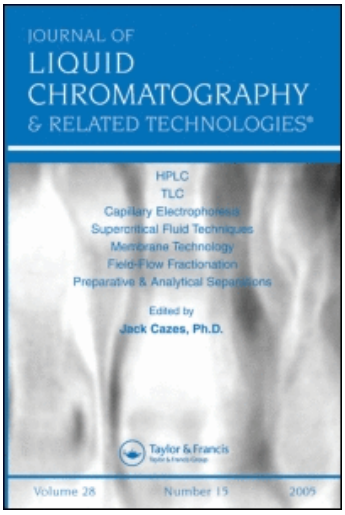

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Simultaneous Determination of Water-Soluble Vitamins in Human Urine by Fluorescence in a Flow-Injection Analysis

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Abstract: Fluorescence in flow-injection analysis is described for the simultaneous determination of thiamine, riboflavin, and folic acid. Its detection limit linearity and reproducibility were examined. The kinetic method is based on the enhancing effect of thiamine and riboflavin on the fluorescence generated by oxidizing to thiochrome with alkaline ferricyanide. Different parameters affecting this reaction were thoroughly studied. The procedure was applied to the determination of thiamine and riboflavin and folic acid in human urine samples.

Keywords: Fluorescence in flow-injection analysis, Water-soluble vitamins, Human urine

INTRODUCTION

Thiamine (vitamin B_1) and riboflavin (vitamin B_2) are biologically and pharmaceutically important compounds. Riboflavin is the precursor of flavin mononucleotide (FMN) and FAD, which serve as cofactors for enzymes involved in the metabolism of amino acids and the maintenance of body cells.[1,2] Many methods for the determination of water-soluble vitamins have been reported, including spectrophotometry,[3,4] chemiluminescence (CL),[5,6] high performance liquid chromatography with ultraviolet,[7–10] fluorometric,[11–14] and mass spectrometric[15] detection. Other reported methods include micellar electrokinetic chromatography[16–19] and

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voltammetry.[20–22] The advantages and disadvantages of these methods have been discussed.

There have been a large number of method studies on determination of vitamins in foods, beverages, drugs, and biological tissues or fluids. Reports on determination of water-soluble vitamins in urine were relatively limited.[6,9,15] Spectrofluorimetry is widely used in quantitative analysis because of its great sensitivity and selectivity.

This technique has not, however, been widely applied to the simultaneous direct determination of many fluorescent components in mixtures, mainly because the fluorescence spectra of individual substances contain broad bands which overlap. HPLC methods with fluorometric (FL) detection are considered the most desirable because of their rapidity, specificity, and sensitivity. In this study, we have developed a LC-FL coupled with a flow injection for determination of thiamine and riboflavin and folic acid in urine without prior sample pretreatment.

EXPERIMENTAL

Apparatus and Procedures

HPLC was performed with Gasukuro Kogyo model 576 pump and model 7125 injector equipped with a 20 μL sample loop, and fluorescence measurements were acquired with a Shimatzu RF-10A_{XL} spectrofluorometer (Japan). Chromatograms were acquired and peak areas calculated by means of an SISC chromatograms Data Integrator.

The flow injection system used in this work is shown in Fig. 1. Mixing tubing (30∼90 cm, 0.5 m, i.d.) was used in the flow system. In the FI manifold, a potassium hexacyanoferrate solution in sodium hydroxide-methanol was continuously pumped and mixed. Finally, the vitamin sample

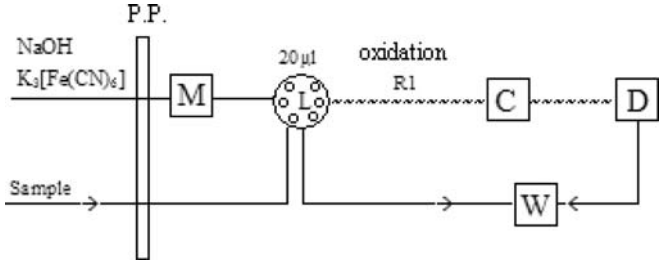

Figure 1. FI manifold. M = Mixer, L = Loop, C = Column, 25 cm × 4.6 mm, P. P. = Peristaltic pump, D = Fluorometric detecetor, W = Waste, R1 = 70 cm (long), 0.5 mm (i.d.).

was injected into the stream of oxidized thiochrome and conducted to the fluorometric detector.

Reagents and Materials

The vitamins tested were thiamine and riboflavin and folic acid from Acros. The pre-column reagent solution was prepared daily.

Human Volunteers

Five normal, healthy, men and women, aged from 20 to 22 years, were selected. The average height was 160 ± 5 cm and the average weight was 50 ± 10 kg. Each received complete multi-vitamin (thiamine 20 mg, riboflavin 10 mg, folic acid 400 mg) per day for 1–5 days.

Sample Preparation

Urine Sample Collection

Urine samples were collected at specific time intervals (0,1, 2, 4, 8, 16, 24, 48, 72, 96, and 120 h, respectively) after vitamin intake. Time 0 values were used as background and subtracted from sample values. The remainder of the samples were kept in high density polyethylene containers and stored in a freezer (-20°C) for further determination of thiamine and riboflavin and folic acid by HPLC.

Extraction of Urinary Metabolites of Water-Soluble Vitamins

Since thiamine is excreted unchanged in urine and riboflavin mostly in the free VB_2 form, therefore, the urine was centrifugated for 10 min. The supernatant was analyzed directly after dilution with water.

Determination by Liquid Chromatography

Stock solutions of standards were prepared by dissolving the appropriate amount of vitamin in water. A set of standard solutions were prepared by appropriate dilution of the stock solutions with water to 10 mL in amber graduated flasks. Reversed-phase LC was performed on a 25 cm $\times$ 4.6 mm i.d., 5 μm particle Hypersil ODS C_{18} column. The isocratic mobile phases were 72 : 28, 62 : 38, 52 : 48, 42 : 58 methanol-water; the mobile phase flow rates were $0.2 \sim 1.0$ mL min^{-1}. After separation on the Hypersil column, detection was achieved by use of a time program in which the detector was

programmed at Ex 358 nm (excitation) and Em 475 nm (emission) from 0 to 8 min to determine riboflavin and folic acid, at Ex 365 nm and Em 435 nm from 8 to 15 min to determine thiamine, then back to Ex 358 nm and Em 475 nm at the end of run.

RESULTS AND DISCUSSION

Optimization Condition for the FI-FL System

There are several physical and chemical variables that influence the fluorescence (FL) intensity of the signal in the FI (flow-injection) system such as length of the reactors, temperature, reaction time, flow rate, pH, and concentrations of the reagent. The FI system was optimized for the proposed procedure by altering each variable in turn, while keeping the others constant. Table 1 shows the ranges and optimal values found. In all the experiments described in Table 1, a solution of 0.1 $\mu g\,mL^{-1}$ thiamine was used.

Performance of the FI System for Vitamins Measurements

A series of standard solutions were injected into the manifold shown in Fig. 1 under the optimized conditions to test the linearity of vitamins. The calibration plots obtained by plotting the peak area against the concentration of thiamine, riboflavin, and folic acid show good linearity over the range of 0.25–8 $mg\,L^{-1}$. For thiamine, riboflavin, and folic acid, the regression equations being $y = -9.1 + 105X$ (correlation coefficient, $r = 0.9998$), $y = 0.1 + 6.35X$ ($r = 0.9999$) and $y = -9.5 + 24.8X$ ($r = 0.9951$). The detection limits were 0.05 $\mu g\,mL^{-1}$, 0.09 $\mu g\,mL^{-1}$, and 0.13 $\mu g\,mL^{-1}$ for thiamine, riboflavin, and folic acid, respectively. Chromatograms of a standard mixture detected with and without gradient of wavelengths are shown in Fig. 2(A) and (B), respectively.

Table 1. Physical and chemical variables optimized

Variable	Studied range	Selected value
Reactor length (cm)	30∼90	70
Temperature (°C)	10∼40	25
Reaction time (min)	5∼30	10
Flow rate (mL min^{-1})	0.2∼1.0	0.5
pH of oxidizing	8.7∼11.2	10.8
$K_3[Fe(CN)_6]$ concentration (mM)	1∼10	2
NaOH concentration (mM)	0.1∼4	2

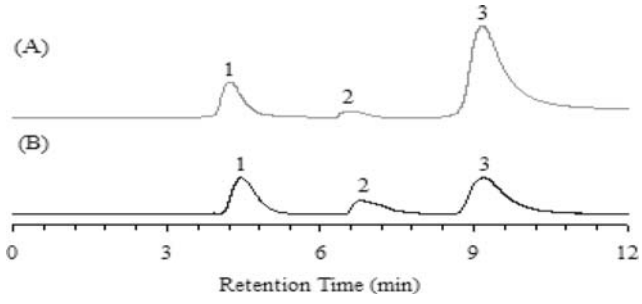

Figure 2. Chromatograms of vitamin standards on a Hypersil ODS C_{18} column. Peaks: 1 = folic acid; 2 = riboflavin; 3 = thiamine. Mobile phase methanol-water (42 : 58, *v*/*v*) containing 1 mM Potassium hexacyanoferate pH = 10.8; flow rate 0.5 mL/min; (A) fluorescene detector gradient of wave-length 358 nm excitation and 475 nm mission, held for 8 min and change to 365 nm excitation and 435 nm emission in the next 7 min; (B) with excitation and emission wavelength of 358 nm and 475 nm, respectively.

Recovery Test

A 400 μL aliquot of thiamine and riboflavin and folic acid were added to 1 mL of diluted urine samples and to urine samples that contained known amounts of endogenous vitamins, and extraction was carried out as described above. To calculate percentage recovery, the amount of endogenous vitamins were subtracted from the measured total amount, divided by the added amount, and multiplied by 100. Table 2 shows the FI-FL traces obtained for volunteer urine samples spiked with vitamins. Recovery and precision were observed (recoveries ranging from 97 ± 3.5% to 105 ± 3.0%).

Table 2. Recovery of thiamine, riboflavin, and folic acid in human urine

Sample, complete multi-vitamin	Volunteers	Added ($\mu g\,mL^{-1}$)	Found ($\mu g\,mL^{-1}$)	Recovery (%, N = 3[a])
Thiamine	Person A	57	56	97 (3.5%)[b]
	Person B	0. 57	0. 60	105 (3.0%)
Riboflavin	Person A	8.57	8.93	104 (0.7%)
	Person B	13.33	13.73	103 (0.9%)
Folic acid	Person A	4.55	4.66	102 (4.8%)
	Person B	8.33	8.47	101 (2.9%)

[a]Number of determination.
[b]Relative standard deviation.

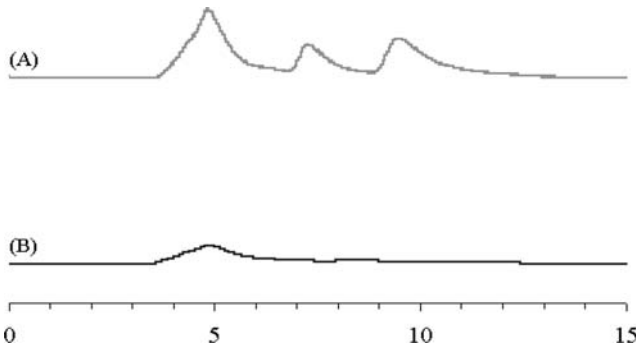

Figure 3. Chromatograms obtained form (A) after (B) before of oral complete multivitamin. Analysis conditions are identical to those listed in Fig. 2.

Application to Human Urine

The proposed method was applied to the determination of thiamine, riboflavin, and folic acid in human urine. Figure 3(A) and (B) compares the chromatogram of pure standard (Fig. 2). Sample constituents with retention characteristics identical to those of folic acid, riboflavin, and thiamine were identified and measured. The measured concentration of vitamins in the urine samples of volunteers collected 4 h after eating 1.28 g/day vitamin pills, are summarized in Table 3. The metabolic profiles of thiamine and riboflavin in urine are shown in Figs. 4 and 5, respectively.

From the curve, it can be seen that thiamine and riboflavin were metabolized rapidly after taking the vitamin pills. Both thiamine and riboflavin

Table 3. The concentrations of vitamins of volunteers in the urine sample taken 4 h after eating 1.28 g/day vitamin pills and determined by the FI-FL system

	Vitamin concentration in human urine ($\mu g\,mL^{-1}$)[a]		
Volunteers	Thiamine	Riboflavin	Folic acid
F1	9.33 (0.4%)[b]	203 (3.8%)	38.3 (4.3%)
F2	12.5 (0.8%)	42.0 (1.9%)	22.5 (2.7%)
M1	9.32 (0.3%)	209 (3.4%)	38.3 (3.3%)
M2	1.94 (1.0%)	—[c]	—[c]
M3	4.40 (1.8%)	30.1 (2.2)	25.5 (4.2%)

[a]Number of determination (N = 3).
[b]Relative standard deviation.
[c]Not determined.

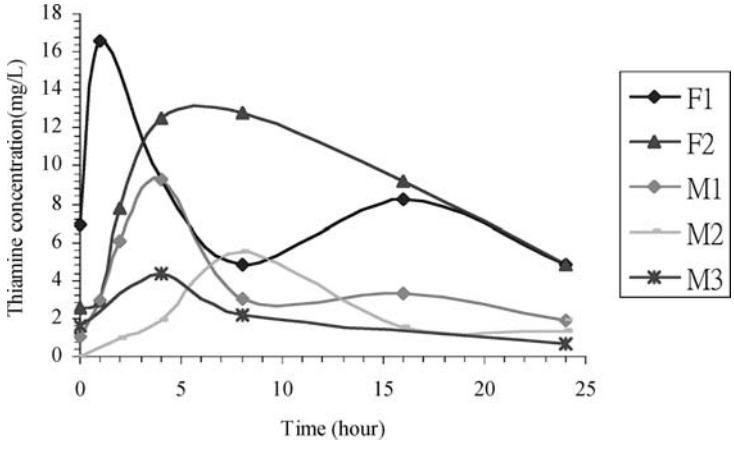

Figure 4. Time-concentration profile after single oral dose 100 mg of thiamine.

concentration reached their maximum after four hours and dropped sharply within a few hours. The total thiamine and riboflavin excreted though urine were 7.05 mg and 86.82 mg in a total volume 1.53 L in 24 h, respectively. Vitamins are quickly metabolized when they exist in excess in a body. Nevertheless, the amount excreted depends on tissue stores and on the amount ingested. Vitamins taken at overdose proved to rapidly show up in the urine. Figures 6, 7, and 8 show the concentration of vitamins tended to increase after an intake of vitamin pills and reached their peak when 3–4 days elapsed, and decreased slowly. The general trend was similar

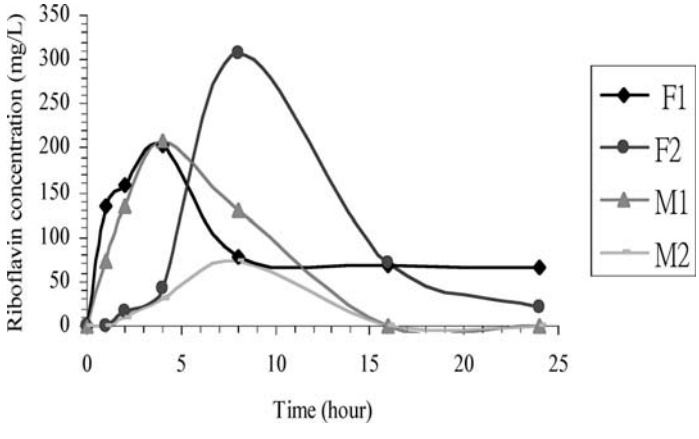

Figure 5. Time-concentration profile after single oral dose 50 mg of riboflavin.

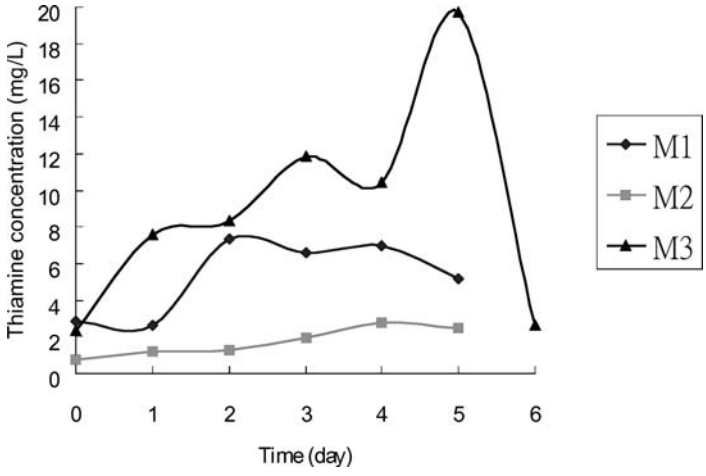

Figure 6. Day course of gastrointestinal absorption of thiamine metabolite after intake of complete multi-vitamin 1.28 g/day; thiamine 20 mg; riboflavin 10 mg.

among the two volunteers, although some variances in concentrations of vitamins in urine were observed.

CONCLUSIONS

We have applied a method of determination of water-soluble vitamins in urine by coupling the FI system to gradient fluorometric (FL) detection without any pretreatment procedures, to simultaneously determine vitamins in urine after intake of vitamin pills. Rapid and easy monitoring of components of biological interest in urine will be important for clinical purposes.

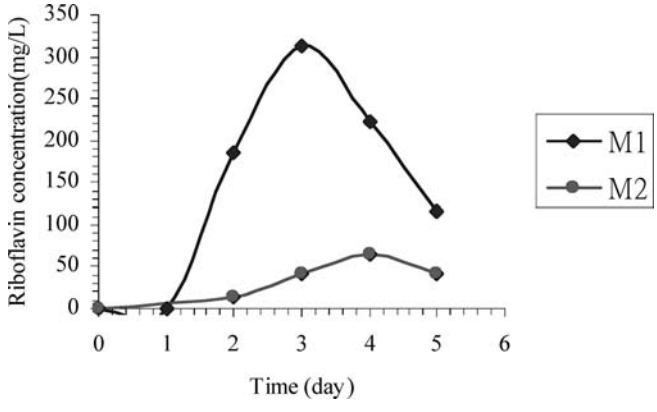

Figure 7. Day course of gastrointestinal absorption of riboflavin metabolite after intake of complete multi-vitamin 1.28 g/day; thiamine, 20 mg; riboflavin, 10 mg.

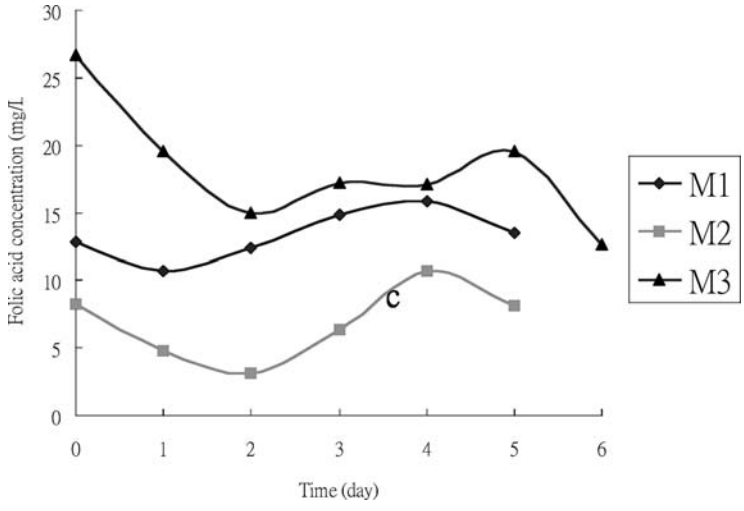

Figure 8. Day course of gastrointestinal absorption of folic acid metabolite after intake of complete multi-vitamin 1.28 g/day; thiamine 20 mg; riboflavin 10 mg.

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