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Simultaneous determination of riboflavin phosphate and other ingredients in a multivitamin pharmaceutical preparation by on-line automated LC coupled with pre-column immobilized enzyme reactor

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Abstract

An automated chromatographic detection system for the simultaneous determination of riboflavin phosphate, caffeine, nicotinamide and pyridoxine hydrochloride in a multivitamin pharmaceutical preparation was constructed. Hydrolytic pretreatment of riboflavin phosphate to riboflavin was carried out using a pre-column enzyme reactor, in which immobilized sweet potato acid phosphatase was packed, and then enzymatically hydrolyzed riboflavin and other ingredients in the pharmaceutical preparation were concentrated in an ODS trap column. The concentrated riboflavin and other ingredients were back-eluted from the trap column using a mobile phase containing 1-decanesulfonate as an ion-pair reagent, and then subsequently chromatographed on an ODS analytical column. It was necessary to wash the ODS trap column with aqueous acetonitrile to remove 1-decanesulfonate in the trap column, which is advantageous to concentrate the riboflavin and other ingredients for the subsequent analysis. The calibration curves for riboflavin phosphate and other ingredients were linear over the concentration ranges tested, and correlation coefficients for standard curves were 0.9999 for all four ingredients. Analytical recoveries of the four ingredients at different levels of concentration added to the ordinary pharmaceutical preparation were also in the range of 99.1–101.2%. The present method was superior to the ordinary manual and batch-wise enzymatic methods in being harmless to the environment, rapid and accurate under continuous autoanalysis.

Keywords: Riboflavin phosphate; Immobilized enzyme reactor; Simultaneous determination; Automation; Acid phosphatase; Multivitamins

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1. Introduction

Riboflavin, vitamin B_2, plays an important role in biochemical redox reactions in the form of two coenzymes, riboflavin monophosphate (FMN) and flavin adenine dinucleotide (FAD). Riboflavin is only slightly soluble in water; therefore, hydrophilic commercial FMN is blended with other vitamins for a pharmaceutical liquid preparation, 'Multivitamin Drink', which is ingested as a supplement for lack of the vitamins in the daily diet. On the other hand, as is described in the introduction section of the preceding paper, commercial FMN preparations are a complex mixture of various riboflavin phosphates and are hygroscopic, making it difficult to obtain commercial FMN in pure form. Furthermore, quantitative determination of an individual riboflavin phosphate is not of fundamental significance for quality control of manufactured pharmaceutical preparations, but that of total riboflavin is essential. In addition, riboflavin standard can easily be obtained [1]. Total riboflavin in a pharmaceutical product is currently determined by manual spectrophotometric or fluorometric methods [2–5], which are time-consuming during the assay procedure and/or harmful to the environment due to chloroform extraction. The batch-wise enzymatic conversion of riboflavin phosphates to riboflavin, using phosphate-hydrolyzing enzymes such as alkaline phosphatase [6], a mixture of takadiastase/β-amylase [7], or wheat germ acid phosphatase [8], prior to high performance liquid chromatographic (HPLC) analysis is a useful technique for the determination of total FMN. These enzymes convert hydrophilic FMN to a single molecular structure of hydrophobic riboflavin. Lam and Lowande pointed out that enzymatic conversion of FMN to riboflavin had two specific advantages: (1) a pure reference standard, riboflavin, can be used, and (2) multiple peaks of FMN, which are poorly resolved from those of other vitamins, for example, pyridoxine hydrochloride, can be eliminated in the chromatographic separation [6].

In the preceding paper, we reported a highly efficient HPLC system for the determination of total riboflavin. Immobilized sweet potato acid phosphatase was used as an immobilized enzyme reactor (IMER), and the IMER was then incorporated in an on-line pre-column analytical system for automatic determination of total riboflavin phosphates [1].

In this study, we have successfully achieved the simultaneous determination of riboflavin phosphate, caffeine, nicotinamide and pyridoxine hydrochloride in a multivitamin pharmaceutical liquid preparation, 'Multivitamin Drink', using an automated pre-column IMER/reversed-phase HPLC system, and a few new findings has been also found.

2. Experimental

2.1. Materials

Riboflavin sodium phosphate (FMNs) raw material was purchased from F. Hoffmann-La Roche (Basel, Switzerland). 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 ammonium sulfate (1.8 M) and magnesium chloride (10 mM) (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 acetonitrile was obtained from Kanto Chemical (Tokyo, Japan). 'Multivitamin Drink', a commercial pharmaceutical liquid preparation, was supplied from Taisho Pharmaceutical Co. All other chemicals used were of analytical-reagent grade.

2.2. Preparation of immobilized acid phosphatase and enzyme reactor

The procedure for preparation of immobilized acid phosphatase and IMER was described in previous papers [1,9]. In brief, sweet potato acid phosphatase was immobilized by covalent coupling with glutaraldehyde to aminopropyl con-

trolled-pore glass (aminopropyl-CPG). 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 50 × 4 mm ID, and used on-line as a pre-column IMER at 30°.

2.3. Preparation of sample solution and standard solution

The preparation of both FMNs and riboflavin standard solution has been described in detail in a previous paper [1]. Standard preparations of caffeine, nicotinamide and pyridoxine hydrochloride were dried sufficiently, and then dissolved in citrate buffer (pH 5.0; 50 mM) prior to use.

2.4. Spectrophotometric, fluorometric and batch-wise enzymatic HPLC methods

The quantitative result of the on-line pre-column IMER/HPLC determination reported here was compared with that obtained by the spectrophotometric method [3], indirect fluorometry (lumiflavin method) [5] and the batch-wise enzymatic HPLC method [8].

2.5. Apparatus and procedures

A schematic diagram of the pre-column IMER/reversed-phase HPLC system is shown in Fig. 1. The flow diagram was substantially the same as given in the previous paper, except that one solvent was replaced. Rinsing of the trap column with a pump connected to a solvent of a weak solution of acetic acid was not necessary, since we have found that washing with a carrier stream, i.e. acetate buffer (pH 5.0; 0.5 M) suffices for rinsing of the trap column. Alternatively, a rinse-solvent, i.e. water–acetonitrile–phosphoric acid (700:300:1, v/v/v), was pumped using this pump.

2.5.1. General design of system

Two Shimadzu LC-10A pumps (Kyoto, Japan), two 'six-ported' switching valves (SCV-14H, Shimadzu), a column oven (CTO-10AC, Shimadzu) and an automatic sample-injector (SIL-10A, Shimadzu) were controlled by a system controller (SCL-10A, Shimadzu).

2.5.2. Sample load and enzymatic reaction mode

The IMER thermostated at 30 °C in a column oven (CTO-10AC, Shimadzu) was pre-equilibrated with the carrier stream of acetate buffer

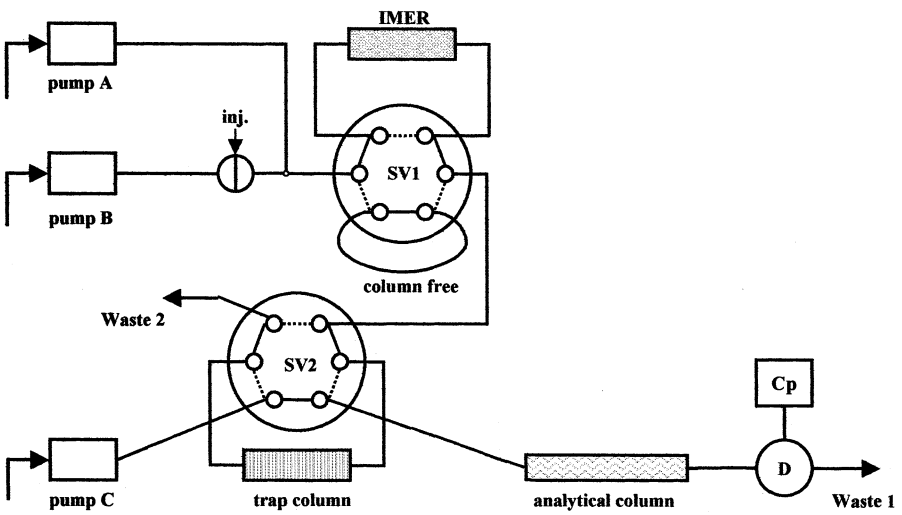

Fig. 1. Flow paths used throughout the study. IMER, immobilized enzyme reactor; pump A, rinse-solvent, i.e. water–acetonitrile–phosphoric acid (700:300:1, v/v/v); pump B, acetate buffer (pH 5.0; 0.5 M); pump C, mobile phase (water–acetonitrile–phosphoric acid containing 1-decanesulfonate); inj., sample injector; SV, switching valve; D, UV detector; Cp, computing integrator.

Table 1
Retention capacity of the trap columns

Column	Retention time (min)			
	Riboflavin	Caffeine	Nicotinamide	Pyridoxine
ODS	>60	>60	15	30
CN	24	15	8	6
Phenyl	>60	>60	14	11

Selection of proper trap column was performed using flow path (b) in Fig. 1. ODS = YMC-Guardpack ODS-AM, 30 × 10 mm ID CN = YMC-Guardpack CN, 30 × 10 mm ID ODS = YMC-Guardpack Ph 30 × 10 mm ID.

(pH 5.0; 0.5 M) prior to sample injection. The sample solution containing FMNs was injected through an automatic sample-injector with a sample volume of 20 μl. Subsequently, the sample solution was brought to IMER through the carrier stream, and FMNs in the sample solution were converted to riboflavin by the reaction of IMER. Hydrolyzed riboflavin and other ingredients (caffeine, nicotinamide, pyridoxine hydrochloride and internal standard) in the sample solution were then passed through a trap column (YMC-pack ODS-50, 30 × 10 mm ID, Tokyo, Japan).

2.5.3. Analysis mode

Ten minutes after sample injection, the switching valve (SV) on this line, SV-2, was switched, and then enzymatically hydrolyzed riboflavin and other ingredients were back-eluted from the trap column using a mobile phase containing acetonitrile. The mobile phase comprised of water–acetonitrile–phosphoric acid containing 1-decanesulfonate as an ion pair reagent was pumped at a flow-rate of 1.0 ml/min. The analytes were subsequently chromatographed on an analytical column (TSK gel ODS-80Ts, 250 × 4.6 mm ID, 5 μm particles, Tosoh), and detected by UV absorption at 280 nm with a spectrophotometer (SPD-10AV, Shimadzu) equipped with an 8 μl flow cell. The analytical column was thermostated at 50 °C in a column oven (TU-300, JASCO). Chromatographic data were recorded on a Chromatopac CR-7A integrator (Shimadzu). During the period of separation and detection, the trap column was programmed to wash with the rinse-solvent via an opposite IMER line, i.e. a column-free line, and then buffered with acetate buffer (pH 5.0), for the next analysis.

3. Results and discussion

3.1. Selection of proper trap column

The trap column plays an important role in the pre-column IMER/HPLC system, i.e. riboflavin and other ingredients (caffeine, nicotinamide and pyridoxine hydrochloride) must be retained onto the trap column during sample load mode. An

Table 2
Comparison of methods for determination of FMNs as total riboflavin

n	FMNs purity as riboflavin(%)			
	Manual methods		HPLC	
	Spectrophotometry	Fluorometry	Batch wise enzymatic	IMER/HPLC
1	97.3	87.0	94.1	97.3
2	96.5	86.3	94.1	97.1
3	96.6	87.3	94.0	97.7
4	96.9	85.7	94.4	97.3
5	96.6	87.2	94.5	97.4
6	96.9	86.8	94.2	97.6
Average ± R.S.D. (%, $n = 6$)	96.8 ± 0.3	86.8 ± 0.7	94.2 ± 0.2	97.4 ± 0.7

R.S.D., relative standard deviation.

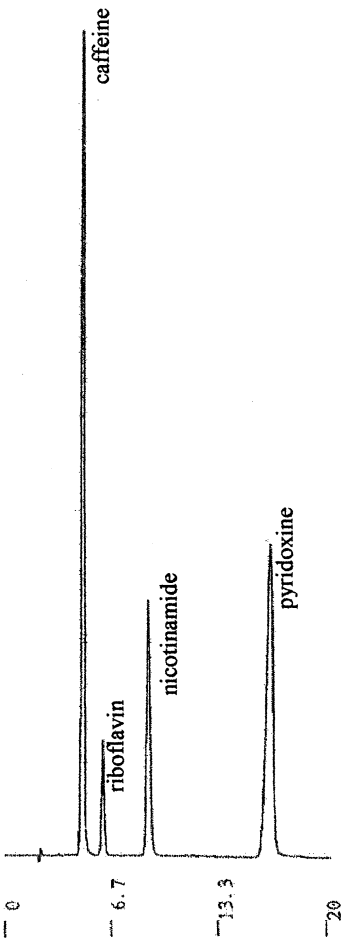

Fig. 2. Typical chromatogram of four standard preparations obtained using ion-pair RP-HPLC. Analytical column, TSKgel ODS80Ts, 150 × 4.8 mm ID; mobile phase, water–acetonitrile–phosphoric acid (860:140:1, v/v/v) containing 1-decanesulfonate (2.5 mM).

ODS trap column (TSK guardgel ODS-80Ts) with a size of 15 × 3.2 mm ID was sufficient for absorption of lipophilic riboflavin [1]. However, hydrophilic vitamins in the pharmaceutical liquid preparations are normally very rapidly eluted from ODS column. The results of previous experiments indicated that a pumping time of 5 min was sufficient for the hydrophilic FMNs to flow through the IMER column and to reach 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 [1]. Therefore, the capacity of the trap column was necessary to retain all of the ingredients during a pumping time of 10 min. The selection of a proper trap column was performed by using the column free line in Fig. 1, and the effluent was monitored by another detector on the waste line (Waste 2). The ODS and phenyl trap columns retained all four ingredients well, and the retention capacity of the ODS trap column for nicotinamide was slightly higher than that of phenyl trap column. As shown in Table 1, the ODS trap column (YMC Guard Pack ODS-AM) with a large size of 30 × 10 mm ID was adequate for simultaneous determination of riboflavin phosphate, caffeine, nicotinamide and pyridoxine hydrochloride in multivitamin pharmaceutical liquid preparations.

3.2. Comparison of analytical methods for the determination of total riboflavin phosphate

At present, spectrophotometric and fluorometric methods are used exclusively for the determination of riboflavin phosphate in pharmaceutical products [2–5]. Spectrophotometric and direct fluorometric methods are based on the absorption of the isoalloxazine structure of the molecule, and therefore, have been successfully applied to pharmaceutical products with a high purity [2–4]. The lumiflavin fluorometric method, in which riboflavin is irradiated in alkaline solution to produce lumiflavin, which is extracted from the acidified solution with chloroform, is specific for riboflavin and its derivatives [5]. The lumiflavin method is more sensitive than the spectrophotometric one, and can be used to determine riboflavin in complex materials. We have compared our on-line IMER/HPLC method with manual methods described above and with the batch-wise enzymatic HPLC method. As shown in Table 2, the IMER/HPLC method was equivalent to the spectrophotometric method and slightly superior to the batch-wise enzymatic HPLC method. The lumiflavin method was somewhat inferior to other methods, and the reason for which is unclear. In addition, the lumiflavin method requires a chloroform extraction step, is therefore, harmful to health and the environment. The on-line IMER/

HPLC method was superior to the batch-wise enzymatic method in terms of detectability of FMNs and the possibility of continuous autoanalysis.

3.3. Analytical system for multivitamin pharmaceutical preparation

Reversed-phase HPLC (RP-HPLC) is commonly used for determining riboflavin and caffeine [10,11], whereas ion-pair RP-HPLC using a mobile phase containing an anionic counter ion, such as heptanesulfonate or octanesulfonate, is utilized to determine nicotinamide and pyridoxine hydrochloride [12,13]. The latter method of HPLC may be used to advantage in simultaneous determination of riboflavin, nicotinamide and pyridoxine hydrochloride in multivitamin pharmaceutical preparations, as found in several papers [6,13,14]. Initially, sample injection was carried out using an ordinary RP-HPLC system, i.e. in the absence of IMER and the trap column. Both nicotinamide and pyridoxine were rapidly eluted and poorly resolved on RP-HPLC, while good chromatographic separation was obtained on ion-pair RP-HPLC using aqueous acetonitrile containing 1-decanesulfonate as an ion pair reagent. The retention of nicotinamide and pyridoxine, which have a pyridine ring, was enhanced with increasing concentrations of 1-decanesulfonate as shown in Fig. 2. When sample was injected into the on-line IMER/HPLC system shown in Fig. 1, only the chromatogram obtained by a first sample injection was satisfactory. Then unusual chromatograms that revealed abnormal retention behavior for nicotinamide and pyridoxine continuously appeared. This phenomenon disappeared when the trap column was washed by a rinse-solvent, i.e. water–acetonitrile–phosphoric acid (700:300:1, v/v/v), and subsequently by acetate buffer (pH 5.0; 0.5 M) prior to the next sample injection. Fig. 3 shows an illustration of possible retention behavior of the analytes in the trap column. After the analytes were back-eluted against the analytical column, merely washing the trap column by the carrier stream of acetate buffer (pH 5.0; 0.5 M) was insufficient to remove ion-pair reagent. Consequently, 1-decanesulfonate in the mobile phase remained along the gel surface of the trap column. When the next sample was injected and then analytes including riboflavin enzymatically converted from FMNs were introduced into the trap column via the carrier stream, nicotinamide and pyridoxine were ion-paired with 1-decanesulfonate and widely diffused along the trap column. Therefore, the retention behavior of neither nicotinamide nor pyridoxine in the trap column was constant. When the sample was injected after removing 1-decanesulfonate from the trap column with the

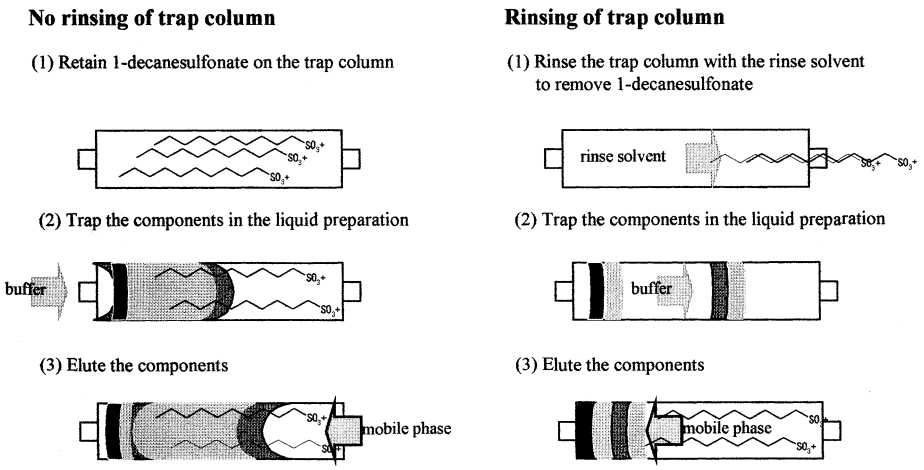

Fig. 3. Scheme of behavior of riboflavin and other ingredients in the trap column.

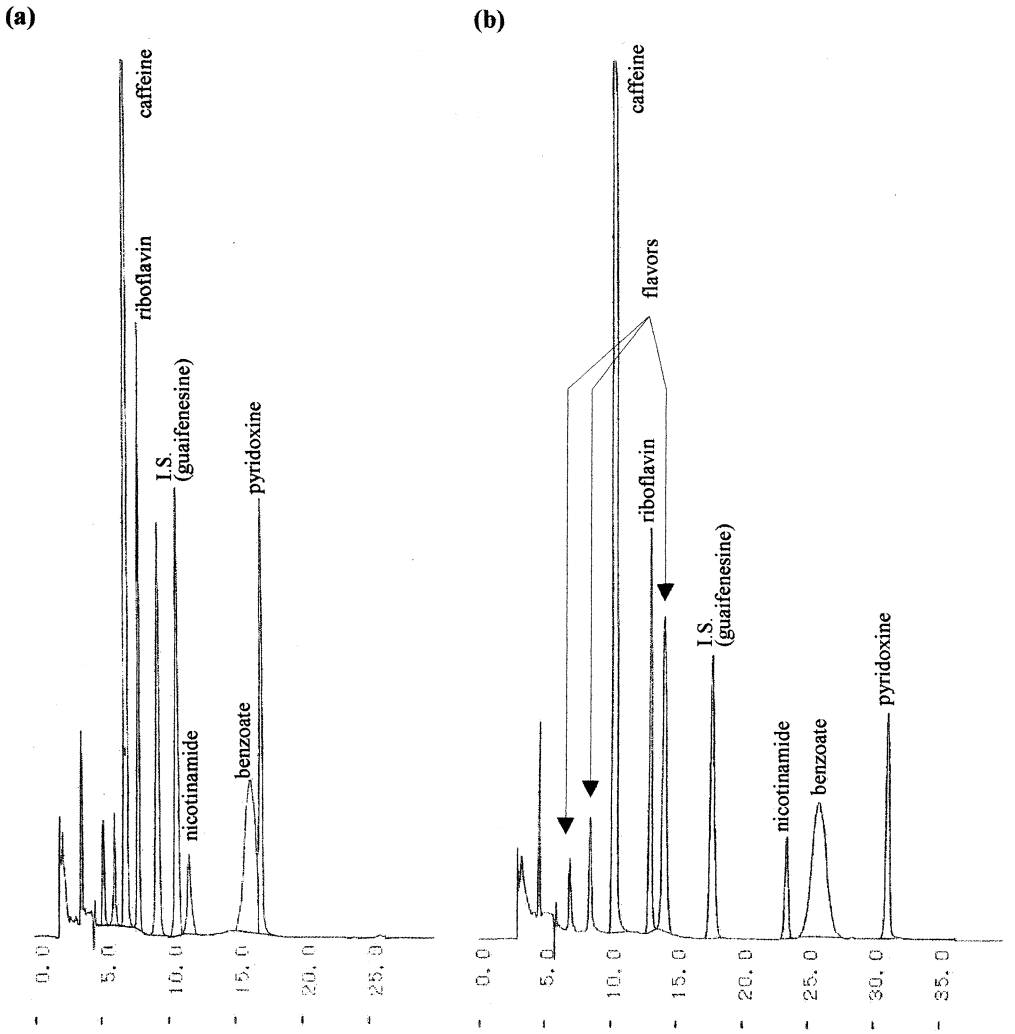

Fig. 4. Chromatograms of FMNs and other ingredients in the commercial liquid preparation 'Multivitamin Drink' obtained using the pre-column IMER/HPLC system. (a) analytical column, TSKgel ODS80Ts, 150 × 4.8 mm ID; mobile phase, water–acetonitrile–phosphoric acid (860:140:1, v/v/v) containing 1-decanesulfonate (5.0 mM); (b) analytical column, TSKgel ODS80Ts, 250 × 4.8 mm ID; mobile phase, water–acetonitrile–phosphoric acid (880/120/1, v/v/v) containing 1-decanesulfonate (7.5 mM).

rinse-solvent and then equilibrating with carrier stream, the four ingredients were concentrated on the trap column with regularity, and reproducible chromatograms were also obtained. The optimization of the proposed procedure can be thus achieved by both the selection of a proper trap column and the maintenance of the condition of the trap column. The chromatogram in Fig. 4(a) shows practical application of this on-line IMER/HPLC technique to a multivitamin formulation, 'Multivitamin Drink', a commercial liquid preparation. The resolution of pyridoxine and benzoate as a stabilizer was not complete. Complete separation was achieved by decreasing the ratio of acetonitrile in mobile phase, and by increasing the size of the analytical column and the concentration of 1-decanesulfonate, as shown in Fig. 4(b).

3.4. Validation of simultaneous determination of pharmaceutical ingredients

Phosphoric salts are known to inhibit phosphtatase activity. Therefore, we performed a comparative study of the effect of phosphoric acid added to substrate, i.e. FMNs, on the rate of enzymatic conversion of FMNs to riboflavin. As shown in Table 3, phosphoric acid suppressed the enzymatic conversion of FMNs to riboflavin in the batch-wise enzymatic HPLC method, whereas no remarkable inhibition was observed with the on-line pre-column IMER/HPLC method. This result indicates that phosphoric salt co-injected with FMNs can be separated from FMNs in the column of IMER, and that can be used for the pharmaceutical liquid preparations containing phosphoric salt. The analytical validity of determination of FMNs, caffeine, nicotinamide and pyridoxine in the pharmaceutical liquid preparations was proven with the internal standard method. Guaifenesin was used as an internal standard (I.S.), and the peak that appeared in the chromatogram can be seen in Fig. 4. The results of validation are summarized in Table 4. The concentrations of the four ingredients in 'Multivitamin Drink', a commercial preparation, were 50, 500, 200 and 50 μg/ml for FMNs, caffeine, nicotinamide and pyridoxine hydrochloride, respectively. A standard solution containing the internal standard was prepared by diluting the above commercial preparation with citrate buffer (pH 5.0; 50 mM) and/or by adding the standard preparations to the required concentrations. Studies on the stability of analytes in standard working solution showed that there were neither decomposition products in the chromatogram nor quantitative difference in peak area during analytical procedure. The ratios of peak areas of four standard preparations to that of I.S. (y) versus the concentration of four ingredients (x, μg/ml) were calculated for the regression equation and correlation coefficient (r). The correlation coefficients for all four ingredients revealed excellent linearity. Recoveries of three different concentrations added to the commercial liquid preparation ranged from 99.1 to 101.2%, with relative standard deviations (R.S.D) less than 1.0%. As a result of the pulse distortion of the benzoate peak, the limit of detection (LOD), based on a signal-to-noise (S/N) ratio of 3, for nicotinamide was inferior to other ingredients. These analytical validation data prove that the on-line automated pre-column IMER/HPLC system exhibit accuracy and precision in the simultaneous determination of ingredients in pharmaceutical products. The IMER retained its original activity even after the repeated use of more than 100 samples of 'Multivitamin Drink', and the reproducibility between different IMERs is also observed.

Table 3
Effect of phosphoric acid in sample solution on acid phosphatase activity

Concentration of phosphoric acid (μmol/ml)	Relative enzyme activity(%)	
	Batch wise enzymatic	IMER/HPLC
0.0	100.0	100.0
1.3	87.8	97.5
2.6	83.5	96.0
5.2	75.7	84.0
26.0	5.7	86.5

The concentration of FMNs in simple solution was determined at 5 μg/ml.

4. Conclusions

In this paper, an automated pre-column IMER/reversed-phase HPLC system has been relevantly developed for the simultaneous determination of four ingredients in a multivitamin pharmaceutical liquid preparation, 'Multivitamin Drink', and a few new findings has been also contained. Simultaneous determination was carried out with the aid of an ion pair reagent, 1-decanesulfonate. Significant aspects of the simultaneous HPLC analysis were selection of a proper trap column and maintenance of the condition of the trap column. Removal of ion pair reagent from the ODS trap column was the most important factor in obtaining reproducible chromatographs for riboflavin and other ingredients under continuous

Table 4
Validation data for determination of the ingredients of 'Multivitamin Drink' on the IMER/HPLC system

Components	Linearity				LOD (μg/ml)	Recovery and precision		
	Concentration range (μg/ml)	r	Intercept	Slope		Added (μg/ml)	Recovery ± R.S.D. (%, $n = 3$)	Mean values (%, $n = 9$)
FMN	0.5–20	1	−0.0051	0.1646	0.05	3.8	99.3 ± 0.4	99.8 ± 0.5
						5.0	100.3 ± 0.4	
						6.3	99.8 ± 0.2	
Caffeine	1–200	1	0.0475	0.1685	0.01	38.1	100.7 ± 0.5	100.9 ± 0.4
						50.9	100.7 ± 0.3	
						63.6	101.2 ± 0.3	
Nicotinamide	2–80	1	−0.0046	0.0141	0.4	15.2	100.0 ± 0.2	99.7 ± 0.6
						20.3	99.6 ± 0.7	
						25.3	100.6 ± 0.2	
Pyridoxine hydrochloride	0.5–20	0.9999	−0.0272	0.2935	0.05	3.8	99.6 ± 0.6	99.5 ± 0.4
						5.0	99.1 ± 0.3	
						6.3	99.6 ± 0.2	

r, Correlation coefficient; LOD, limit of detection; R.S.D., relative standard deviation.

analysis. It was demonstrated that enzymatic conversion of riboflavin phosphate to riboflavin by IMER was not affected in the presence of phosphoric salts and/or other ingredients in the pharmaceutical preparation. The unique method described here is superior to other methods in terms of accuracy, precision, and harmless to the environment and making possible to continue autoanalysis.

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