A Cloud Point Extraction-Spectrofluorimetric Method for Determination of Thiamine in Urine

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A simple and efficient cloud point extraction-spectrofluorimetric method for the determination of thiamine in human urine is proposed. The procedure is based on the oxidation of thiamine with ferricyanide to form thiochrome, its extraction to Triton X-114 micelles and spectrofluorimetric determination. The variables affecting oxidation of thiamine, extraction and phase separation were studied and optimized. Under the experimental conditions used, the calibration graphs were linear over the range 2.5-1000 ng mL^{-1}. The limit of detection was 0.78 ng mL^{-1} of thiamine and the relative standard deviation for 5 replicate determinations of thiamine at 400 ng mL^{-1} concentration level was 2.42%. Average recoveries between 93-107% were obtained for spiked samples. The proposed method was applied to the determination of thiamine in human urine.

Key Words : Thiamine, Cloud point extraction, Spectrofluorimetry, Urine

Introduction

Thiamine (vitamin B1), a water-soluble vitamin, is a biologically and pharmaceutically important compound. It is necessary for carbohydrate metabolism, the maintenance of normal neural activity, prevention and also treatment of beriberi disease.[1] The daily recommended intake of thiamine is generally 8-15 mg, although such an amount may easily exceeded by oral consumption. When the intake is lower than the minimum requirement thiamine is not excreted in urine, while any excess appears in the urine as thiamine or pyrimidine when the intake is higher.[2] Thiamine is found as its free form (T), as well as various phosphorylated derivatives such as thiamine monophosphate (TMP), thiamine diphosphate (TDP) and thiamine triphosphate (TTP). Dietary thiamine is extensively converted to free T in digestive tract and absorbed.[3] Erythrocytes transport small amounts of T and TMP and 80% TDP present in blood, while T and TMP are the main thiamine content of plasma. In urine, T can be detected, as well as minor molecules derived from thiamine degradation.[4] The quantification of total thiamine is normally advised in whole blood, serum and erythrocytes. Though, whole blood is the preferred specimen for thiamine assessment, plasma and urine can also be useful for evaluating nutritional status of thiamine or diagnostic purposes.[5]

Several analytical techniques have been used for determination of thiamine. The most common procedure for thiamine determination is the alkaline oxidation of thiamine and its phosphate esters to thiochrome, a highly fluorescent compound. Generally its formation and detection is associated with a chromatographic separation.[4,5] High performance liquid chromatography (HPLC)[2,4-7] and capillary electrophoresis[8] were usually developed to determine the thiamine in complicated samples. Spectrofluorimetry[9,10] and spectrophotometry[11] can be used for analysis of thiamine in relatively simple solutions or pharmaceuticals. Chemiluminescence,[1,12] electrochemical[13,14] and turbidimetric[15] methods have also been proposed for determination of thiamine.

Applications of HPLC, the most widely used method, for the determination of thiamine and its esters in biological fluids and foods, have been reviewed.[16] But, due to matrix effects, sample pretreatment and clean-up steps such as liquid-liquid extraction (LLE) (or solid phase extraction) is usually necessary. This process not only involves the use of organic solvents which are toxic and expensive but also requires a long sample preparation time.

The use of extraction steps based on phase separation by the cloud point extraction (CPE) methodology offers a convenient alternative to more conventional extraction systems. The principles, advantages and applications of CPE have been well-established and identified in recent years and comprehensive reviews of the theory and applications of surfactant-mediated separations in analytical chemistry are available in the literature.[17-19] The CPE methodology has been applied to the extraction and preconcentration of a wide range of organic compounds and metallic ions in different samples,[20-26] but little attention has been paid to the extraction of pharmaceuticals from biological fluids and to the best of our knowledge, only two reports were presented so far.[27,28]

In the present work, we report on the results obtained from applying the CPE-spectrofluorimetric method for the determination of thiamine in urine. The method is based on chemical oxidation of thiamine in aqueous or urine sample to thiochrome by using ferricyanide, extraction of thiochrome to non-ionic micelles and its determination by spectrofluorimetry. This technique permits analysis and quantification of the thiamine in urine samples with simple spectrofluorimetric method instead of time-consuming and tedious HPLC method and without further sample clean-up steps. Thus, time and cost of the analysis can be significantly

decreased in addition to other well-known advantages of CPE methodology.

Experimental Section

Instrumentation. All fluorescence measurements were made using a Shimadzu RF-5301 PC spectrofluorophotometer equipped with a 150 W Xenon lamp and 1.00 cm quartz cells. Instrument excitation and emission slits both were adjusted to 5 nm. A thermostated bath (636 Friedberg/Hessen, Germany), was used for CPE experiments. A Hettich centrifuge (EBA 20) with 25 mL calibrated centrifuge tubes was used to accelerate the phase separation process. A Corning M120 pH-meter was used for pH measurements.

Reagents. The non-ionic surfactant Triton X-114 (Sigma, St. Louis, MO, USA) was used without further purification. An aqueous solution of 1% (v/v) Triton X-114 was prepared by dissolving 0.5 mL of this surfactant in doubly-distilled water and diluted up to 50 mL in a volumetric flask. Stock standard solution of thiamine at a concentration of 500 μg mL^{-1} was prepared from thiamine hydrochloride (E. Merck) and was kept at 4 °C for one week. Working standard solutions were obtained by appropriate dilution of this stock standard solution.

A 0.01 mol L^{-1} solution of ferricyanide was prepared by dissolving appropriate amount of potassium hexacyanoferrate (III) (E. Merck) in distilled water and diluting to 25 mL with double distilled water. A 1.0 mol L^{-1} NaOH solution was also prepared.

All other reagents were of analytical-reagent grade (E. Merck) and all solutions were prepared using doubly-distilled water.

Recommended procedure for calibration. Aliquots of 25 mL solution containing thiamine in the range of 2.5-1000 ng mL^{-1}, 1.25 mL ferricyanide solution (0.01 mol L^{-1}), 2.5 mL NaOH solution (1.0 mol L^{-1}) and 2.5 mL Triton X-114 solution (1.0% v/v) were kept in a controlled temperature bath for 10 min at 40 °C. Separation of the two phases was achieved by centrifugation for 5 min at 3800 rpm. After cooling in an ice bath for 5 min, the surfactant-rich (SR) phase became viscous, and the supernatant aqueous phase was easily decanted. The SR phase was diluted to 2.5 mL with ethanol-water (1 : 1 v/v) and the fluorescence intensity was measured at 438 nm with the excitation wavelength set at 368 nm.

Procedure for the urine samples. Urine samples were obtained from an apparently healthy male volunteer who took single oral dose of 100 mg thiamine tablet (Daru Pakhsh, Tehran, Iran) in the morning and before the breakfast. Urine samples were collected for 10 h after administration of thiamine and the urinary volumes were recorded. Each urine sample was stored at 4 °C. Aliquots of these samples were centrifuged at 4000 rpm for 7 min and 0.2 mL portions of upper clear solution were subjected to the CPE and thiamine determination by spectrofluorimetry as described above.

Also, drug-free urine samples obtained from healthy volunteer were used for recovery experiments. For this purpose, aliquot of 0.2 mL of urine sample was spiked with thiamine at concentrations of 50, 200 and 400 ng mL^{-1} and subjected to the CPE and spectrofluorimetric determination.

Results and Discussion

Thiamine reacts with ferricyanide in alkaline medium and forms thiochrome, which is subsequently trapped in the surfactant micelles and separated from the aqueous phase or biological matrix. The excitation and emission spectra of thiamine as thiochrome in micellar media are given in Figure 1 with maxima at 368 ± 3 and 438 ± 3 nm, respectively. CPE conditions were studied and suitably adjusted as follows:

The concentration of oxidizing agent. Different oxidizing agents such as $Fe(CN)_6^{3-}$, Hg^{2+}, Cu^{2+}, potassium permanganate, hydrogen peroxide etc. have been proposed for thiamine oxidation in the literature.[9,10] The effect of some of these reagents such as $Fe(CN)_6^{3-}$, Hg^{2+} and Cu^{2+} on thiamine oxidation was examined. The results showed that when Hg^{2+} was used as oxidizing agent, the maximum yield of thiochrome and therefore, the highest fluorescence intensities were obtained in aqueous solution. However, for urine sample, $Fe(CN)_6^{3-}$ gave higher fluorescence intensities as compared with others using the same concentration. In addition, $Fe(CN)_6^{3-}$ is safer than Hg^{2+} from practical point of view. Therefore, ferricyanide was selected as the oxidizing agent for thiamine. The effect of ferricyanide concentration on the fluorescence intensities was studied and results were shown in the Figure 2. It was found that 0.2-2.5 × 10^{-4} mol L^{-1} from ferricyanide led to the constant and maximum signals. At concentrations lower than this range, the fluorescence intensity decreased due to insufficient concentration of thiamine for oxidation, whereas higher amounts of ferricyanide can probably quench the fluorescence and decrease

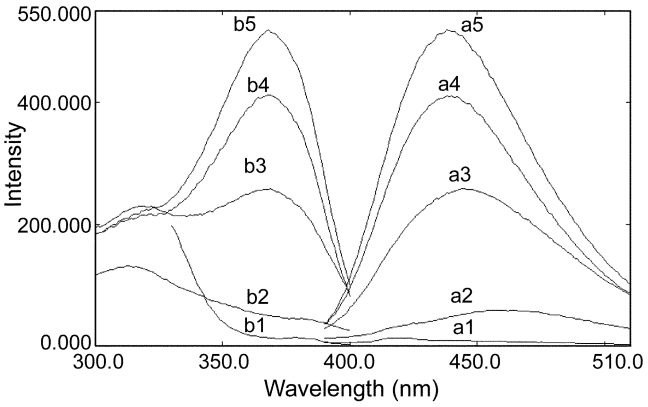

Figure 1. Emission and excitation spectra: a1 & b1 Emission and excitation of blank; a2 & b2: Emission and excitation of urine blank; a3 & b3: Emission and excitation of thiochrome after administration of thiamine tablet; a4 & b4: Emission and excitation of thiochrome when 400 ng mL^{-1} thiamine spiked to the urine; a5 & b5: Emission and excitation of thiamine standard solution (500 ng mL^{-1}) as thiochrome; 5.0 × 10^{-4} mol L^{-1} $Fe(CN)_6^{3-}$; 0.1 mol L^{-1} OH^-, 0.1% (v/v) Triton X-114.

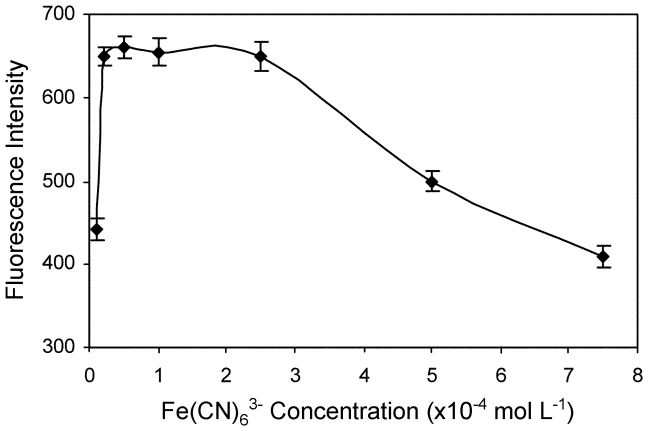

Figure 2. Effect of $Fe(CN)_6^{3-}$ concentration on the spectrofluorimetric responses: 500 ng mL^{-1} thiamine, 0.1 mol L^{-1} OH^-, 0.1% (v/v) Triton X-114.

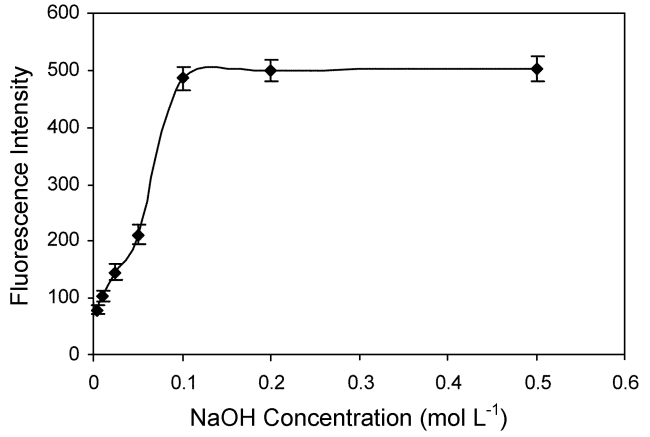

Figure 3. Effect of OH^- concentration on the spectrofluorimetric responses: 500 ng mL^{-1} thiamine, 5.0×10^{-4} mol L^{-1} $Fe(CN)_6^{3-}$, 0.1% (v/v) Triton X-114.

the fluorescence intensity. The quenching effect of ferricyanide has been reported by others.[29]

On the other hand, results of preliminary experiments showed that ferricyanide concentrations more than 5.0×10^{-4} mol L^{-1} were adequate for efficient oxidation of thiamine in urine and achievement of higher fluorescence intensities. Therefore, this concentration (5.0×10^{-4} mol L^{-1}) was used in the rest of work. Although, the sensitivity in this concentration is less than that of optimum concentration range.

The concentration of NaOH. The Oxidation yield of thiamine depends on the pH and the time taken to reach equilibrium which decreases with increasing OH^- concentration. The influence of OH^- concentration on the fluorescence intensity was studied. As shown in Figure 3 the fluorescence rapidly increased up to 0.1 mol L^{-1} from NaOH and then remained constant. Hence, this concentration (2.5 mL from 1.0 mol L^{-1} NaOH) was chosen for further experiments. A comparison between OH^- and different buffer systems on analytical signals was performed. For this purpose, pH of a series of solutions containing certain amount

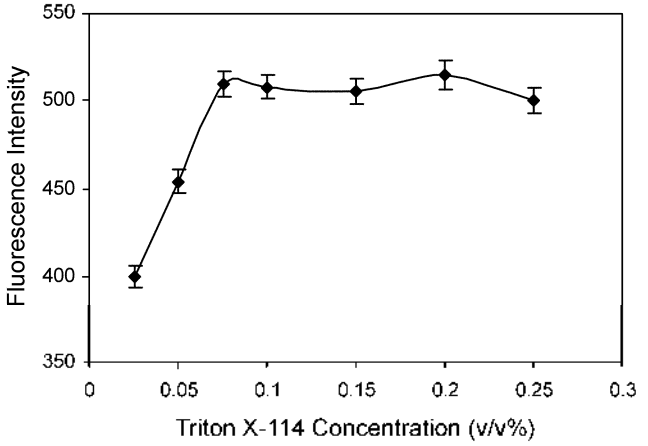

Figure 4. Effect of Triton X-114 concentration on the spectrofluorimetric responses: 500 ng mL^{-1} thiamine, 5.0×10^{-4} mol L^{-1} $Fe(CN)_6^{3-}$; 0.1 mol L^{-1} OH^-.

of thiamine and other reagents was adjusted in 12.0 by using OH^- or different buffer systems (such as ammonia, phosphate or borate) and solutions were subjected to the CPE and spectrofluorimetric determination. The results showed that higher analytical signals were obtained by using OH^- as buffering agent. Therefore, the pH adjustment was not performed by using buffer systems and addition of proper amount of OH^- is sufficient for adjustment of pH and achievement of higher sensitivity.

The concentration of Triton X-114. Figure 4 shows the effect of concentration of Triton X-114 on the analytical signals. The fluorescence intensity increased with increasing the concentration of the surfactant and reached to a maximum value at 0.075% v/v of the surfactant and then remained approximately constant. A 0.1% v/v surfactant concentration was chosen for other experiments.

Effects of other experimental factors. Optimal incubation time and equilibration temperature are necessary to complete extraction and to achieve easy phase separation. The temperature at which the sample solution is heated was examined at 30-60 °C and an optimum of 40 °C was selected. The dependence of extraction efficiency upon equilibration time was studied within a range of 10-60 min. An equilibration time of 10 min was chosen as the best. The effect of the centrifugation time on extraction efficiency was the other parameter that was studied within a range of 5-25 min. A centrifuge time of 5 min at 3800 rpm was selected for the entire procedure. Also, the effect of ethanol-water ratio as diluting agent was studied and the results showed that the ratios above 1 : 1 (v/v) ratio resulted to higher analytical signals.

Analytical performance. Calibration graphs were obtained by CPE of 25 mL of standard solutions containing known amounts of the thiamine in the presence of 0.1% (v/v) Triton X-114 and under the experimental conditions explained in the procedure. The SR phase (≈ 0.2 mL) was diluted to 2.5 mL with ethanol-water (1 : 1 v/v) and the fluorescence was measured. In all cases, linear relationships between the

Table 1. Results of recoveries of spiked samples

Sample	Thiamine added (ng mL^{-1})	Thiamine found* (ng mL^{-1})	Recovery (%)
Urine			
	50	46.7 ± 1.12	93.4
		47.5 ± 1.11	95.0
		47.2 ± 1.13	94.4
	200	210.0 ± 4.72	105.0
		213.4 ± 4.95	106.7
		204.4 ± 4.55	102.2
	400	392.0 ± 9.41	98.0
		400.8 ± 9.62	100.2
		406.8 ± 9.76	101.7

*Average of three determinations ± standard deviation. Biological samples were obtained from one individual and three aliquots of them were analyzed.

fluorescence intensity and the concentration of the thiamine in the solution were obtained. The linear concentration range of method was between 2.5-1000 ng mL^{-1} of thiamine which was wider than other sensitive methods.[2,4,5] The relative standard deviation (RSD) obtained for 5 replicate determinations of the same sample at 400 ng mL^{-1} level was 2.42%. The limit of detection (LOD) calculated as three times the standard deviation of the blank signals was 0.78 ng mL^{-1} which was comparable with other sensitive techniques.[2,4,5,8] This low detection limit allowed quantification of vitamin levels even in those patients with the lowest normal values (66-346 ng mL^{-1}).[4]

The validation and application of the method. Proposed method was applied to the determination of thiamine in urine samples. Accuracy of the proposed method was proven by spike-recovery test. Aliquots of 0.2 mL urine sample were spiked with three different concentrations of thiamine at 50, 200 and 400 ng mL^{-1} and recovery experiments were conducted for these samples as well. The results are summarized in Table 1 and calculated amounts of recoveries varied between 93-107%. These recoveries along with coincidence of excitation and emission spectra of spiked and administered thiamine to those of standard thiamine (see Figure 1), indicated that no significant matrix effect was observed in the proposed procedure.

For practical application of this method, urine samples were collected for 0 to 10 h after administration of a single oral dose of 100 mg thiamine and the urinary volumes were recorded. A 0.2 mL portion of each sample was used for thiamine determination and they were sometimes supplemented with thiamine to test the recovery of the method. The results of trial determinations were summarized in Table 2 which shows the excretion of the excess of thiamine in the body. A total thiamine excreted through urine was 9.4% of that taken in a total volume of 0.65 L urine. The thiamine concentration was increased sharply to reach its maximum within 4 to 6 h and then decreased within few hours as reported by others.[2,30]

Conclusion

A CPE-spectrofluorimetric method was proposed to quantification of thiamine as its oxidized fonn, thiochrome, and was applied to a real urine sample. There are some advantages of the method, including:

Table 2. Determination of excreted thiamine through urine

Time for urine sampling (h)	Volume of urine (mL)	Thiamine Added (μg mL^{-1})	Thiamine found* (μg mL^{-1})	Recovery	Thiamine in sample (μg mL^{-1})	The excreted thiamine (mg)
0.8	41	0	0.18 ± 0.004	–	2.25	0.09
		0.4	0.57 ± 0.013	98		
1.5	70	0	0.21 ± 0.005	–	2.62	0.18
		0.4	0.59 ± 0.014	95		
2.8	57	0	0.42 ± 0.006	–	5.25	0.30
		0.4	0.81 ± 0.012	98		
3.2	83	0	0.44 ± 0.007	–	5.50	0.46
		0.4	0.85 ± 0.013	102		
4.5	92	0	2.12 ± 0.032	–	26.50	2.44
		0.4	2.50 ± 0.037	95		
6	119	0	2.42 ± 0.036	–	30.25	3.60
		0.4	2.82 ± 0.042	100		
7.5	97	0	1.03 ± 0.015	–	12.87	1.25
		0.4	1.44 ± 0.019	102		
10	91	0	0.93 ± 0.013	–	11.62	1.06
		0.4	1.35 ± 0.018	105		

*Average of three determinations ± standard deviation. Biological samples were obtained from one individual and three aliquots of them were analyzed.

1. Compared with HPLC methods for determination of thiamine in biological fluids, which require a previous step of clean-up, the proposed procedure allows carrying out the analysis in a simple and quick way and without long and tedious clean-up steps.

2. The proposed method represents a promising approach in the area of pharmaceutical monitoring with low operation cost, simplicity of instrumentation and non-polluting respect.

3. The method was verified with real samples and applied to the determination of thimaine in urine.

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