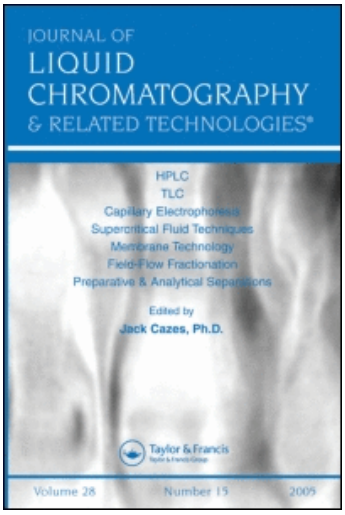

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RAPID DETERMINATION OF WATER-SOLUBLE B GROUP VITAMINS IN URINE BY GRADIENT LC/MS WITH A DISPOSABLE HOME-MADE MICROCOLUMN

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ABSTRACT

A rapid and simple analytical method without pretreatment, based on gradient LC/MS with a disposable microcolumn, has been developed to determine B group vitamins in urine. Urine samples were directly injected to the disposable home-made microcolumn. A 5 cm silica capillary with a sintered silica frit was placed in the microcolumn outlet. A 1/32 inch coupling union with graphite Vespel ferrules was used to give a safe and void volume free connection between the 5 cm capillary and a long capillary to the electrospray interface. The microcolumn

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can be emptied after being used for a series of urine samples, and repacked with fresh stationary phase. Healthy volunteers swallowed an overdose of vitamin pills, and the urine samples were collected 1,2,3,5, and 8 hours after swallowing. Vitamins immediately showed up in urine, hit the maximum, and disappeared swiftly. This technique is expected to have some application for clinical purposes.

Key Words: B group vitamins; Urine; LC/MS; Disposable microcolumn

INTRODUCTION

Vitamins are essential food constituents that are required in small amounts for maintaining life. Vitamins are generally divided into two main groups; fat- and water-soluble vitamins. While fat-soluble vitamins have been analyzed by both GC and LC techniques, water-soluble vitamins have been determined exclusively by HPLC techniques.[1–6] In most HPLC analyses of water-soluble vitamins, pretreatment procedures were required to concentrate vitamin components and/or to eliminate interfering materials.[4–8] Determination of water-soluble vitamins in complex biological samples, such as blood and urine, by HPLC suffers from low sensitivity and residual matrix interferences in spite of pretreatment. In addition, vitamins are often unstable in such biological matrices.[9]

In this study, we have developed a rapid and sensitive method for determining B group vitamins in urine sampled 1, 2, 3, 5, and 8 hours after taking an overdose of vitamin pills, without pretreatment by the gradient LC/MS system. Various studies on the determination of vitamins by LC/MS were reported.[10–14] Determination of water soluble vitamins in urine by LC/MS has not yet been reported. We believe that this is the first report on pseudo-on-time LC/MS determination of water soluble vitamins excreted in urine. This pseudo-on-time determination method was enabled by incorporation of a cheap disposable home made microcolumn.

EXPERIMENTAL

Apparatus

Two Shimadzu (Tokyo, Japan) 10AD pumps, a Shimadzu DGU-14A membrane degasser, a Tee union with a 1/16 inch I.D. stainless steel frit (as a

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micromixer), a Rheodyne (Cotati, CA, USA) 7520 injector with a 0.5 μL injection loop, and a home-made 0.5 mm I.D. microcolumn were combined to construct the LC part of the system.[15]

A glass-lined stainless steel packed microcolumn (30 cm long × 0.5 mm I.D.) was prepared as before.[15–18] The Alltima C18 stationary phase from Alltech (Deerfield, IL, USA) was used for packing. A piece of 5 cm × 50 μm I.D. (400 μm O.D.) deactivated silica capillary from J & W (Folsom, CA, USA) was cut, treated on a propane burner to have a sintered-silica frit at the tip, and placed in the column outlet union (a 1/16 to 1/32 inch reducing union). The stainless steel ferrule at the 1/32 inch side of the union was replaced with a graphite Vespel ferrule to secure safe fitting for the silica capillary. The schematic drawing for the connection between the column outlet and the electrospray interface is shown in Figure 1. A long piece of 80 cm × 25 μm I.D. (400 μm O.D.) deactivated silica capillary from SGE (Sydney, Australia) was cut and coupled to the silica capillary of the column outlet through a 1/32 inch coupling union with graphite Vespel ferrules. This union was originally designed for GC purposes, but it worked nicely for our purpose, too. It enabled leak-free and void-volume-free connection between the two silica capillaries. The two capillaries are of the same O.D. (0.4 mm) and are aligned to be in tight and exact contact each other in the union by the graphite Vespel ferrules. The long silica capillary can be easily coupled to the electrospray interface with a finger-tight nut/ferrule.

The mass spectrometer was a VG Biotech (Manchester, UK) Quattro triple quadrupole system with a nitrogen-flow assisted electrospray interface. The electrospray voltage was set at 3.5 kV, and the cone voltage, at 20 V. The source temperature was controlled at 90°C. The nitrogen gas flow rate was adjusted to

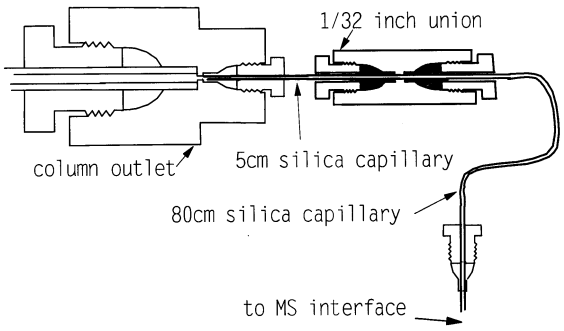

Figure 1. The scheme of connection between the column outlet and the electrospray interface of mass spectrometer. The silica capillary of column outlet and the silica capillary to the electrospray are of the same O.D. (0.4 mm) and are in direct contact in the 1/32 inch union.

0.3 L/min. The mass spectrometer was operated in the selective ion recording (SIR) mode for detection of individual vitamins and internal standard. The m/z values of selected ions were 265 (vitamin B1), 377 (vitamin B2), 170 (vitamin B6), 184 (4-pyridoxic acid), and 112 (cytosine).

Chemicals

Methanol and water were of HPLC grade and purchased from Fisher (Pittsburg, PA, USA), and used without purification. Pyridoxine (vitamin B6), 4-pyridoxic acid. Thiamine (vitamin B1), riboflavin (vitamin B2), and cytosine (internal standard) were obtained from Fluka (Buchs, Switzerland).

Preparation of Standard Solutions in Blank Urine

The blank urine was collected a week in advance and filtered through a 0.45 μm membrane filter. The blank urine was found to have no vitamins at any recognizable level. Each vitamin was exactly measured (10 mg) and dissolved in 50 mL blank urine on the same day of the excretion experiment. The mother solutions of individual vitamins were mixed in appropriate amounts and diluted with blank urine to give standard solutions over a proper range of concentration covering the whole concentration range of the real urine samples. The concentration ranges of real urine for individual vitamins were determined by preliminary experiment. The standard concentrations of vitamin B1 and B2 were chosen to be 5, 10, 15, 20, and 40 μg/mL, and those of 4-pyridoxic acid, 10, 20, 30, 40 and 80 μg/mL. The mother solutions and standard solutions were stored in a refrigerator at 4°C. We found that the chromatographic signals of vitamins of the mother solution, and standard solutions stored in the refrigerator, were stable within 3% RSD for repeated measurements during a period of 24 hours. The standard solutions were used to obtain calibration curves and to serve as external standards for real urine samples. We used the blank urine to prepare standard solutions since we had found that standard solutions made in pure water gave faulty results.

The internal standard was measured (cytosine 50 mg) and dissolved in 50 mL blank urine. A proper amount of the internal standard solution was added to the external standards and the urine samples so that the concentration of the internal standard was exclusively 50 μg/mL.

Urine Sample Preparation

Three commercial vitamin pills were taken with 200 mL of water by a healthy man in the morning, without eating breakfast. He ate lunch 5 hours later.

Each pill contained 50 mg vitamin B1, 2.5 mg vitamin B2, 2.5 mg vitamin B6. He took water continuously, little by little, at a rate of 100 mL/hr. His urine samples (about 30 mL each time) were taken 1, 2, 3, 5, and 8 hours after eating the pills. Each urine sample was filtered immediately, through a 0.45 μm membrane filter. A 19 mL aliquot of filtered urine was taken and 1 mL of internal standard stock solution was added. The urine sample was then injected for analysis by Gradient LC/MS.

The exact same procedure was repeated for two other healthy men, to check if the excretion trends are variable among different persons.

Chromatographic Procedures

The home-made Alltima C18 column (300×0.5 mm) was used. The gradient elution flow rate was fixed at 10 μL/min. The solvent A was 0.1% TFA (trifluoroacetic acid) in methanol, and B, 0.1% TFA in water. The eluent composition was initially 30% A + 70% B, held for 2 min, and was linearly changed to 90% A + 10% B in 4 min, held for 4 min, then swiftly returned to the initial composition. All the peaks were eluted within 15 min, and the system was reequilibrated with the initial eluent within additional 15 min, thus, it took 30 min to run a sample. A standard solution was injected 30 min before the first urine sample. Right after analysis of the first urine sample, another standard solution was injected, followed by the next urine sample, and such steps were repeated for all the urine samples.

Quantitative Determination

Two different methods were used for analysis of urine samples from the first volunteer as follows.

1. Determination based on direct comparison of peak areas between a urine sample and the standard solution injected right before it. The following equation was used.

$$C_X = C_{STD}(M_X/M_{STD})$$

In the above equation, C_X represents the component concentration in the urine, C_{STD}, the concentration in the standard solution, M_X, the area count of the component in the urine sample, and M_{STD}, the area count of the component in the standard solution.

2. Determination based on comparison of peak areas between the component and the internal standard. First, the response factors were determined for individual components from the data of the standard solution as follows:

$$R_f = M_{STD}/C_{STD}$$

Next, the response factor ratio was computed. The response factor ratio(R_R) is defined as the response factor of a vitamin component divided by the response factor of internal standard. Then, the concentration of a vitamin component(C_X) in the urine sample was determined as follows:

$$C_X = C_{IS}(M_X/M_{IS})/R_{R,X}$$

In the above equation, C_{IS} is the concentration of internal standard in the urine sample, M_X, the area count of the vitamin component, M_{IS}, the area count of internal standard, and $R_{R,X}$, the pre-determined response factor ratio of the vitamin component ($R_{f,X}/R_{f,IS}$) based on the standard solution data.

Only the first method was used for the next volunteers since the results obtained by the two methods were generally in good agreement.

RESULTS AND DISCUSSION

We have analyzed vitamins in urine using a LC/MS system with a home-made disposable microcolumn. The merit of our method is elimination of pretreatment of urine samples. What we mean by the disposable microcolumn is that the contaminated column can be easily emptied after being used for a series of urine samples and repacked. Contamination of the column is monitored by appearance of interference of unknown peaks and baseline drift and variation of retention time. Such anomalies are not significant during the whole set of vitamin excretion experiment for 8 hours after eating vitamin pills.

If a conventional HPLC method with a UV detector is used, tedious pretreatment is necessary to concentrate the components and to eliminate matrix interferences. Pretreatment steps, such as solid phase extraction, will require a large amount of time, and uncertainty in determination owing to instability and incomplete recovery of the component will be involved. Using an electrospray mass spectrometer as a detector enables leaving out pretreatment, since it secures high sensitivity and selectivity via selective ion recording. Thus, rapid, almost on-time analysis is possible. Contamination of the column and consequent reduction of column lifetime had retarded development of direct injection techniques of urine, since a commercial micro- or regular column is expensive. However, we were able to produce home-made microcolumns at an expense of 1/5–1/10 of the market price. Furthermore, the contaminated column can be easily emptied and repacked at an even lower expense. The amount of stationary phase required for

packing a microcolumn is only 200 mg or less. The stationary phase was actually disposed of, and a disposable column costs only a few dollars in such a case. Thus pseudo-on-time LC/MS analysis with direct injection of urine was enabled in this study.

The SIR chromatograms for individual components obtained by the gradient LC/MS system for a standard solution, and the urine sample collected 3 hours after eating vitamin pills are shown in Figures 2 and 3, respectively. The m/z values of selective ions were 265 for vitamin B1, 377 for vitamin B2, 184 for 4-pyridoxic acid, and 112 for cytosine. The peak of cytosine was overlapped with the peak of vitamin B1, but it does not cause any problem in quantitative determination since selective ion recording was used. It was confirmed by the fact that the determined concentrations of vitamin B1 by the internal and external standards were in good agreement.

The calibration curves of individual components by standard solutions are shown in Figure 4. Good linearity was observed for each calibration curve over the concentration range examined. The statistic results of calibration curves are summarized in Table 1.

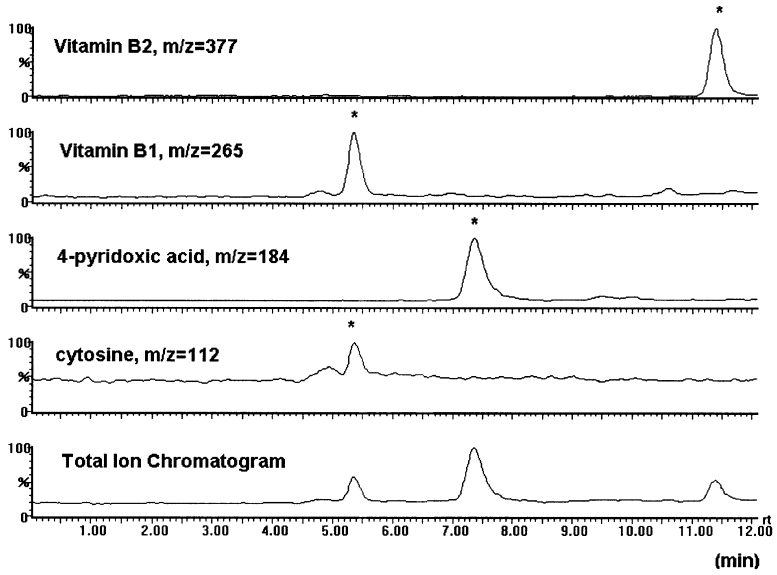

Figure 2. The SIR chromatograms of individual components obtained by gradient LC/MS for a standard solution injected right after the urine sample, which was taken 3 hours after eating vitamin pills. The m/z values of the selected molecular ions for vitamin B1, B2, 4-pyridoxic acid, and cytosine (internal standard) were 265, 377, 184, and 112. The component peak of interest in each SIR chromatogram is marked with an asterisk.

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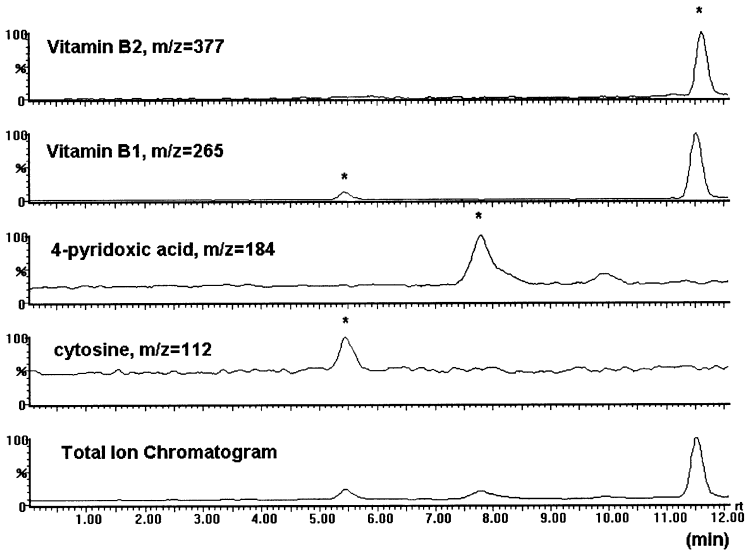

Figure 3. The SIR chromatograms of individual components obtained by gradient LC/MS for the urine sample taken 3 hours after eating vitamin pills. The component of interest in each SIR chromatogram is marked with an asterisk.

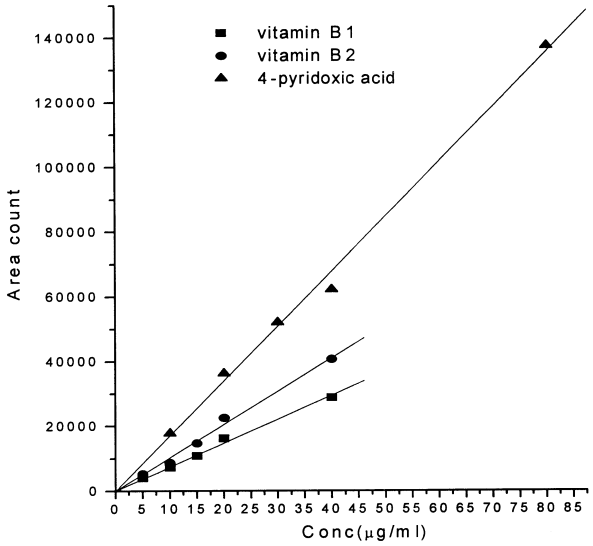

Figure 4. The calibration curves obtained from the standard solutions.

Table 1. Statistical Results of Calibration Curves (X = Concentration, Y = Area Count)

	Vitamin B1	Vitamin B2	4-Pyridoxic Acid
Calibration curve equation	Y = 736.8*X	Y = 1026.5*X	Y = 1695.3*X
Correlation coefficient (r)	0.997	0.996	0.997

Table 2. The Concentrations of Vitamins of a Volunteer (Person A) in the Urine Samples Taken 1, 2, 3, 5, and 8 Hours after Eating 3 Vitamin Pills and Determined by the Gradient LC/MS System[a,b]

		Vitamin Concentration in Urine (μg/mL)		
Elapsed Time		B1	B2	4-Pyridoxic Acid
1	IS.[c]	18.5	5.15	15.3
	ES.[d]	18.1	5.03	15.0
	Av.(diff.)[e]	18.3(0.4)	5.09(0.12)	15.1(0.3)
2	IS.	28.1	7.68	20.4
	ES.	27.7	7.56	20.1
	Av.(diff.)	27.9(0.4)	7.62(0.12)	20.2(0.3)
3	IS.	28.9	10.2	27.0
	ES.	29.7	10.4	27.8
	Av.(diff.)	29.3(0.8)	10.3(0.2)	27.4(0.8)
5	IS.	5.90	5.80	9.40
	ES.	5.10	5.02	8.13
	Av.(diff.)	5.50(0.8)	5.41(0.78)	8.76(1.27)
8	IS	2.72	1.39	3.70
	ES.	2.67	1.36	3.64
	Av.(diff.)	2.70(0.05)	1.38(0.03)	3.67(0.06)

[a]The differences between the computed values based on the internal and external standards are given in parentheses.
[b]Each vitamin pill contains 50 mg B1, 2.5 mg B2, and 2.5 mg B6.
[c]The concentrations of vitamins calculated by the internal standard.
[d]The concentrations of vitamins calculated by the external standard.
[e]The average and difference (in parentheses) of the two data based on the internal and external standard.

The measured concentrations of vitamins in the urine samples of a volunteer (person A) collected 1, 2, 3, 5, and 8 hours after eating 3 vitamin pills are summarized in Table 2. The concentrations determined by the internal and external standards were in good agreement. The excretion patterns of vitamin B1, B2, and 4-pyridoxic acids with regard to elapsed time for the three volunteers, are compared in Figure 5. The concentrations of vitamin B1, B2, and 4-pyridoxic acids in urine increased rapidly after eating vitamin pills and reached the peak

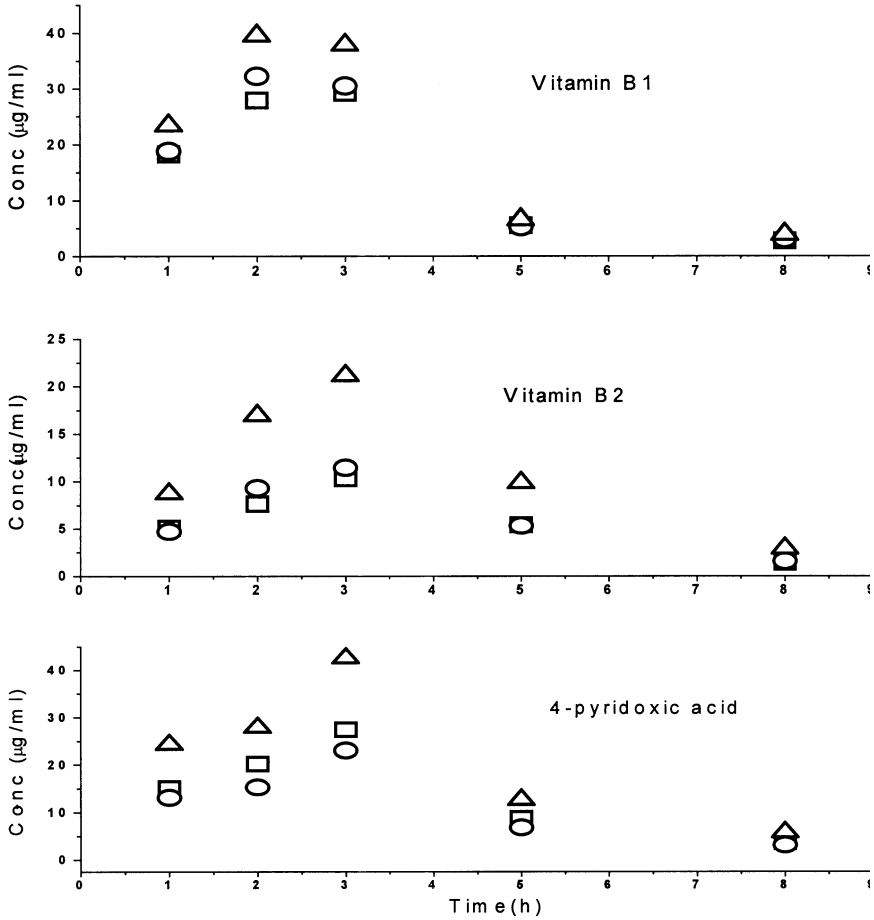

Figure 5. The trends of concentrations (with their reliability ranges) of vitamin B1, B2, and the metabolite of vitamin B6 (4-pyridoxic acid) in urine of three volunteers, with respect to elapsed time after swallowing 3 vitamin pills. Person A, person B, person C.

when 3 hours elapsed, and decreased rapidly. The general trends were similar among the three volunteers, although some variances in concentrations of vitamins in urine were observed. Vitamin B6 was not found in the urine samples. Vitamin B6 is quickly metabolized to 4-pyridoxic acid, thus, its concentration in urine would be negligible.

Direct excretion of a vitamin component was found insignificant compared to decomposition by metabolism. For example, the total amount of vitamin B1 excreted in the urine of person A was estimated ca 2 mg, while the total amount of vitamin B1 swallowed was 150 mg. The swallowed amount of vitamin B2 was 7.5 mg, but only 0.6 mg was excreted in its original form in urine. Nevertheless, the concentration of a vitamin component in urine should be a measure of level of the component in the body.

Rapid and easy monitoring of components of biological interest in urine will often be important for clinical purposes, for example, in examining drug abuse. This study shows that LC/MS techniques can serve nicely for such purposes, when a cheap disposable home made microcolumn is incorporated.

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