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On-line automated high-performance liquid chromatographic determination of total riboflavin phosphates using immobilized acid phosphatase as a pre-column reactor

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Abstract

An automated chromatographic detection system for the determination of total riboflavin phosphates using immobilized sweet potato acid phosphatase as a pre-column reactor is reported on. An immobilized enzyme reactor, incorporated in the on-line analytical system, hydrolysed riboflavin phosphates to riboflavin, and then lipophilic riboflavin was concentrated at the top of an ODS trap column. Enzymatically hydrolysed riboflavin was back-eluted from the trap column using a mobile phase containing methanol, and then subsequently chromatographed on an ODS analytical column. The effluents were monitored by UV absorption at 280 nm. The calibration graph for total riboflavin phosphates, determined by this method, was linear over the range 0.5–500 nmol/ml, with a correlation coefficient of 0.9999. The detection limit at a signal-to-noise ratio of 3 was 25 pmol/ml. The average conversion rate of riboflavin phosphates to riboflavin was estimated at 97%. The relative standard deviations of the intra- and inter-assay precision were 1.2 and 2.6%, respectively.

Keywords: Immobilized enzymes; Automation; Riboflavin phosphates; Phosphates; Phosphatases; Enzymes

1. Introduction

Immobilized enzyme reactors (IMERs) can be used not only as post-column reaction detectors but also be used with to the pre-column tools for clean up purposes or enzymatic pretreatment of analytes [1], even though there are fewer examples of pre-column than post-column applications. We recently prepared a highly efficient immobilized acid phosphatase, and the preparation with the highest retention of enzyme activity was obtained by use of sweet potato acid phosphatase as an enzyme source. The immobilized sweet potato acid phosphatase was active toward three different substrates, i.e., *p*-nitrophenyl phosphate, β-glycerophosphate and riboflavin phosphate. The stability, reusability and utility of the immobilized acid phosphatase were verified by using it as an IMER in a flow-injection system and a pre-column high-performance liquid chromatography (HPLC) reaction system. The IMER exhibited high stability and utility after repeated use, and was active and stable in methanol solution [2].

Riboflavin, vitamin B2, plays a role in biochemical redox reactions in the form of two coenzymes, riboflavin monophosphate (FMN) and flavin adenine dinucleotide (FAD). An adequate amount of riboflavin is usually provided from dietary sources, and

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any insufficiency is made up by pharmaceutical preparations containing FMN sodium salt. Phosphoric acid esters of riboflavin were easily separated by reversed-phase HPLC. Nielsen et al. reinvestigated the chromatographic separation of riboflavin phosphates and riboflavin analogs using HPLC [3], and demonstrated that commercial FMN preparations contained seven different flavin phosphates, i.e., riboflavin 5′-phosphate (5′-FMN), riboflavin 4′-phosphate (4′-FMN), riboflavin 3′-phosphate (3′-FMN), riboflavin 4′,5′-diphosphate, riboflavin 3′,4′-diphosphate, riboflavin 3′,5′-diphosphate and riboflavin, and the compositions of riboflavin phosphoric acid esters and riboflavin were also different among various commercial FMN preparation sources. Moreover, riboflavin phosphate is an extremely hygroscopic compound. Consequently, it is very difficult to obtain commercial FMN in pure form. On the other hand, the quantitative determination of an individual riboflavin phosphate is not of fundamental significance for the quality control of manufactured pharmaceutical preparations but that of total riboflavin is essential. In addition, riboflavin standard can be easily obtained. Various methods for determining total riboflavin in pharmaceutical products have been reported [4–6]. Enzymatic conversion of riboflavin phosphoric acid esters to riboflavin is a useful technique in the determination of total riboflavin [7,8].

The immobilized acid phosphatase is active toward riboflavin phosphate substrate, and is extremely stable and methanol-resistant, therefore it appears to be useful in the on-line enzymatic hydrolysis HPLC system [2]. In the present report, immobilized sweet potato acid phosphatase was incorporated in the on-line pre-column analytical system for automatically determining total riboflavin sodium phosphate in pharmaceutical preparations, and the analytical conditions of this system were carefully examined.

2. Experimental

2.1. Materials

Riboflavin sodium phosphate (FMNs) was purchased from E. Merck (Darmstadt, Germany). Riboflavin standard, the Japanese Pharmacopeia standard, was a product of the Japanese National Institute of Health (Tokyo, Japan). Acid phosphatase (orthophosphoric monoester phosphohydrolase (acid optimum), EC 3.1.3.2) from sweet potato (Type XA, suspension in 1.8 *M* ammonium sulfate and 10 m*M* magnesium chloride, pH 5.3, 18 U/mg protein) was obtained from Sigma (St. Louis, MO, USA). Aminopropyl controlled-pore glass (aminopropyl-CPG, 1400 Å pore diameter, 120–200 mesh) was purchased from CPG (Lincoln Park, NJ, USA). HPLC-grade methanol was obtained from Kanto (Tokyo, Japan). All other chemicals used were of analytical-reagent grade.

2.2. Preparation of immobilized acid phosphatase and enzyme reactor

The procedure for the preparation of immobilized acid phosphatase has been described in detail in a previous paper [2]. In brief, sweet potato acid phosphatase was immobilized by covalent coupling with glutaraldehyde to aminopropyl controlled-pore glass (aminopropyl-CPG) in the presence of phosphate as an enzyme inhibitor. The Shiff base double bond and the residual aldehyde groups were reduced with sodium borohydride. The immobilized acid phosphatase thus obtained was packed into a stainless steel column of 10×4 mm I.D. or 50×4 mm I.D., and used on-line as a pre-column IMER at room temperature. A blank column was prepared in a similar manner as the IMER preparation, except that the aldehyde-activated glass was directly treated with sodium borohydride, and then packed into another column (50×4 mm I.D.).

2.3. Measurement of enzyme activity

The procedure for the measurement of immobilized acid phosphatase activity has also been described in detail in a previous paper [2]. In brief, the activity of immobilized acid phosphatase was determined by a batchwise operation. The preparation of immobilized enzyme was weighed into 10-ml plastic test-tubes with conical bottoms and the assays were run using three different substrates, i.e., *p*-nitrophenyl phosphate, β-glycerophosphate and riboflavin phosphate. When *p*-nitrophenyl phosphate was

used as a substrate, the assay is as follows: 2.0 ml of 0.1 *M* acetate buffer, pH 5.6, was added to the reaction tube containing the gel, and was pre-incubated for 3 min at 40°C. The enzyme reaction was started by adding 0.06 ml of 0.6 *M* disodium *p*-nitrophenyl phosphate. The reaction was allowed to continue for 10 min at 40°C by momentary mixing the suspension with a vortex-mixer at 1 min intervals. After incubation for 10 min, an adequate volume of the supernatant of the reaction mixture was transferred into a test tube, and the total volume was adjusted to 1.0 ml. The *p*-nitrophenol liberated was color-developed by the addition of 2 ml of 0.5 *M* NaOH, and the absorbance was measured at 405 nm by a spectrophotometer using *p*-nitrophenol as a standard. The assay process for β-glycerophosphate or riboflavin sodium phosphate was similar to that described above, except as follows: 2.5 ml of 0.1 *M* acetate buffer, pH 5.6, was added to the reaction tube, and the substrate concentration and the volume were 20 mg/ml in 0.1 *M* acetate buffer, pH 5.6, and 0.5 ml, respectively. After 10 min incubation, aliquots of the supernatant were transferred to a test tube, and the total volume was adjusted to 2.0 ml. The inorganic phosphate enzymatically liberated was color-developed and determined by the measurement of the absorbance at 660 nm by a spectrophotometer using KH_2PO_4 as a standard. The activity of immobilized acid phosphatase was expressed by nmol amounts of product liberation per min per mg of the moist gel [2].

2.4. Preparation of sample solution and standard solution

Water content in the preparations of both FMNs and riboflavin standard used throughout this experiment was measured by the Karl Fischer moisture titrator (MKC-510, coulometric type, Kyoto Electronics Manufacturing, Kyoto, Japan), and the mean values were estimated to be 6.5%, and 0.4% for FMNs and riboflavin standard, respectively. On the basis of these values, FMNs and riboflavin standard were weighed and dissolved as follows. A FMNs stock solution at a concentration of 1.25 m*M* was prepared by dissolving FMNs in water, and a riboflavin standard stock solution at a concentration of 0.125 m*M* was prepared by dissolving riboflavin in a dilute solution of phosphoric acid (1→1000). Working solutions were then diluted with doubly demineralized, distilled water to the required concentrations. Before analysis, the working solutions were filtered using a membrane filter, 0.45 μm pore size, from Millipore (Bedford, MA, USA). The solutions were preserved in light-resistant containers.

2.5. Apparatus and procedures

A schematic diagram of the pre-column IMER/reversed-phase HPLC system for the determination of total riboflavin phosphates (FMNs) is shown in Fig. 1. Three Shimadzu LC-6A pumps (Kyoto, Japan) and two ''six-ported'' switching valves (FCV-2AH, Shimadzu) were controlled by a system controller (SCL-6A, Shimadzu). Three different solutions, specifically two carrier streams, i.e., a weak solution of acetic acid (5 m*M*) and a specific concentration of acetate buffer, pH 5.0, and a mobile phase, were pumped through the individual pumps. Sample solutions containing a specific amount of FMNs were injected through either a Rheodyne Model 7125 injector (Cotati, CA, USA) or an automatic sample injector (SIL-6B, Shimadzu) with a sample volume of 20-μl. The sample solution was passed via a carrier stream containing acetate buffer, pH 5.0, and the flow was then passed through a trap column (TSK guardgel ODS-80Ts, 15×3.2 mm I.D., Tosoh, Tokyo, Japan). After a specific time, the switching valve (SV) on this line, SV-2, was switched, and then the unaltered FMNs and enzymatically hydrolysed riboflavin were back-eluted from the trap column using a mobile phase containing methanol. Chromatographic conditions were set in conformance with those described by Nielsen et al. [3]. The methanol content of the mobile phase was increased to shorten the analytical time. The mobile phase comprised of 0.1 *M* ammonium formate buffer, pH 3.7–methanol (73:27, v/v), was pumped at a flow-rate of 1.0 ml/min. FMNs and riboflavin were subsequently chromatographed on an analytical column (TSK gel ODS-80Ts, 150×4.6 mm I.D., 5-μm particle, Tosoh), and detected by UV absorption at 280 nm with a spectrophotometer (SPD-6AV, Shimadzu) equipped with an 8-μl flow cell. The trap column and analytical column were thermostated at 50°C in a column oven (CTO-6A,

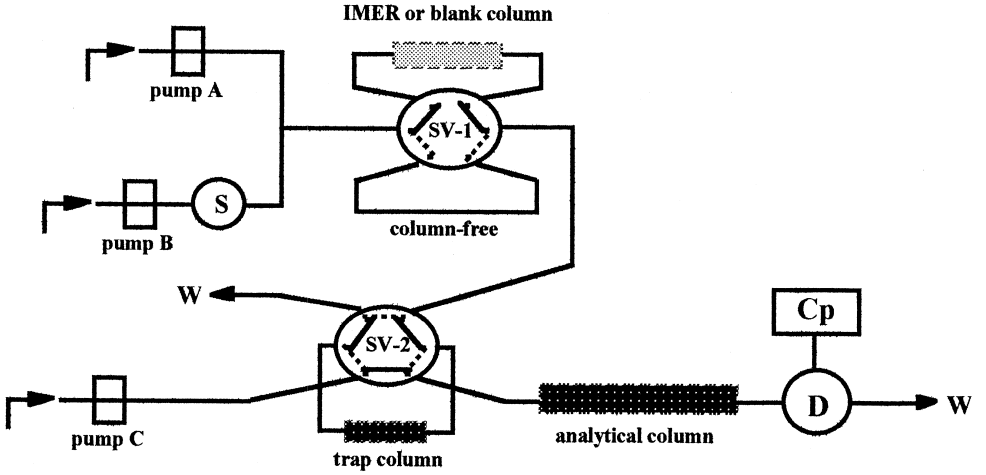

Fig. 1. Schematic diagram of the pre-column IMER/HPLC system for the determination of total FMNs. IMER=immobilized enzyme reactor; pump A=5 m*M* acetic acid; pump B=acetate buffer, pH 5.0; pump C=mobile phase (0.1 *M* ammonium formate buffer, pH 3.7–methanol, 73:27); S=sample injector; SV=switching valve; D=detector; Cp=computing integrator; W=waste.

Shimadzu). Chromatographic data were recorded on a Chromatopac CR-4A integrator (Shimadzu). During the period of the separation and detection, the trap column was programmed to wash with 5 m*M* acetic acid via an opposite IMER line, i.e., a column-free line, and then buffered with acetate buffer, pH 5.0, for the next analysis. The detailed flow paths involved in the series of the experiments are shown in Fig. 2.

3. Results and discussion

3.1. Design of the analytical system

The effectiveness of pre-column reactions using enzymes, regardless of whether the system on-line or off-line and/or soluble or insoluble, has been widely recognized. Enzymes have been used as tools for not only clean-up purposes or pretreatment of analytes but also the identification or peak-shift chromatography of the analytes [9,10]. In spite of this interesting technique, few examples of pre-column, on-line and IMER application have been described in the literature. Bowers and Johnson first demonstrated the concept of an on-line and pre-column modification with the IMER [11–13]. They described a peak corresponding to the retention time of estriol glucuronide which was enzymatically shifted to that of estriol catalysed by immobilized β-glucuronidase. An example regarding on-line pretreatment of analytes with the IMER was reported by Tsai et al. [14]. They used a pre-column IMER of choline oxidase and catalase to eliminate the choline peak, which interferes with the specific determination of acetylcholine using a similar post-column IMER (acetylcholine esterase and choline oxidase enzyme reactor). The enzymatic peak-shift technique with the IMER seems to be useful in performing the automated and on-line determination of total FMNs in pharmaceutical preparations. The fundamental design of the analytical system was based on the principle reported by Johnson and Bowers [13], i.e., the injecting sample was passed through the IMER, and then enzymatically hydrolysed lipophilic riboflavin was trapped onto the top of an ODS trap column (see Fig. 1). The complete conversion of the substrate to product is necessary for pre-column reaction purposes. The effect of flow-rate on the conversion of FMNs to riboflavin was carried out using the IMER of 10×4 mm I.D. and the carrier stream of 0.1 *M* acetate buffer, pH 5.0. The sample solution (1.25 nmol FMNs per 20-μl injection) was injected through an injector and passed through using the flow path shown in (a) in Fig. 2. The switching valve of SV-2 was switched at 10 min after the sample

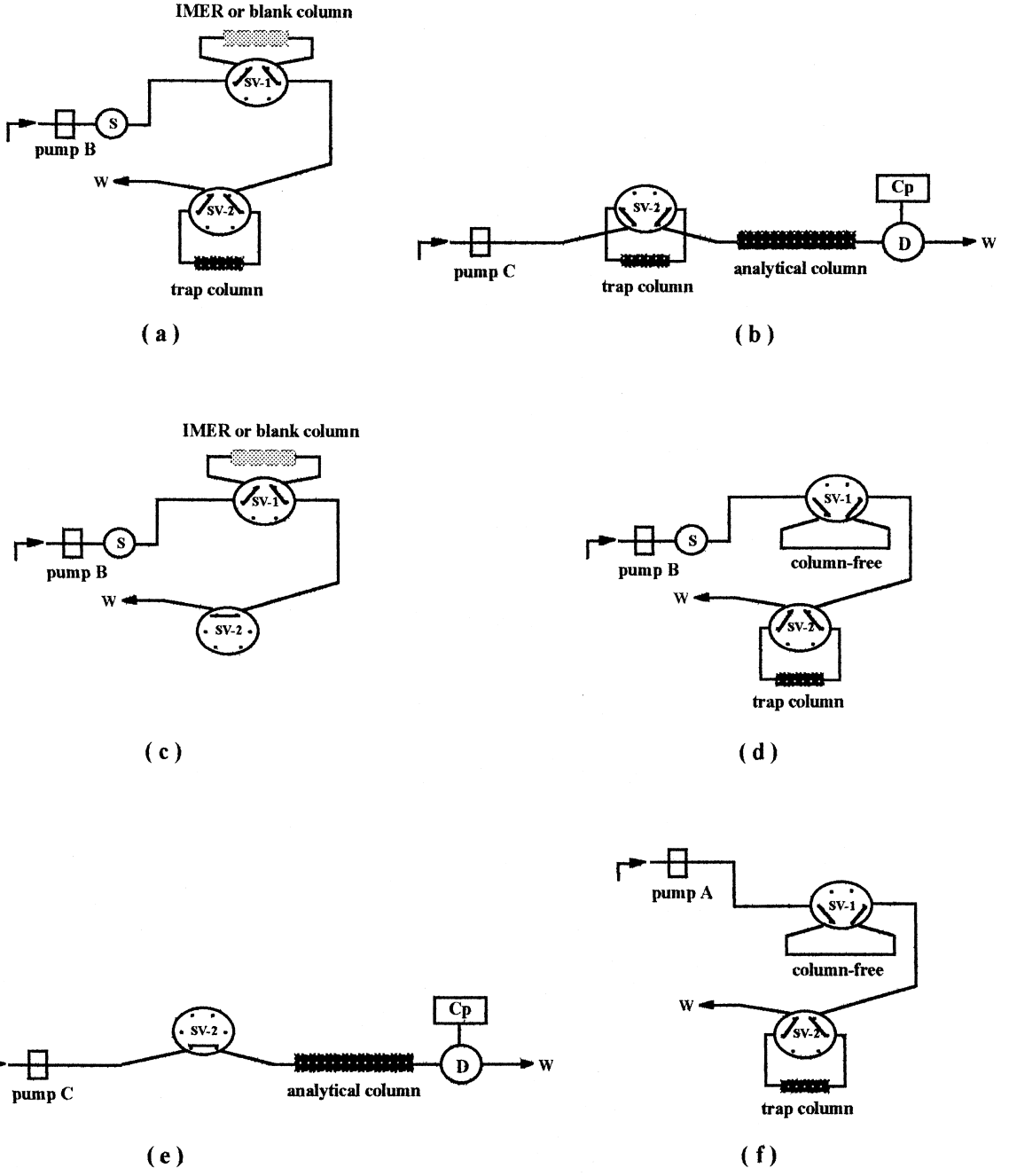

Fig. 2. Detailed flow paths used at various stages throughout the study. (a) Sample load mode, (b) analysis mode, (c) IMER or blank column rinse mode, (d) riboflavin standard load mode, (e) analysis exclusive mode and analytical column rinse mode, (f) trap column rinse mode. Abbreviations as in Fig. 1.

injection, and then the analyte was separated on the analytical column (Fig. 2b). The carrier stream was allowed to continue to flow during analysis. Another detector (D-2) was connected to the waste line of the flow path (c) in Fig. 2 for monitoring slow moving analytes. As shown in Fig. 3, slower flow-rates increased the rate of conversion. The conversion rate of FMNs to riboflavin at the flow-rate of 0.1 ml/min was about 80%, however, a small peak was concomitantly detected by D-2. On the other hand, when riboflavin standard was injected, the resulting peak area exhibited a constant value, regardless of the flow-rate and of the analytical line. The dead volumes, from injector to the top of the trap column, of this system were estimated to be less than 0.23 ml and about 0.1 ml for the lines with the IMER (Fig. 2a) and without the IMER (Fig. 2d), respectively. The eluting time of 10 min (i.e., eluting volume of 1.0 ml at the flow-rate of 0.1 ml/min) should be enough to elute the analytes from the IMER. This means that FMNs must possess a biospecific adsorption to the IMER [13,15], and/or a moderate affinity for the hydrophilic environment of CPG. The flow-rate of 0.1 ml/min required too much time and

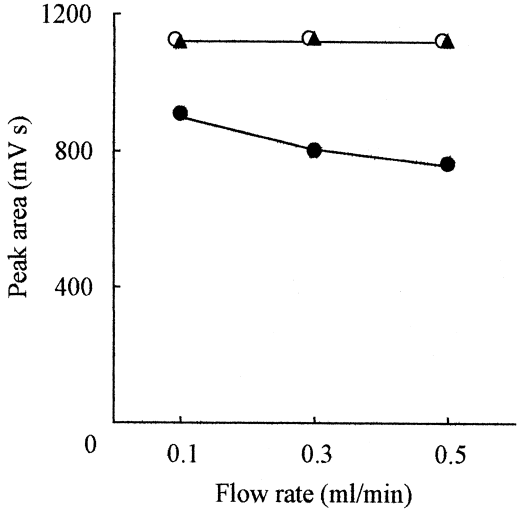

Fig. 3. Effect of flow-rate on the conversion of FMNs to riboflavin, together with that of peak area obtained from riboflavin standard using a different flow path. Samples of FMNs (●) or riboflavin standard (○) were injected using flow path (a) shown in Fig. 2, and samples of riboflavin standard (▲) were injected using flow path (d) shown in Fig. 2. Sample amounts of FMNs and riboflavin standard: 1.25 nmol per 20-μl injection.

was not accurate enough, therefore, we chose the 0.5 ml/min flow-rate. Instead of increasing the flow-rate, the IMER with a column size of 50×4 mm I.D. was used for further research.

3.2. Improvements of the system and the conversion rate

The effects of ionic strength and of pumping time on the adsorption of the analytes to the trap column and/or to the hydrophilic matrices, the IMER, were examined by using the flow paths shown in (d) and (a) in Fig. 2, respectively. The flow path (d) was used for experiments with the trap column. After the sample solution of FMNs was flowed through with either 0.1 *M* or 0.5 *M* acetate buffer at pH 5.0 for a specific time at the flow-rate of 0.5 ml/min, SV-2 was switched over, and the flow path was changed from (d) to (b) as shown in Fig. 2. The analytes on the trap column were then back-eluted and chromatographed on the analytical column. Peaks for 5′-FMN, 4′-FMN, 3′-FMN and riboflavin in the commercial FMNs preparation were identified on the basis of the data reported by Nielsen et al. [3], and riboflavin diphosphates were negligible. Fig. 4 shows the results of this experiment. In the flow path of the column-free line, all substances in the FMN-preparation appeared and they were adsorped to the trap column at the pumping time of 1 min when either 0.1 *M* or 0.5 *M* acetate buffer was used as the carrier (Fig. 4a and b). An increase in ionic strength caused the rapid release from the trap column (Fig. 4b). The blank column was used in the place of the IMER to examine the adsorption to the hydrophilic matrices (Fig. 2a). Not all of the FMNs appeared in the trap column when the ionic strength was low even with a flow time of 10 min (Fig. 4c). The results prove hydrophilic FMNs were retained in a CPG hydrophilic environment under low ionic strength. Hydrophilic FMNs were adsorbed to the hydrophobic trap column at a lower level, whereas they had a moderate affinity for the IMER with a CPG hydrophilic environment. Increasing the ionic strength, however, suppressed the adsorption of FMNs to the hydrophilic matrices and a pumping time of 5 min was sufficient for the FMNs to flow through the hydrophilic matrices (Fig. 4d). Lipophilic riboflavin was not adsorbed at all to the hydrophilic matrices, but

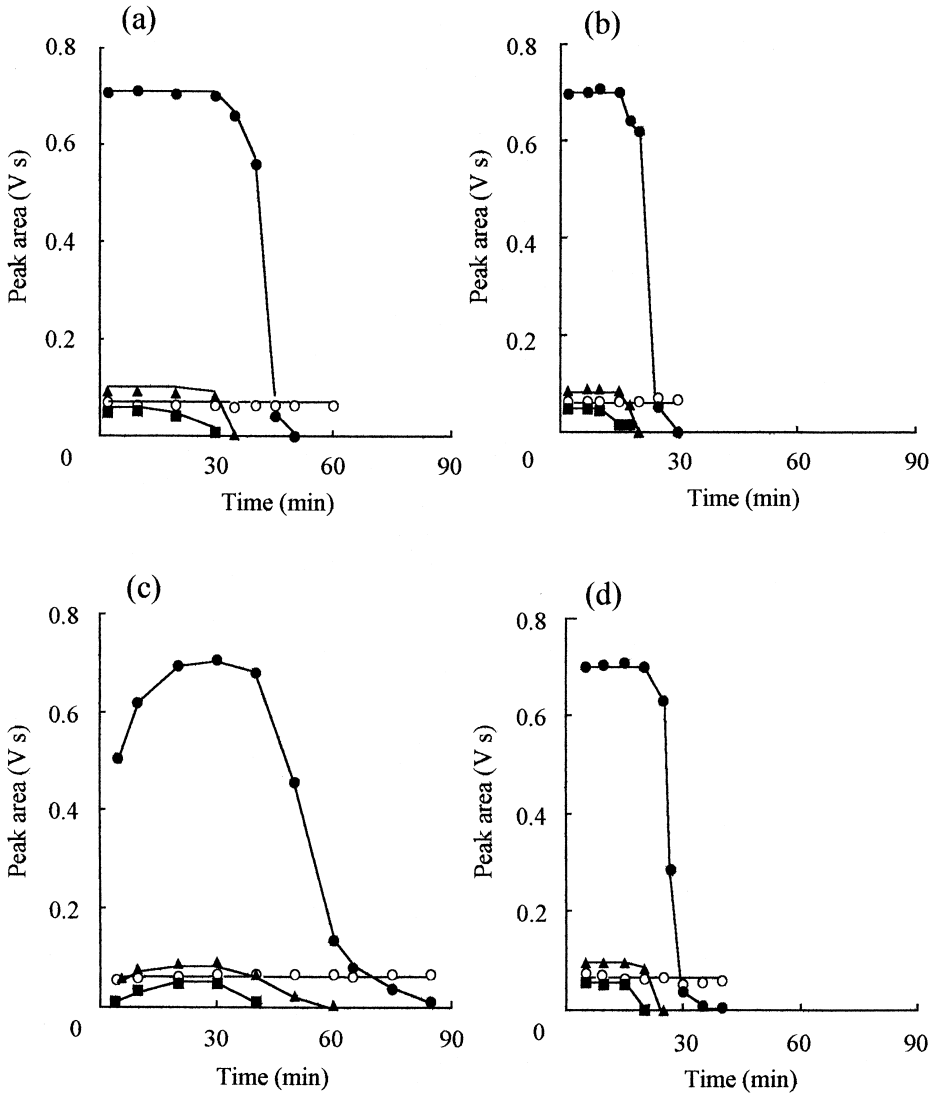

Fig. 4. Effects of ionic strength and of pumping time on the adsorption of the analytes to the trap column and/or to the hydrophilic matrices. Flow path (d) shown in Fig. 2 was used in (a) and (b). Flow path (a) with the blank column shown in Fig. 2 was used in (c) and (d). 0.1 *M* Acetate buffer, pH 5.0 was used in (a) and (c), and 0.5 *M* acetate buffer, pH 5.0 was used in (b) and (d). Flow-rate of pump B=0.5 ml/min. Symbols: ●=5′-FMN; ▲=4′-FMN; ■=3′-FMN; ○=riboflavin.

well adsorbed to the trap column. In order to secure the conversion of FMNs to riboflavin by the IMER, a pumping time of 10 min at a flow-rate of 0.5 ml/min was used for further research. This procedure increased the conversion rate with the IMER up to 90%, however, a small 4′-FMN peak was still observed on the chromatogram. The influence of the added compounds on the conversion of FMNs to riboflavin was further examined. We have tried to increase the conversion rate by adding KCl, citrate buffer (pH 5.0), ethylenediaminetetraacetic acid disodium salt (EDTA), ethanol or methanol to a sample solution. The final concentration in the sample solution was 150 m*M* and 50% for salts and alcohols, respectively. The addition of KCl, ethanol or methanol brought the conversion rate to 91–93%.

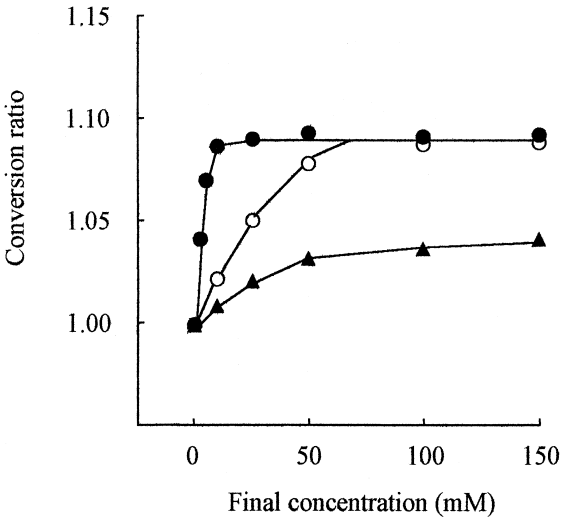

Fig. 5. Effects of additives and their concentrations on the conversion of FMNs to riboflavin. Symbols: ●=citrate; ○=EDTA; ▲=lactic acid.

When the sample solutions containing citrate or EDTA were analyzed, the 4′-FMN peak almost completely disappeared, and a conversion rate of approximately 96% was reached. The addition of citrate or EDTA in the carrier stream was shown to be ineffective. Fig. 5 shows the result of an experiment which measured the conversion ratio as a function of the concentration of citrate, EDTA or lactic acid. In particular, the addition of citrate in the sample solution was extremely effective, and 50 m*M* at the final concentration was sufficient. Citrate has a weak chelating action on metals like that of EDTA. However, the effective concentration of citrate was lower than that of EDTA, and hence the chelating effect may not have been as important in increasing the conversion rate. Certain phosphatases had associated phosphotransferase, which transfer the phosphate group from phosphomonoesters to suitable hydroxy compounds as phosphate acceptors [16]. The shift of the reaction equilibrium may have been

Table 1
Time program of the on-line pre-column IMER/HPLC analytical system for the determination of total FMNs[a]

Step (min)	Duration (min)	Flow path (see Fig. 2)	Flow-rate (ml/min)	Action
1 (0)	10	a	0.5	Inject sample and flow with pump B
2 (0)	10	e	0.1	Rinse analytical column with pump C
3 (10)				Switch the flow path by SV-2 to analyze with analytical column
4 (10)	10	b	1.0	Elute from the trap column and followed by HPLC analysis with pump C
5 (10)				Start Cp and record the chromatogram
6 (10)	10	c	1.0	Rinse the IMER with pump B
7 (20)				Switch the flow path by SV-1 to prepare for the next analysis
8 (20)				Switch the flow path by SV-2 to prepare for the next analysis
9 (20)	10	e	1.0	Continue HPLC analysis
10 (20)			0	Stop pump B
11 (20)	5	f	1.0	Rinse the trap column with pump A to remove mobile phase solvent
12 (25)			0	Stop pump A
13 (25)				Switch the flow path by SV-1 to prepare for the next analysis
14 (25)	5	a	1.0	Rinse both the trap column and the IMER with pump B
15 (30)			0	Stop pump B
16 (30)			0	Stop pump C
17 (30)				Stop Cp and complete a cycle of analysis

[a] Pump A=5 m*M* acetic acid, pump B=0.5 *M* acetate buffer, pH 5.0, pump C=mobile phase. Cp and SV as in Fig. 1. For determining riboflavin standard, flow path (d) was used in the place of flow path (a) at step 1.

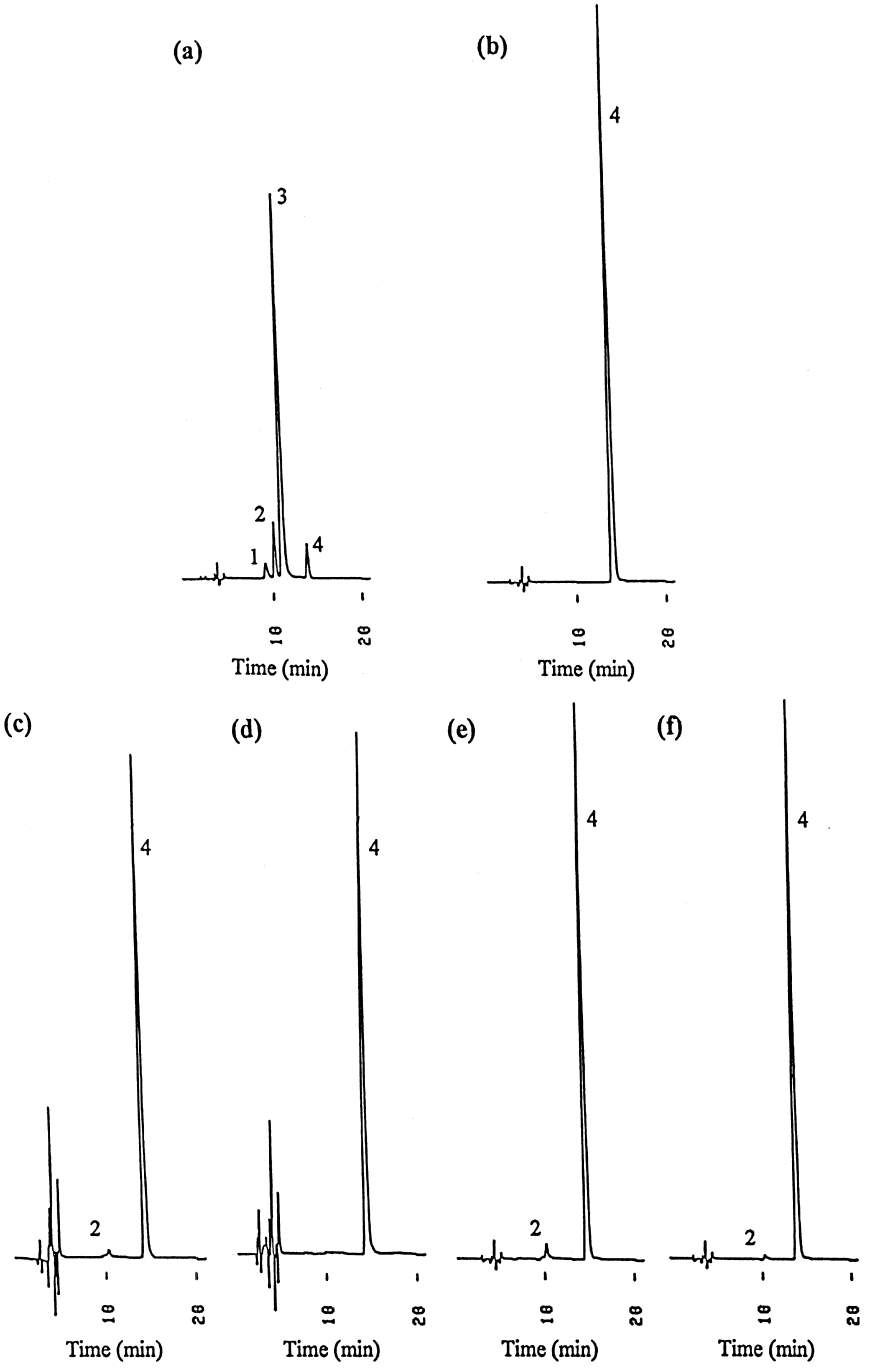

Fig. 6. Chromatograms of FMN preparations and riboflavin standard obtained using the on-line automated pre-column IMER/HPLC system. (a) and (b) were measured without the IMER. (c) through (f) were measured with the IMER. Sample and injection amounts: (a) FMNs, 1.25 nmol per 20-μl injection; (b) riboflavin, 1.25 nmol per 20-μl injection; (c) FMNs in the absence of citrate, 0.125 nmol per 20-μl injection; (d) FMNs in the presence of citrate, 0.125 nmol per 20-μl injection; (e) FMNs in the absence of citrate, 1.25 nmol per 20-μl injection; (f) FMNs in the presence of citrate, 1.25 nmol per 20-μl injection. Peaks: 1=3′-FMN; 2=4′-FMN; 3=5′-FMN; 4=riboflavin.

a cause of the increased conversion rate. Compounds, with a hydroxy group, i.e., lactic acid, ethanol and methanol, did not substantially increase the conversion rate. The hydroxy group of citrate does not appear to be essential. Further experimental research concerning the effect of citrate on the configurational relationship between enzyme, the IMER, and substrate, 4′-FMN, remains to be done in the future.

3.3. On-line automated system operation

It was found based on careful study that the optimal conditions for the conversion of FMNs to riboflavin by the pre-column IMER system are an IMER size of 50×4 mm I.D., carrier stream with a 0.5 *M* acetate buffer, pH 5.0, addition of citrate to the sample solution at the final concentration of 50 m*M*, pumping time of 10 min at a flow-rate of 0.5 ml/min. Table 1 shows the essential steps of a pump program for performing the repeated use of the on-line analysis with automated sample injector. One pre-column IMER/HPLC analysis cycle is completed in 30 min, and the IMER and the trap column can be prepared during analysis for the next analysis. Peak areas when riboflavin standard solutions were injected and flowed through either the flow path with the IMER (Fig. 2a) or that without the IMER, i.e., the column-free line (Fig. 2d), were consistent with each other. The same data is shown in Fig. 3. The flow path shown in Fig. 2d was used for the calibration of riboflavin standard to avoid damaging the IMER. The immobilized acid phosphatase was freshly packed and used as the IMER in order to obtain typical chromatograms and the data for functional evaluation of this system. The results appear in Fig. 6, which shows the chromatograms obtained from the IMER reactions of FMNs preparations in the presence or absence of citrate, together with those obtained from the column-free line.

3.4. Functional evaluation of the pre-column IMER/HPLC system

A calibration graph for total FMNs, obtained by this IMER/HPLC method and determined in the presence of citrate, was linear over the wide range 0.5–500 nmol per ml (255 ng–255 μg per ml). The regression equation and the correlation coefficient (r) for the peak area (y, mV s) versus the concentration of FMNs preparations (x, nmol/ml) was $y=17.268x-2.651$ and $r=0.9999$ ($n=48$), respectively. The detection limit was 25 pmol per ml at a signal-to-noise ratio of 3. The calibration graph for riboflavin standard ranging from 0.5 to 125 nmol per ml also showed excellent linearity. The regression equation was $y=17.823x-2.910$ ($n=24$, $r=0.9995$). Consequently, the average conversion rate of FMNs to riboflavin was estimated at 97%. The intra-assay precision was determined five times each on the same day with an injection amount of 1.25 nmol (62.5 nmol per ml). For determining the inter-assay precision, a sample identical with that employed for intra-assay was measured once on each of 7 days. The relative standard deviations (RSDs) of the intra-assay and the inter-assay precision were 1.2% and 2.6%, respectively. The conversion rate in the presence of citrate, even after the repeated use of 600 samples, was over 95% at the injecting amount of 1.25 nmol. The addition of citrate increased the conversion rate, however, the increment was slightly different at the stage of the repeated use of the IMER. The analytical system described herein appears to be attractive in terms of analytical methodology for the determination of total FMNs, and should be applicable to a variety of multivitamin preparations with a slight modification.

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