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Flow injection on-line photochemical reaction coupled to spectrofluorimetry for the determination of thiamine in pharmaceuticals and serum

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The photochemical reaction of thiamine was studied with a photochemical reactor made by coiling a knotted PTFE reactor around a low-pressure mercury lamp. Acetone, which was previously reported to be a sensitizer for the photochemical reaction that took place *in situ* in a flow-through cell, severely depressed the fluorescence signal of the photochemical reaction that took place on-line in the knotted PTFE reactor when sodium sulfite was involved in the photochemical reaction. Experiments revealed that the effect of acetone on the photochemical reaction was dependent on the intensity of the irradiation that was used to induce the photochemical reaction, and that acetone might impair the photochemically induced fluorescence if strong UV irradiation was applied to induce the photochemical reaction and sodium sulfite was used to enhance the fluorescence signal. Based on these observations, a flow injection on-line photochemical–spectrofluorimetric method for the determination of thiamine was developed without using acetone. With the proposed method, a detection limit of 0.11 μg l^{-1} thiamine, a relative standard deviation of 0.36% for 11 determinations of 1 mg l^{-1} thiamine and a sampling frequency of 100 h^{-1} were achieved. The developed method was successfully applied to the determination of the thiamine content in various pharmaceutical preparations and serum.

Keywords: *Photochemical reaction; spectrofluorimetry; thiamine; pharmaceutical preparation; serum*

Thiamine (vitamin B_1) is an essential nutrient for humans to maintain normal neural activity and to prevent beriberi. People usually obtain the nutrient from natural and fortified foods. When needed, the vitamin can also be obtained from various pharmaceutical preparations containing thiamine.

Among the various methods[1] for the determination of thiamine, the most widely used is the so-called thiochrome method which was first reported by Jansen.[2] This approach involves oxidation of thiamine by hexacyanoferrate(III) in aqueous alkaline solution to the fluorescent thiochrome, extraction of the latter into an organic phase, and measurement of the fluorescence intensity of the organic extract. The extraction of thiochrome is necessary to separate it from hexacyanoferrate(III) which may quench the fluorescence of thiochrome. Apart from potassium hexacyanoferrate(III), other oxidizing agents such as cyanogen bromide[3,4] and mercury[5] have also been used to oxidize thiamine to fluorescent thiochrome. With the use of these two reagents, solvent extraction was avoided. However, the highly toxic Hg^{II} and cyanogen bromide would cause problems as regards environmental pollution.

The thiochrome fluorescence methods have been adapted to flow injection analysis (FIA). Karlberg and Thelander,[6] using potassium hexacyanoferrate(III) as oxidizing agent, developed an FI extraction approach for the determination of thiamine in various pharmaceutical preparations. Martinez-Lozano *et al.*[7] employed Hg^{II} for the FI spectrofluorimetric determination of thiamine in vitamin–mineral preparations. Moreover, an FI electrochemical oxidation system has been coupled to spectrofluorimetry for pharmaceutical analysis.[8]

Owing to their inherent advantages over ordinary chemical reactions, photochemical reactions have been widely used for post-column derivatization coupled to fluorimetric detection in high-performance liquid chromatography (HPLC).[9,10] In the last few years, this type of reaction has also been applied in FI systems coupled to spectrofluorimetry. Thus, FI on-line photochemical–spectrofluorimetric approaches for the determination of pharmaceuticals such as chlorpromazine,[11–14] promethazine,[11,12,15] perphenazine,[11] reserpine[16] and sulfamethazine[17] have been developed. Guo *et al.*[18] reported that thiamine could be converted into an intensely fluorescent compound in alkaline medium by a photochemical reaction and that the reaction was greatly sensitized by acetone. Based on this discovery, they developed an *in situ* photochemical–spectrofluorimetric method for the determination of thiamine in pharmaceutical preparations and serum. In their method, the test solution containing the analyte and acetone, after being merged on-line with a sodium hydroxide–sodium sulfite solution, was pumped into a flow-through cell and held there for irradiation at 280 nm so that the photochemical reaction took place in the flow-through cell (so-called *in situ* photochemical reaction). After 60 s, the fluorescence intensity of the photochemically induced fluorescent compound was measured at 440 nm (excitation at 370 nm). With this approach, the use of the toxic reagent potassium hexacyanoferrate(III) was avoided, and solvent extraction was no longer necessary, which is beneficial to both the operator and the environment. Nevertheless, acetone—an inflammable solvent with an annoying odor—was used in the method. Moreover, the excitation monochromator of the spectrofluorimeter had to be frequently switched back-and-forth during the measurement, causing excessive wearing of the mechanical system of the monochromator.

In this work, the photochemical reaction of thiamine was studied in the continuous-flow mode. The photochemical reaction took place on-line in a photochemical reactor made of knotted PTFE tubing, which was coiled around a low-pressure mercury lamp.[14] It was found that if sodium sulfite was present in the reagent solution, acetone significantly depressed, rather than enhanced, the fluorescence signals of the on-line photochemical reaction, and that the reaction without acetone was more sensitive than the acetone-sensitized *in situ* photochemical reaction. Based on this observation, an FI photochemical–spectrofluorimetric method for the determination of thiamine was developed. The method was successfully applied to the determination of thiamine in pharmaceutical preparations and serum.

Experimental

Apparatus

A Model RF-540 spectrofluorimeter (Shimadzu, Kyoto, Japan) equipped with a xenon lamp was used to measure fluorescence

intensity. A Model LZ 1010 peristaltic pump (Zhaofa Institute for Lab. Automation, Shenyang, China), a Rheodyne (Cotati, CA, USA) 5020 six-port PTFE injector with a 250 μl sampling loop, and a 120 μl rectangular flow-through cell (Shimadzu) were used to assemble the FIA manifold as illustrated in Fig. 1. A chart recorder (Dahua Instrument Co., Shanghai, China) was used to record the peak traces. The photochemical reactor was constructed by coiling a knotted reactor, which was fabricated in-house from 50 cm × 0.5 mm id PTFE tubing as described in ref. 19, around a low-pressure mercury lamp (od 1.5 cm, length 22 cm). PTFE tubing of 0.5 mm id was used for all connections, and PVC pump tubing of appropriate diameter was employed to propel the aqueous carrier, and also the reagent and sample solutions.

Reagents

A thiamine hydrochloride stock solution (1000 μg ml^{-1}) was prepared by dissolving the reagent (Sigma, St. Louis, MO, USA) in 0.01 mol l^{-1} HCl, and was stored in the dark. A sodium sulfite (0.4%)–sodium hydroxide (2%) mixed reagent solution was prepared daily by dissolving 0.4 g of Na_2SO_3 in 100 ml of 2% NaOH solution. All chemicals used were of analytical-reagent grade or better, and doubly distilled water was used throughout.

Sample preparation

Tablets

An appropriate amount (0.01 g for vitamin B_1 tablets, 0.04 g for compound vitamin B tablets and 0.1 g for Ershiyi Jinweita Pian) of the previously powdered sample was accurately weighed into a 100 ml calibrated flask. Then, 10 ml of 0.1 mol l^{-1} HCl were pipetted into the flask before water was added to the mark. After sonication for 20 min, the remaining insoluble residue was filtered off. A 2 ml aliquot of the filtrate was pipetted into a 25 ml calibrated flask and diluted to the mark with water.

Injections

A sample vial (volume 2 ml) was totally transferred into a 100 ml calibrated flask, in which 10 ml of 0.1 mol l^{-1} HCl had been placed, and the mixture was diluted to the mark with water. The solution was then diluted stepwise to about 1 μg ml^{-1} with water for measurement.

Serum

A pooled human serum was prepared by simply mixing serum samples taken from five healthy adults. It was then diluted 100-fold with water for the determination.

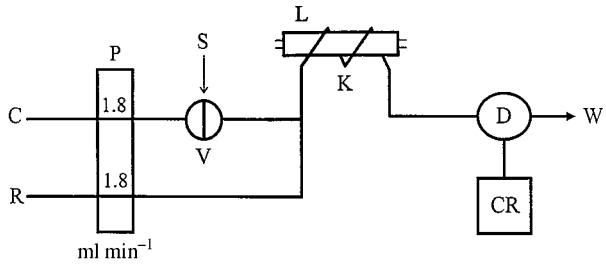

Fig. 1 Experimental set-up for FI on-line photochemical reaction–spectrofluorimetry. C, Carrier (H_2O); R, Na_2SO_3–NaOH mixed reagent solution; S, sample solution; D, spectrofluorimetric detector; K, knotted PTFE reactor; L, low-pressure mercury lamp; P, peristaltic pump; CR, chart recorder; V, injection valve; and W, waste. When used for investigation into the effect of acetone, the valve was removed and sample solutions were continuously pumped through the probe C to merge with reagent solution.

Procedure

The excitation and emission wavelengths of the spectrofluorimeter were set at 370 and 440 nm, respectively, both with a bandpass of 5 nm. Before measurement, the low-pressure mercury lamp was turned on, and the aqueous carrier and mixed reagent solution were continuously pumped through the knotted PTFE reactor and the flow-through cell to waste until a stable baseline was obtained. After sampling a test solution for 15 s, the valve was switched to the injection position. Twenty seconds later, the valve was switched back to the sampling position for the next cycle. Three replicates were run for each test solution, and a calibration graph based on peak height was used for quantification.

Results and discussion

Effect of acetone on the photochemical reaction

Guo *et al.*[18] have reported that the *in situ* photochemical reaction of thiamine was significantly sensitized by acetone and that the reaction rate of the acetone-sensitized photochemical reaction was further accelerated on addition of sodium sulfite to the sodium hydroxide solution. In a preliminary test with an unsegmented continuous-flow assembly modified from the manifold illustrated in Fig. 1 (see the legend to Fig. 1), it was unexpectedly found that acetone severely depressed the photochemically induced fluorescence signal of the on-line photochemical reaction on addition of sodium sulfite to the alkaline solution. This interesting observation prompted us to carry out a systematic investigation into the role played by acetone in the photochemical reaction which took place both *in situ* in the flow-through cell as described in ref. 18 and on-line in the knotted PTFE photochemical reactor. The results are presented in Table 1. As can be seen, when Na_2SO_3 was absent from the alkaline solution, acetone did sensitize the photochemical reaction in both reaction modes (see columns 2–4 in Table 1), and the optimum acetone concentration in the thiamine solution was in the range 0.5–1.0%. When Na_2SO_3 was present in the NaOH solution to sensitize the photochemical reaction, however, the effect of acetone became complicated. Thus, it depressed the fluorescence signal of the on-line photochemical reaction (see column 5 in Table 1), but enhanced the signal of the *in situ* photochemical reaction if the flow-through cell was irradiated at 280 nm using a 5 nm bandpass (see column 6 in

Table 1 Influence of acetone on photochemically induced fluorescence

Acetone concentration in sample solution (%)	Fluorescence intensity* obtained with 2% NaOH containing					
	No Na_2SO_3			0.5% Na_2SO_3		
	A†	B_1‡	B_2§	A†	B_1‡	B_2§
0	7.6	0.9	5.0	40.7	2.4	43.6
0.5	18.2	16.9	22.9	22.0	31.4	33.0
1.0	18.3	18.4	21.6	18.7	32.6	29.9
2.0	13.7	15.4	16.0	14.4	27.6	19.9

* The fluorescence intensity was measured at 440 nm (λ_{em}) with 370 nm being used as the excitation wavelength (λ_{ex}). † A; Thiamine solution was continuously pumped at 1.8 ml min^{-1} to merge with a flow of alkaline reagent solution (also at 1.8 ml min^{-1}) and irradiated on-line in a knotted PTFE reactor (50 cm × 0.5 mm id) by the radiation emitted from a low-pressure mercury lamp, around which the knotted PTFE reactor was coiled. ‡ B_1: Thiamine solution, after being pumped at 1.8 ml min^{-1} to merge with a flow of alkaline solution (1.8 ml min^{-1}), was stopped in the flow-through cell and irradiated *in situ* for 60 s at 280 nm (5 nm bandpass) using the excitation monochromator of the spectrofluorimeter. § B_2: As for B_1 except that the bandpass was 20 nm.

Table 1). The opposing effects were suspected to be related to the difference in the intensity of the irradiation which induced the photochemical reaction: the knotted PTFE photochemical reactor (for on-line photochemical reaction) coiled directly around the low-pressure mercury lamp might be exposed to more intense radiation than the flow-through cell (for *in situ* photochemical reaction). To verify this suspicion, the bandpass of the excitation monochromator was set to 20 nm (the highest setting) to increase the radiation intensity from the monochromator, and the influence of acetone on the *in situ* photochemical reaction was again observed. As expected, acetone now showed a negative effect (see column 7 in Table 1). When the central wavelength of the excitation monochromator was set at 254 nm (the same wavelength as the maximum emission of the low-pressure mercury lamp) for the *in situ* photochemical reaction, the same tendency was observed. Thus, it is clear that the effect of acetone is dependent on the intensity of the radiation which induces the photochemical reaction when sodium sulfite is involved in the photochemical reaction.

Since acetone was reported to sensitize the *in situ* photochemical reaction through acceleration of the reaction rate,[18] its influence on the kinetic behavior of the on-line photochemical reaction was studied in both the presence and absence of sodium sulfite. In the test, the irradiation time was controlled by changing the flow rate of the sample solution (note: the alkaline solution always had the same flow rate as the sample solution). Fig. 2 shows the relative fluorescence intensity plotted against the sample flow rate. When Na_2SO_3 was absent from the NaOH solution, acetone significantly accelerated the reaction rate of the on-line photochemical reaction as the maxima of the curves of fluorescence intensity *versus* sample flow rate gradually shifted to higher flow rates with an increase in the acetone concentration [Fig. 2(*a*)]. However, the values of the maximum fluorescence intensity of these curves decreased with an increase in the acetone concentration from 0.5 to 2.0%. Thus, it was considered that acetone played two different roles in the photochemical reaction, *i.e.*, although it accelerated the photochemical reaction, an excess of acetone might decrease the fluorescence intensity of the photochemical reaction product. The depressive effect of acetone on the fluorescence intensity of the photochemical reaction product is further illustrated in Fig. 2(*b*) [note the difference in the ordinate scale between (*a*) and (*b*)], which shows the kinetic behavior of the on-line photochemical reaction in the presence of sodium sulfite. Under these conditions, the photochemical reaction rate became so rapid that the maxima of all four curves appeared at the highest flow rate regardless of the presence of acetone, while at a given flow rate the fluorescence intensity consistently decreased with an increase in the acetone concentration. Hence, the accelerating effect of acetone on the rate of the on-line photochemical reaction was masked by sodium sulfite, while its depressing effect on the fluorescence intensity of the photochemical reaction product was clearly displayed. Therefore, it can be concluded that acetone is detrimental to the on-line photochemical reaction of thiamine when sodium sulfite is used to enhance the sensitivity.

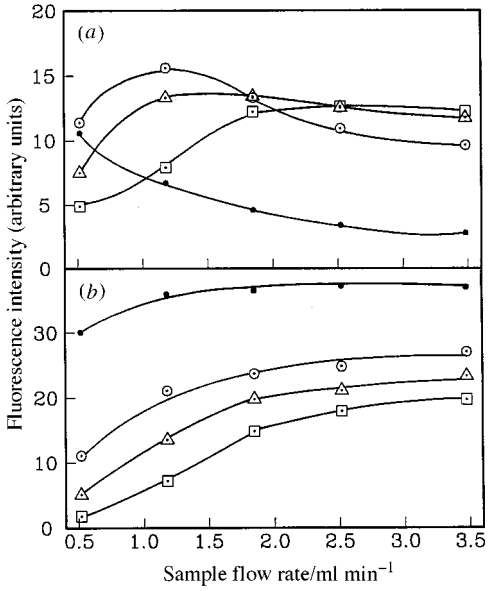

Fig. 2 Kinetic behavior of the on-line photochemical reaction of thiamine. Immediately after being merged on-line with an alkaline solution containing no Na_2SO_3 (*a*) or 0.5% Na_2SO_3 (*b*), thiamine solution (1 mg l^{-1}) was continuously pumped at different flow rates to pass a 0.5 m knotted PTFE reactor that was coiled around a low-pressure mercury lamp. Acetone concentrations (%): ●, 0; ⊙, 0.5; △, 1.0; ⊡, 2.0. The lowest (0.5 ml min^{-1}) and highest (3.4 ml min^{-1}) flow rate of the thiamine solution corresponds to an irradiation time of 6 and 0.9 s, respectively.

Effect of other organic solvents and surfactants

Other organic solvents miscible with water, and surfactants, were also examined. It was found that methanol, ethanol and acetonitrile had no effect on the on-line photochemical reaction in the absence of sodium sulfite, and that methanol and ethanol at a concentration level of 0.5% severely inhibited the reaction, whereas acetonitrile up to a concentration of 30% only slightly depressed the photochemically induced fluorescence signal if the on-line photochemical reaction was sensitized by sodium sulfite. Among the surfactants tested (sodium lauryl sulfate, cetyltrimethylammonium bromide, Triton X-100 and β-cyclodextrin), only Triton X-100 in the concentration range tested (0.02–0.5%) enhanced the on-line photochemically induced fluorescence signal, by a factor of 2, in the absence of Na_2SO_3. If sodium sulfite was present in the alkaline solution, none of the surfactants had a significant influence on the photochemically induced fluorescence signal.

Based on these observations, organic solvents and surfactants were excluded in the following study of the FI on-line photochemical reaction.

Influence of NaOH *and* Na_2SO_3 *concentrations on the FI on-line photochemical reaction*

Guo *et al.*[18] reported that an alkaline medium was favored for the photochemical reaction of thiamine but the photochemically induced fluorescence intensity decreased rapidly with an increase in the time interval between mixing the thiamine solution with the alkaline solution and irradiating the mixture. This was attributed to the decomposition of thiamine in the alkaline medium before it was converted into the corresponding fluorescent compound. When the manifold illustrated in Fig. 1 was used, thiamine in the injected sample zone underwent a photochemical reaction immediately after the sample zone had been mixed on-line with the Na_2SO_3–NaOH solution, resulting in no significant analyte decomposition. It was found that the order of addition of sodium sulfite was critical. If sodium sulfite was first added manually to a thiamine solution and the resulting solution was injected and merged on-line with an NaOH solution or even with an NaOH solution containing sodium sulfite, the photochemically induced fluorescence signal was very poor. Only when an injected zone of thiamine solution containing no sodium sulfite was merged on-line with the mixed Na_2SO_3–NaOH solution was a high fluorescence signal observed. Fig. 3 shows the influence of the Na_2SO_3 and NaOH concentrations in the mixed reagent solution on the fluorescence signals. Thus, 2% NaOH containing 0.4% sodium sulfite was finally chosen.

Optimization of FI variables

For FIA, it is desirable that the maximum yield of the chemical reaction be obtained while the dispersion of the reaction product is kept as small as possible. The knotted reactor has been shown to cause much smaller dispersion of a sample zone than a straight reactor or a coiled reactor of the same length.[20] In the present FI on-line photochemical reaction system, a 10–30% increase in the fluorescence signal, depending on the sample volume and the length of the reactor, could be obtained when a knotted reactor was substituted for a reaction coil of the same length.

For the FI on-line photochemical reaction, the time for which the sample zone is irradiated is dependent on both the length of the knotted reactor and the flow rate of the aqueous carrier. In addition, the dispersion of the sample zone is also affected by these two experimental parameters. Thus, the influence of the knotted reactor length on the fluorescence intensity was studied at carrier flow rates of 1.8 and 3.7 ml min^{-1} (keeping the ratio of the carrier flow rate to the reagent flow rate constant), respectively. It was found that the highest fluorescence signal was obtained when a 50 cm knotted reactor and a carrier flow rate of 1.8 ml min^{-1} were used. Thus, these values were adopted in further tests.

Owing to the large dead volume (120 μl) of the rectangular flow-through cell, a fairly large sample volume was favorable. It was observed that the relative fluorescence intensity signals corresponding to injection volumes of 0.5, 0.25 and 0.1 ml were 1.10, 1.00 and 0.82, respectively. Therefore, a sample volume of 0.25 ml was used as a compromise between sensitivity and sampling frequency.

Analytical performance

The analytical performance of the proposed FI on-line photochemical–spectrofluorimetric method is shown in Table 2. The detection limit, precision and sampling frequency of the present system are significantly better than those obtained with *in situ* photochemical–spectrofluorimetry.[18] Moreover, the frequent change of the wavelength of the excitation monochromator required by the *in situ* photochemical reaction[18] was avoided, resulting in greater ease of operation and less wear of the mechanical system of the spectrofluorimeter.

Interference study

The potential interferences from the ingredients that might be present in pharmaceutical formulations were tested and the results are summarized in Table 3. The tolerance level for other water-soluble vitamins and metal ions allows the proposed method to be used to determine the thiamine content in compound vitamin B and vitamin–mineral preparations.

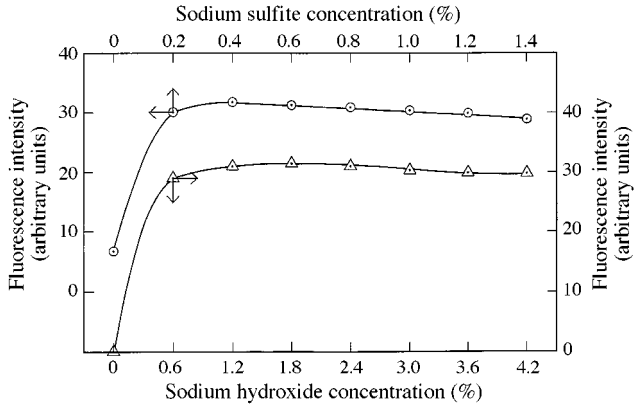

Fig. 3 Influence of NaOH and Na_2SO_3 concentrations in the mixed alkaline solution on the fluorescence signals of the FI on-line photochemical reaction. Thiamine concentration, 1 mg l^{-1}; flow rates of carrier and mixed reagent solution, 1.8 ml min^{-1}; length of knotted PTFE reactor, 0.5 m. ⊙, 2% NaOH with variable concentrations of Na_2SO_3; △, 0.4% Na_2SO_3 with variable concentrations of NaOH.

Applications

The proposed method was applied to assay the thiamine content in several pharmaceutical preparations. The accuracy of the proposed method was first checked by performing recovery tests on solutions prepared from the thiamine formulations. For the vitamin B_1 tablet, vitamin B_1 injection and compound vitamin B tablet formations, aliquots of the sample solutions that would give a final thiamine concentration of 1 mg l^{-1} were spiked with the same amount of standard thiamine. The recoveries of the added thiamine were 99.5% for vitamin B_1 tablets, 101.3% for vitamin B_1 injections and 100.3% for compound vitamin B tablets. For Ershiyi Jinweita Pian (a multivitamin–mineral tablet formation, in which the mass ratio of ascorbic acid to thiamine is 10 : 1), the ascorbic acid concentration would be higher than the tolerance level if the thiamine concentration in the sample solution was 1 mg l^{-1}. Tests indicated that the recovery of thiamine added to a sample solution (made from Ershiyi Jinweita Pian) containing 0.57 mg l^{-1} thiamine was 95.2%, and that added to a sample solution containing 0.3 mg l^{-1} thiamine was 100%. Thus, the thiamine concentrations in the final sample solutions (for measurement) were made to be about 1 mg l^{-1} for the vitamin B_1 tablet, vitamin B_1 injection and compound vitamin B tablet formulations, and 0.3 mg l^{-1} for the Ershiyi Jinweita Pian formulation. The analytical results obtained by the proposed method were compared with those obtained by the manual thiochrome extraction (TCE) method.[21,22] As can be seen in Table 4, there is a good agreement between the mean values obtained by the

Table 2 Analytical performance of the FI on-line photochemical–spectrofluorimetric method

Detection limit/μg l^{-1}	0.11 (3σ)
Relative standard deviation (%)	0.36 (1.0 mg l^{-1}, $n = 11$)
Regression equation	$F = 0.2 + 29.7 \times C^*$
Linear dynamic range/mg l^{-1}	0.01–10
Determination coefficient (R^2)	1.000 (0.9999997)
Sampling frequency/h^{-1}	100
Sample consumption/ml per run	0.4 (including to waste)

* F, Fluorescence intensity in arbitrary units; C, thiamine concentration in mg l^{-1}.

Table 3 Tolerance levels (mg l^{-1}, error < 5%) towards compounds that might be present in pharmaceutical formulations*

Pyridoxine	1000
Nicotinamide	200
Sodium pantothenate	10.0
Ascorbic acid	5.0
Tartaric acid	1.0†
Riboflavin	1.0
Menadione sodium bisulfite	0.2
K^+	100†
Ca^{2+}	100†
Mg^{2+}	100†
Zn^{2+}	100†
Fe^{3+}	20
Cu^{2+}	20
Mn^{2+}	2
I^-	100†

* Thiamine concentration, 1 mg l^{-1}. † Maximum level tested.

two methods, but the precision of the proposed method is significantly better than that of the manually operated TCE method. The proposed method was also used to determine the thiamine concentration in human serum, and satisfactory results were obtained, as shown in Table 5.

Conclusion

The proposed FI on-line photochemical–spectrofluorimetric method for the determination of thiamine possesses several advantages over the previously reported *in situ* photochemical reaction.[18] Firstly, acetone, which was previously employed to sensitize the *in situ* photochemical reaction,[18] is not required for the present on-line photochemical reaction. This reduces the analysis cost and prevents the operator from exposure to an inflammable solvent with an offensive odor. Secondly, the change in the wavelength of the excitation monochromator needed during the *in situ* photochemical reaction is avoided, lessening the wear and tear of the mechanical system of the spectrofluorimeter. Thirdly, the analytical performance, in terms of the detection limit, precision and sampling frequency, of the present system is significantly better than that of the previous *in situ* photochemical–spectrofluorimetric method. The developed method is suitable for the determination of thiamine in pharmaceutical preparations and human serum. The on-line photochemical reaction might also be adapted to post-column photochemical derivatization and fluorimetric detection for the HPLC determination of thiamine in complex samples such as natural and fortified foods. This work is currently in progress in our laboratory.

Table 4 Determination of thiamine in pharmaceutical preparations

		Found* (mean ± *s*, *n* = 3)	
Sample	Labeled*	Proposed method	TCE method[21,22]
Vitamin B_1 tablets	10	10.2 ± 0.2	9.79 ± 0.66
Vitamin B_1 injections	100	106 ± 2	100 ± 5
Compound vitamin B tablets	3	2.76 ± 0.01	2.80 ± 0.30
Multivitamin–mineral tablets	2.5	2.50 ± 0.08	2.50 ± 0.19

* Expressed in mg per tablet or mg per injection.

Table 5 Determination of thiamine in human serum*

Thiamine concentration†/mg l^{-1}		
Added	Found (mean ± *s*, *n* = 3)	Recovery (%)
0	0.022 ± 0.003	—
0.20	0.22 ± 0.01	99
0.50	0.52 ± 0.01	100

* Original serum sample was diluted 100-fold before analysis. † Concentrations in the diluted serum.

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