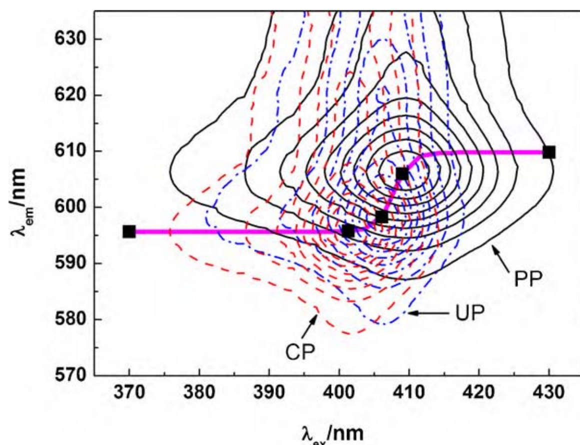

Fig. 2. Contour map of 20 nmol L^{-1} PP (thin solid line), 25 nmol L^{-1} UP (dash dot line), 10 nmol L^{-1} CP (dash line) and the NLVASF scanning path (thick solid line).

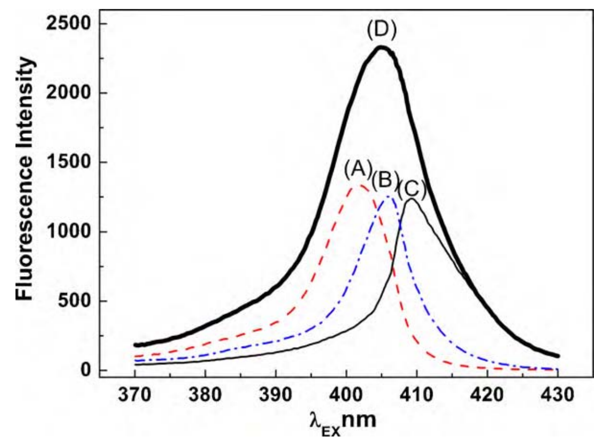

Fig. 3. NLVASF spectra of (A), CP (50 nmol L^{-1}, dash line); (B), UP (70 nmol L^{-1}, dash dot line); (C), PP (60 nmol L^{-1}, solid line) and (D), the mixture with the same concentration as before (solid line).

3.2. Series of standard samples and validation

According to the low level of porphyrins in blood, the concentrations of PP, UP and CP were selected with the concentration ranges of 2–80, 1–28 and 0.5–24 nmol L^{-1}, respectively. Considering the number and superposition of analyzed mixture, a large number of calibration samples were used to build the model by the PLS1 algorithm. According to Thomas and Haaland's method [14,25], a calibration set of 36 samples was applied. The compositions were listed in Table 1 and worked as Y variables of PLS1 model.

Table 1
Composition of training set samples (nmol L^{-1}).

Samples	C_{PP}	C_{UP}	C_{CP}
1	2	1	0.5
2	40	14	12
3	80	28	24
4	10	28	15
5	10	16	24
6	50	4	15
7	50	28	3
8	80	4	24
9	80	16	3
10	30	12	23.5
11	78	12	9
12	30	27	9
13	2	19	18
14	60	1	18
15	60	19	0.5
16	40	8	21
17	40	23	6
18	70	8	12
19	20	14	21
20	20	23	12
21	70	14	6
22	50	12	0
23	30	0	15
24	0	16	9
25	78	1	0
26	2	0	23.5
27	0	27	0.5
28	2	0	0
29	0	1	0
30	0	0	0.5
31	40	0	0
32	0	14	0
33	0	0	12
34	80	0	0
35	0	28	0
36	0	0	24

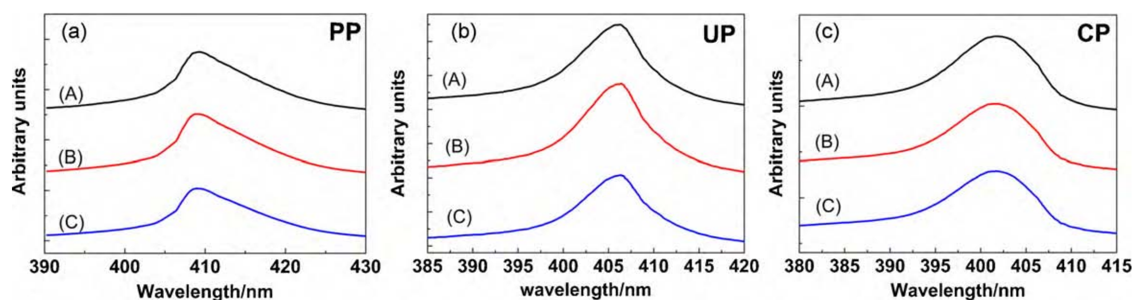

Fig. 4. Analysis of PP, UP and CP simulated data without noise: (A) pure-component spectrum, (B) first PLS1 weight loading vector, w1 and (C) PLS1 vector of regression coefficients, b1.

Table 2
Models parameters using the PLS algorithm.

Analysis	Factors	RMSD	R^2	Wavelength (nm)
PP	3	2.31	0.994	390–430
UP	3	1.08	0.988	385–420
CP	3	0.84	0.991	380–415

In order to determine the optimal factors to avoid the over fitting in the model, and to study the models prediction ability with the various calibration data, the full cross-validation method was applied and the correlation between prediction residual error sum of squares and factors were calculated. The internal cross-validation of the proposed calibration models was performed, and some parameters for the models in this study are shown in Table 2. A total of 251 signal values distributed within the intervals of 390–430 nm for PP, 226 signal values distributed within the intervals of 385–420 nm for UP and 210 signal values distributed within the intervals of 380–415 nm for CP were selected as X variables of PLS1 models, respectively. The first weight loading vector (w1) and the regression coefficients (b1) could be useful in making assignments of spectral bands [26]. It is clear that pure-component spectra, the first weight loading vector and the regression coefficients were very similar (Fig. 4). The consistency of the curves in Fig. 4 indicated that the established models were fit well, and the first weigh loading vector (w1) and the regression coefficient (b1) can be used to assign the spectral bands.

In order to test the prediction ability of the proposed models, four synthetic samples were prepared and their compositions were given in Table 3. The NLVASF prediction data for each sample was calculated by using PLS1 models. The LOD was estimated by the method proposed in reported literature [27,28], through scanning 13 blank samples. The calculated value of LOD for PP, UP and CP are 0.18, 0.29 and 0.24 nmol L^{-1}, respectively.

3.3. Analysis of blood samples

In order to test the validity of the proposed models, the simultaneous determination of the three porphyrins was carried out under the experimental conditions in 15 random blood samples. The results were shown in Table 4. The data showed that PP was found in blood, UP and CP was hardly measurable. In this measurement zinc protoporphyrin was changed into protoporphyrin owing to acidic solvent. The recoveries of four blood samples were studied (Table 5). The recoveries were between 80% and 100% and satisfied the requirement for quantitative analysis. This method can be used to analysis the individual porphyrin components in human whole blood simultaneously. Five identically spiked blood samples were analyzed and obtained relative standard deviations for PP, UP and CP were 4.6%, 4.3% and 4.8%, respectively.

Table 3
Composition and recovery of synthetic mixtures (nmol L^{-1}).

Samples	Added (nmol L^{-1})			Predicted (nmol L^{-1})			Recovery (%)		
	PP	UP	CP	PP	UP	CP	PP	UP	CP
1	15	15	6.5	14	16	6.5	93	107	100
2	55	5	25	53	4.7	21	96	94	84
3	2.5	25	13	2.8	25	13	112	100	100
4	25	5	15	24	5	4	95	100	91

Table 4
Composition of porphyrins in 100 μL blood samples (nmol L^{-1}).

Sample	PLS1 predicted value		
	PP	UP	CP
1	6.1	0.05	−0.17
2	6.0	−0.71	0.37
3	6.1	−0.27	0.03
4	7.0	−0.37	0.09
5	7.2	−0.37	0.12
6	8.0	−0.18	−0.17
7	7.2	−0.10	−0.08
8	5.9	−0.05	0.09
9	7.4	−0.56	0.17
10	6.4	−0.40	0.05
11	8.0	−0.16	−0.04
12	5.2	−0.01	−0.13
13	7.0	−0.26	−0.03
14	8.6	−0.74	0.48
15	6.3	−0.02	−0.09

Table 5
Recoveries in blood samples.

Samples	Added (nmol L^{-1})			Predicted (nmol L^{-1})			Recovery (%)		
	PP	UP	CP	PP	UP	CP	PP	UP	CP
1	50	–	10	44.3	0.4	9.3	88.6	–	93
2	12.5	10	10	11.2	8.7	8.5	89.6	87	85
3	20	3.5	5	17.5	3.2	4.4	87.5	91.4	88
4	30	15	10	24.1	13.6	9.3	80.3	90.5	93

4. Conclusions

A simple and rapid non-linear variable-angle synchronous technique combined with partial least squares (NLVASF/PLS) was applied to the simultaneous determination of protoporphyrin IX, uroporphyrin III and coproporphyrin III in human whole blood. The results indicated that this method could resolve the spectral overlapping problem well and yield favorable results after establishing good models with simple sample pretreatment and that the highest peak of each porphyrin can be acquired in a single scan using NLVASF with maximum sensitivity. This method provided a potential tool for the determination of porphyrins in whole blood and the differential diagnosis of porphyria, especially for rapid routine screening of large number of samples.

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