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Simultaneous determination of water-soluble vitamins in a vitamin-enriched drink by an in-capillary enzyme reaction method

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Abstract

The in-capillary enzyme reaction method was used to determine riboflavin phosphate in a vitamin-enriched drink based on its conversion to riboflavin (vitamin B_2) with alkaline phosphatase. Simultaneously, three water-soluble vitamins [thiamine nitrate (vitamin B_1 mononitrate), pyridoxine hydrochloride (vitamin B_6 hydrochloride) and nicotinamide (vitamin PP)] and anhydrous caffeine in the drink were subjected to quantitative analysis. In the system, electrophoretic migration was used to mix zones containing the substrate (riboflavin phosphate) and the enzyme (alkaline phosphatase). The reaction was then allowed to proceed in the presence of a weak electric field and, finally, the product (riboflavin) of enzyme reaction and other water-soluble vitamins migrated under the influence of an applied electric field to the detector. All the active ingredients and the formulation excipients were successfully separated by micellar electrokinetic chromatography with 135 m*M* sodium dodecyl sulfate. To prevent inhibition of enzyme reaction by the addition of sodium dodecyl sulfate to the reaction zone, sandwich mode injection, in which plugs of sandwich solution without sodium dodecyl sulfate were introduced into the capillary on both sides of the reaction zone, was utilized as a barrier to protect the enzyme reaction from the inhibitor. The relationship between the peak area of the product and the concentration of the substrate was calculated in the in-capillary enzyme reaction method. Excellent linearity was obtained, with correlation coefficients of 0.9999. The established method was validated and demonstrated to be applicable to the determination of the five active ingredients, including riboflavin phosphate, in a commercial vitamin-enriched drink. No interference from the formulation excipients was observed. Good linearities were obtained, with correlation coefficients above 0.999. Recoveries and precisions ranged from 99.3 to 101.8%, and from 0.1 to 2.5% RSD, respectively. Good agreement was obtained between the established method and traditional high-performance liquid chromatographic methods. These results suggest that the in-capillary enzyme reaction method can be used for the simultaneous determination of riboflavin phosphate and other water-soluble vitamins in pharmaceuticals.

Keywords: Pharmaceutical analysis; Derivatization, electrophoresis; In-capillary enzyme reaction; Food analysis; Vitamins; Riboflavin phosphate

1. Introduction

Vitamins are essential for the development and normal growth of human and animal bodies, and their lack or excess causes serious physiological problems. Multi-vitamin preparations are becoming widely used to compensate for possible lack of these nutrients in the daily diet. Water-soluble vitamins are available in many pharmaceutical dosage forms, such

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as drinks, tablets, gelatin capsules and syrups. The widespread use of multi-vitamin preparations has stimulated research on accurate, efficient and easy methods for quality control. Use of CE for the purpose of simultaneous determination of water-soluble vitamins in preparations has been undertaken with capillary zone electrophoresis [1–5] or micellar electrokinetic chromatography (MEKC) [4–12]. Methods based on CE have the capability of rapid, high-resolution separation of analytes from extremely low sample volumes and are suitable for simultaneous determination.

One of the water-soluble vitamins, riboflavin (RF, vitamin B_2), plays a central role in biochemical redox reactions. In principle, it can exist naturally in the form of two coenzymes, riboflavin monophosphate (FMN, riboflavin phosphate) and flavin adenine dinucleotide (FAD). Furthermore, Nielsen et al. demonstrated that there were also several riboflavin phosphoric acid esters (FMNs) in commercial FMN preparations, and that composition of FMNs differed among various commercial sources [13]. For the determination of RF, several techniques, principally microbiological [14], fluorimetric [15], spectrophotometric [14], electrochemical [16] and photochemical [17] methods, have been developed. FMN has been widely used for the liquid preparations because of its high solubility in water compared to RF. Nevertheless, there is no reference standard for FMN since it is very difficult to obtain the compound in pure form. However, RF standard can be easily obtained. Therefore, several HPLC methods have been developed for the determination of total riboflavin after conversion of FMN to RF by reaction with enzyme [18–25]. The method using phosphatase as an enzyme was the most rapid, accurate and convenient [22–25]. Lam and Lowande proposed the HPLC method for simultaneous determination of four water-soluble vitamins, including riboflavin phosphate, with alkaline phosphatase used for the conversion of FMN to RF [22]. Ozone et al. demonstrated the utility of acid phosphatase from wheat germ for this conversion, and applied the method to the analysis of pharmaceuticals [23]. Moreover, Yamato and co-workers succeeded in constructing an on-line analytical system using immobilized acid phosphatase as a pre-column reactor [24,25].

Generally, enzymes are expensive and sometimes only a very small amount of catalyst is available. Since the CE system requires only a small quantity of material, application of CE to an enzyme microreactor has evoked increased interest. Free solution analysis with CE allows in-situ enzyme reaction in a buffer filled in a capillary. Recently, the CE system combined with the in-capillary enzyme reaction method has been applied to the evaluation of enzyme activity [26–33]. Differences in electrophoretic mobility among solutes have been widely used in CE not only as a separation technique but also as a mixing technique. In the method, plugs of substrate and enzyme are first introduced into the capillary. After mixing substrate and enzyme, reaction product is subsequently determined by CE. Each analytical process, that is, mixing, reaction, separation and detection, occurs within the capillary using commercial equipment without complicated modifications. This on-line analytical system reduces the volume of the reaction solution from milliliters to nanoliters, compared to off-line batchwise operation. The present study was performed to demonstrate the applicability of the in-capillary enzyme reaction method to analysis of FMN in pharmaceutical preparations. When the method is used for evaluation of enzyme activity, it is not necessary to convert the substrate to product completely. However, in the proposed system, all FMNs must be completely converted to RF. We have recently shown that all the active ingredients and the formulation excipients in a vitamin-enriched drink could be successfully separated by MEKC with sodium dodecyl sulfate (SDS) in an alkaline separation solution [12]. Accordingly, alkaline phosphatase, which has an alkaline optimum and high activity compared with acid phosphatase, was selected for use as the enzyme in the system. Furthermore, to obtain complete conversion of FMN to RF, attention was focused on a way to prevent the inhibition of enzyme reaction caused by SDS. In this study, we succeeded in simultaneous determination of FMN, based on its conversion to RF with alkaline phosphatase by use of in-capillary enzyme reaction method, other three water-soluble vitamins [thiamine nitrate (vitamin B_1 mononitrate), pyridoxine hydrochloride (vitamin B_6 hydrochloride) and nicotinamide (vitamin PP)] and anhydrous caffeine in a vitamin-enriched drink by subsequent MEKC. Validation of this method for the determination of active

ingredients in a commercial preparation was also performed.

2. Experimental

2.1. Equipment

CE was performed on an Agilent CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detection system operating at 270 nm. The capillary compartment temperature was maintained at 37 °C. Hydrodynamic injection (3.45 kPa) at the anodic end of the capillary was used to introduce samples. Fused-silica capillaries [48.5 cm (40 cm to the detector)×50 μm I.D., 375 μm O.D.] were obtained from Agilent Technologies. A bubble cell capillary arrangement was employed to increase sensitivity. Prior to each day of use, the capillary was conditioned by rinsing with 1 *M* NaOH (100 kPa, 15 min), 0.1 *M* NaOH (100 kPa, 15 min), deionized water (100 kPa, 15 min) and finally the separation solution (100 kPa, 15 min). The capillary was rinsed between runs with 1 *M* NaOH (100 kPa, 3 min), 0.1 *M* NaOH (100 kPa, 2 min), deionized water (100 kPa, 5 min) and finally the separation solution (100 kPa, 5 min). All data were collected and analyzed using ChemStation software (Agilent Technologies).

2.2. Chemicals

The chemical structures of the active ingredients are shown in Fig. 1. Riboflavin sodium phosphate (a commercial FMN) and thiamine nitrate were purchased from F. Hoffman-La Roche (Basel, Switzerland), reference standards for RF, thiamine hydrochloride, pyridoxine hydrochloride, nicotinamide and caffeine from the Society of Japanese Pharmacopoeia (Tokyo, Japan) and phenylephrine hydrochloride as an I.S. for CE analysis from Iwaki Seiyaku (Tokyo, Japan).

Alkaline phosphatase (EC 3.1.3.2) from bovine intestinal mucosa (Type VII-L, affinity purified) and acid phosphatase (EC 3.1.3.2) from wheat germ (Type I) were purchased from Sigma (St. Louis, MO, USA).

Gallic acid monohydrate, Tris, glycine, SDS, boric acid and sodium tetraborate were obtained from Wako (Osaka, Japan). All chemicals were of analytical grade.

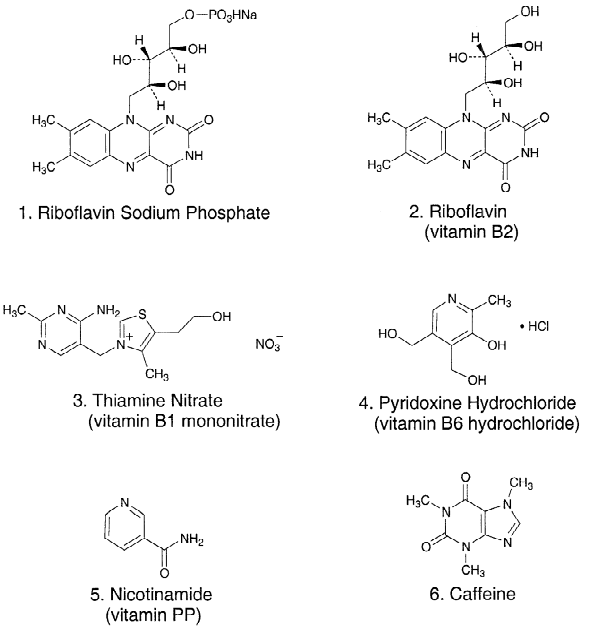

Fig. 1. Active ingredients in vitamin-enriched drink.

FMN solution and alkaline phosphatase solution were each prepared by dissolving the vitamin and the lyophilized protein in 25 m*M* Tris–195 m*M* glycine solution. To prepare the separation solution for MEKC, SDS was dissolved in a buffer solution prepared by mixing 0.2 *M* boric acid solution with appropriate volumes of 50 m*M* sodium tetraborate solution to obtain the required pH values. The solution was filtered through a polysulfone 0.45-μm membrane filter (Nihon Pall ltd., Tokyo, Japan) prior to use.

2.3. Procedure for off-line enzyme reaction method

To determine the rate of conversion of FMN to RF with alkaline phosphatase by an off-line batchwise operation, 2 ml of the riboflavin sodium phosphate solution (50 μg ml^{-1}) was placed in a 10-ml volumetric flask. A 5 ml volume of Tris–glycine solution with/without 135 m*M* SDS and 1 ml of alkaline phosphatase solution (50 units ml^{-1}) was added to the solution in the volumetric flask, and the

mixture was incubated for 10 min at 37 °C. After the incubation, the mixture was diluted to the volume with water. The sample solution was immediately applied to CE analysis using the separation solution described in Section 2.2. Hydrodynamic injection (3.45 kPa, 5 s) was utilized for sample introduction. The reliability of the operation was demonstrated by five repeated assays. The rate of conversion was determined as percentage of the corrected peak area of RF relative to total corrected peak area of RF and FMNs observed on the electropherogram.

2.4. Procedure for in-capillary enzyme reaction method

An illustration of the conversion of FMN to RF by in-capillary enzyme reaction method is shown in Fig. 2. Plugs of substrate solution and enzyme solution were introduced to the inlet of a capillary separately by pressure injection (3.45 kPa, 5 s). The reaction was then allowed to proceed with 1 kV applied for 3 min, and electrophoresis was finally performed using the separation solution described in Section 2.2 with 12 kV applied. In sandwich mode injection, a plug of sandwich solution (borate buffer, pH 8.0) was introduced into the capillary by pressure injection (3.45 kPa, 20 s), followed by plugs of substrate solution, enzyme solution and once again sandwich solution continuously by pressure injection (3.45 kPa, 5, 10 and 20 s, respectively). Mixing time was 10 min. The rate of conversion was calculated as described above.

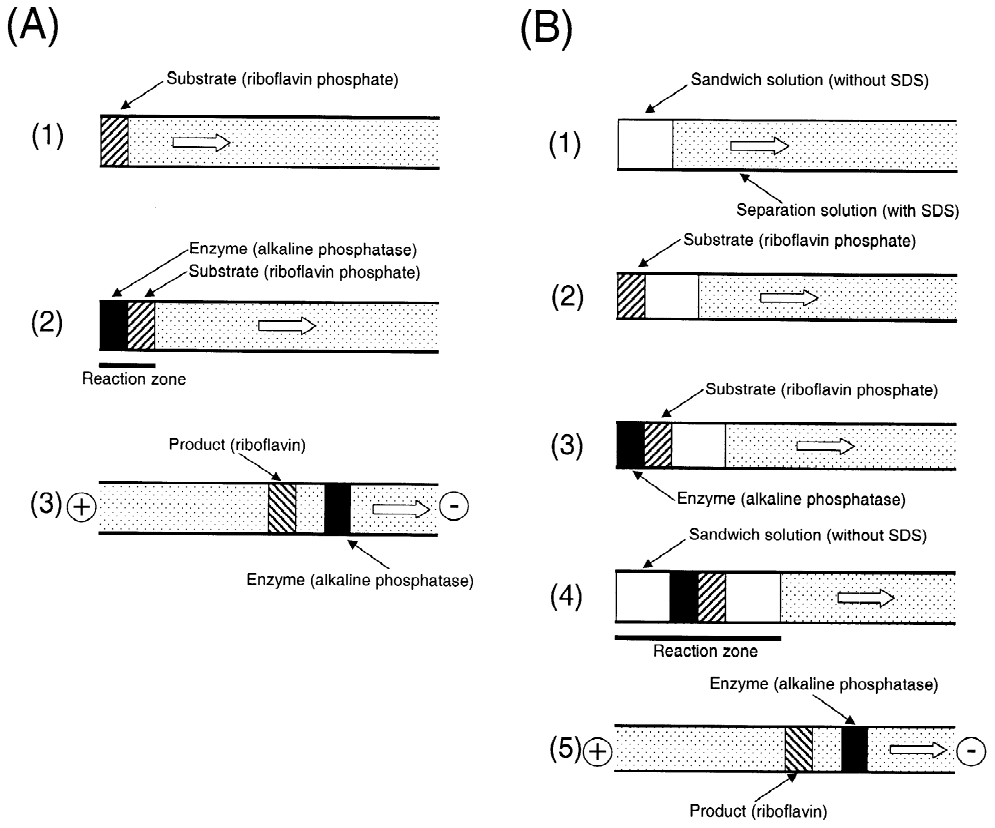

Fig. 2. An illustration of the conversion of riboflavin phosphate to riboflavin by the in-capillary enzyme reaction method. (A) In normal mode injection, plugs of substrate solution (1) and enzyme solution (2) were introduced to the inlet of a capillary separately by pressure injection. After mixing substrate and enzyme, reaction product was subsequently determined by CE (3). (B) In sandwich mode injection, a plug of sandwich solution without SDS (1) was introduced into the capillary, followed by plugs of substrate solution (2), enzyme solution (3) and sandwich solution again (4). After mixing substrate and enzyme within the reaction zone, reaction product was subsequently determined by micellar electrokinetic chromatography with SDS (5).

To determine the ingredients in a commercial vitamin-enriched drink, 2 ml of the preparation was placed in a 10-ml volumetric flask. An internal standard solution was prepared by dissolving 20 mg of phenylephrine hydrochloride in 100 ml of water. A 2 ml volume of the internal standard solution was added to the solution in the volumetric flask and the mixture was diluted to the volume with Tris–glycine solution. Standard compounds (RF, thiamine hydrochloride, pyridoxine hydrochloride, nicotinamide and caffeine) were weighed and diluted in Tris–glycine solution to concentrations similar to those in the prepared sample solution. The same volume of internal standard solution was also added to the standard solution. Gallic acid monohydrate was required to assure the stability of each test solution. All test solutions were passed through a polysulfone 0.45-μm membrane filter (Nihon Pall).

2.5. Procedure for HPLC analysis

HPLC was performed on an Alliance HPLC system instrument (Waters, MA, USA).

FMN and other active ingredients except thiamine nitrate in a vitamin-enriched drink was determined by HPLC analysis after conversion of FMN to RF by a batchwise reaction with acid phosphatase from wheat germ, as reported by Ozone et al. [23] but with slight modification in the composition of the mobile phase to optimize column separation of FMN and other active ingredients. Thiamine nitrate was determined by the JP14 method. The composition of the mobile phase was slightly modified to optimize separation.

3. Results and discussion

3.1. Optimization of in-capillary enzyme reaction method

3.1.1. Study of introduction order to the system

In the in-capillary enzyme reaction method, different electrophoretic mobilities of substrate and enzyme are used to merge each zone. To initiate the reaction, the fast migrating plug is required to traverse across the slow migrating plug under an electric field. In order to optimize the order of introduction in the in-capillary enzyme reaction method, the apparent mobilities of substrate and enzyme within the reaction zone at the inlet of the capillary illustrated in Fig. 2 were examined. Electrophoresis was performed with separation solution composed of Tris–glycine solution of composition equal to that in the reaction zone. As shown in Fig. 3A, peaks of riboflavin 5′-phosphate (5′-FMN), the main component of FMNs [13], and several FMNs were observed. FMNs might be mostly negatively charged in this condition and migrated slowly since they were attracted to the anode. Alkaline phosphatase is also predominately negatively charged at pH values above 4.4 [34], but migrated faster than FMNs (Fig. 3B) since its electrophoretic mobility toward the anode is smaller than that of FMNs because of its higher molecular mass.

Since alkaline phosphatase apparently migrated faster than FMNs within the reaction zone, the order of introduction into the capillary used was as follows; first, a plug of substrate solution was introduced, followed by enzyme solution. After the enzyme reaction, a large amount of FR converted from FMN was observed on the electropherogram, although mostly 5′-FMN was observed before the reaction. As expected, the rates of conversion shown in established order and in reversed order were 86.7

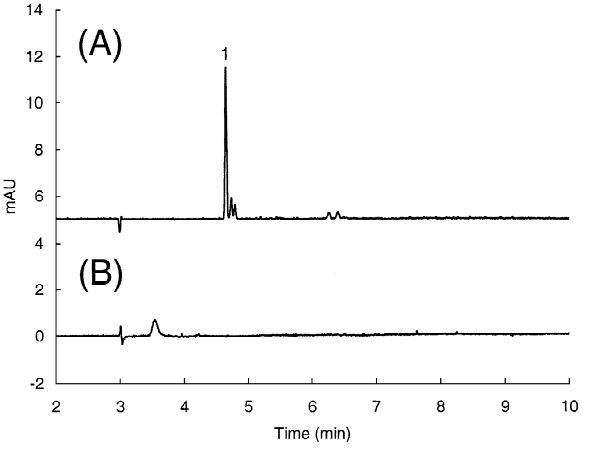

Fig. 3. Difference of apparent mobilities between riboflavin phosphate (A) and alkaline phosphatase (B) within the reaction zone. Separation solution, 25 m*M* Tris–195 m*M* glycine; applied voltage, +12 kV; temperature, 37 °C; detection wavelength, 270 nm; capillary, fused-silica (40 cm×50 μm I.D.); sample, 10 μg ml^{-1} of riboflavin sodium phosphate in Tris–glycine solution and 500 units ml^{-1} of alkaline phosphatase in Tris–glycine solution. Solutes were as noted in Fig. 1.

and 75.2%, respectively. The unforeseen conversion observed in reversed order indicated that some mixing occurred in the stage of introduction of enzyme and substrate.

3.1.2. Study of sandwich mode injection

We have recently shown that SDS micelles in alkaline separation solution were needed for successful separation of all the active ingredients and the formulation excipients in a vitamin-enriched drink [12]. In the in-capillary enzyme reaction, it is necessary to convert FMNs to RF completely for quantitative analysis of FMN in pharmaceuticals. However, inhibition of the enzyme reaction by SDS was a possible reason for incomplete conversion. Consequently, the effect on conversion of SDS addition to the reaction solution was examined by an off-line batchwise procedure. More than 80% of the conversion was observed in the reaction solution without SDS, but only 40% of the conversion occurred in the reaction solution with SDS. SDS may have denatured alkaline phosphatase in the reaction solution.

To prevent permeation of SDS into the reaction zone in the in-capillary enzyme reaction, sandwich mode injection, as shown in Fig. 2B, was used. The sandwich mode injection established by Taga and Honda [35] was a method in which the reagent solution for derivatization was introduced on both sides of the sample solution to obtain high yields of derivatives. In this study, the proposed injection mode was applied for the first time as a barrier against the inhibitor. That is, the aim of the sandwich was to build a safety zone for the enzyme reaction by holding the reaction zone between the sandwich solutions without SDS to produce complete reaction. With both injection modes, conversion to RF was enhanced when the amount of enzyme was increased from 15 to 500 units ml^{-1} for 10 $\mu g\ ml^{-1}$ portion of FMN solution. Sandwich mode injection yielded a high rate of conversion at every point of examination compared with normal mode. Complete conversion to RF was obtained with 500 units ml^{-1} of alkaline phosphatase in sandwich mode injection, but some non-converted FMNs were detected on the electropherogram at the same point in normal mode injection. These examinations revealed that the sandwich mode injection prevented inhibition of enzyme reaction caused by SDS.

The linearity of conversion of FMN to RF in the range from 5 to 100 $\mu g\ ml^{-1}$ was examined. The relationship between relative corrected peak area of converted RF and concentration of FMN was calculated. Straight regression lines passing through the origin ($y = 0.1296x - 0.0293$) with correlation coefficients (r) of 0.9999 were obtained.

3.2. Determination of the active ingredient

3.2.1. Validation of the method

Finally, the combination of the in-capillary enzyme reaction method and the sandwich mode injection was established to obtain complete conversion of FMN to RF. The application and validation of this technique were demonstrated for determination of FMN in a commercial preparation according to the ICH guidelines [36]. Other active ingredients, that is, the water-soluble vitamins and anhydrous caffeine, were also analyzed simultaneously.

To assess specificity, a standard, a sample of a commercial vitamin-enriched drink and placebo mixtures that were prepared in the absence of each active ingredient in the drink base were analyzed according to the established method. Analysis was performed by an internal standard method. Representative electropherograms of each sample solution in Fig. 4 show the separation between the main peaks and the I.S. No interference with the formulation excipients was observed at the migration times of the active ingredients. The peaks of FMNs were observed on the electropherogram before the enzyme reaction (Fig. 4B), but disappeared completely and the peak of RF appeared instead of them after the reaction (Fig. 4C).

The detection limit observed as a peak with a signal-to-noise ratio of 3 is shown in Table 1. There were determined by injecting sample solutions with known low concentrations of analytes.

The quantitation linearity of active ingredients in standard solution was examined at five concentration levels in the range from 50 to 150% of the normal concentration. For each ingredient, the relationship between relative corrected peak area and concentration was calculated and is given in Table 1. In all cases, straight regression lines with correlation co-

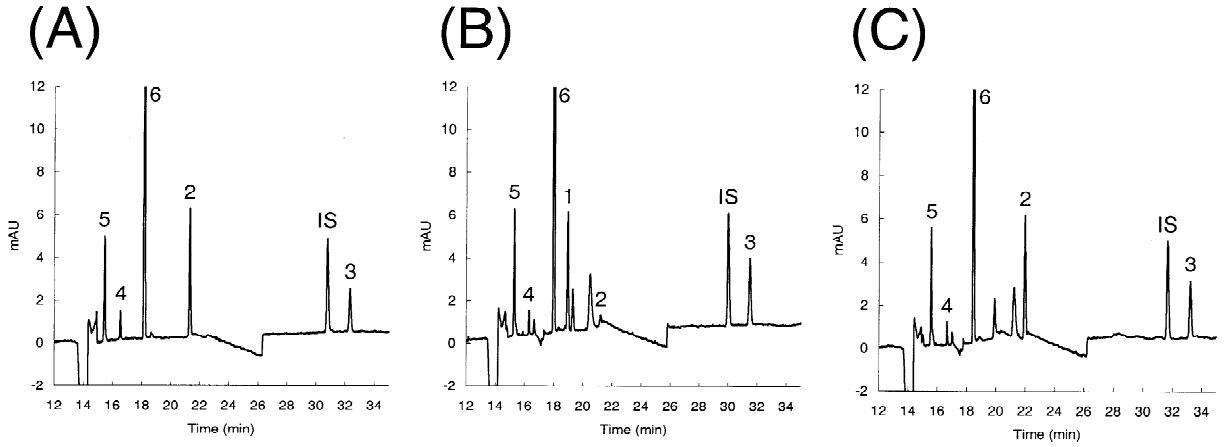

Fig. 4. Typical electropherograms in assay of commercial vitamin-enriched drink. (A) Standard solution; (B) sample solution treated with Tris–glycine solution instead of enzyme solution in the in-capillary enzyme reaction method; (C) sample solution treated with alkaline phosphatase in the in-capillary enzyme reaction method. Separation solution, 135 m*M* SDS in borate buffer (pH 8.0); applied voltage, +12 kV; temperature, 37 °C; detection wavelength, 270 nm; capillary, fused-silica (40 cm×50 μm I.D.); mixing time, +1 kV for 10 min; sandwich mode injection, a plug of sandwich solution was introduced into the capillary by pressure injection (20 s), followed by plugs of substrate solution, enzyme solution and once again sandwich solution continuously by pressure injection (5, 10 and 20 s, respectively). Subsequently, 1000 units ml^{-1} of alkaline phosphatase solution were used for complete conversion in the assay of a commercial vitamin-enriched drink. Solutes were as noted in Fig. 1.

efficients (*r*) above 0.999 were obtained. The intercept values were not significantly different from zero (95% confidence).

Accuracy was assessed over the entire concentration range (80, 100 and 120%) by analyzing placebos spiked with active ingredients at three concentration levels. The solutions were replicated three times each, and the amounts determined were

Table 1
Validation data for determination of active ingredients in a commercial vitamin-enriched drink

	Riboflavin sodium phosphate (Riboflavin)	Thiamine nitrate (Thiamine hydrochloride)	Pyridoxine hydrochloride	Nicotinamide	Anhydrous caffeine
Linearity					
Concentration range (μg ml^{-1})	2.5–12.5	5–15	5–15	20–60	50–150
r	0.9995	0.9998	0.9995	0.9996	0.9999
Intercept	−0.0433	0.0032	0.0019	−0.0151	−0.0663
Slope	0.1622	0.0453	0.01648	0.02901	0.0782
Recovery (*n* = 3, %)					
80%	100.0	100.5	100.8	100.9	101.5
100%	100.1	101.1	99.9	99.5	101.8
120%	99.3	101.3	99.6	100.1	100.7
Precision (*n* = 3, *RSD*%)					
80%	1.1	1.0	2.5	1.0	0.4
100%	1.3	0.7	1.2	1.3	0.1
120%	1.2	0.5	1.3	0.6	0.5
Detection limit (*S*/*N* = 3) (μg ml^{-1})	0.1	1.0	1.0	1.5	0.5

Table 2
Comparison of the in-capillary enzyme reaction method with HPLC determination of active ingredients in a commercial vitamin-enriched drink ($n=6$)

	Riboflavin sodium phosphate	Thiamine nitrate	Pyridoxine hydrochloride	Nicotinamide	Anhydrous caffeine
CE					
Content (mg per 100 ml)	5.04	5.30	4.94	19.57	49.5
(RSD, %)	(1.7)	(1.3)	(1.9)	(1.7)	(2.0)
HPLC					
Content (mg per 100 ml)	5.00	5.32	4.98	19.60	49.9
(RSD, %)	(0.3)	(0.5)	(0.7)	(0.9)	(0.4)

compared to the theoretical amounts. Adequate results for recovery were obtained for all ingredients studied (Table 1).

Precision was determined by measuring ($n=3$) each active ingredient in spiked placebos at the three concentration levels. RSD was estimated from the established method. Good results for precision were obtained for all ingredients examined (Table 1).

3.2.2. Quantitative analysis of active ingredients in a commercial vitamin-enriched drink

Results of the quantitation of active ingredients in a commercial vitamin-enriched drink performed in Fig. 4 are given in Table 2. On the whole, good agreement was obtained between the in-capillary enzyme reaction method and traditional HPLC methods. Regarding precision, the RSD of the established method was not as good as those for the HPLC methods.

4. Conclusion

The application of the in-capillary enzyme reaction method to the simultaneous determination of FMN and other water-soluble vitamins in pharmaceuticals was demonstrated for the first time. The sandwich mode injection was developed as a barrier to protect the enzyme reaction from the inhibitor even with use of the separation solution with SDS micelles. Compared with the HPLC method, slightly less precision was obtained, but the in-capillary enzyme reaction method offered numerous advantages to conventional HPLC method: (i) it required only small quantities of enzyme and reduced cost; (ii) it was readily automated; (iii) it reduced organic solvent consumption. The established method gave simultaneous determination of all the ingredients including thiamin nitrate with on-line enzyme reaction although two separation systems and complicated batchwise enzyme reaction for each sample were required in conventional HPLC method. The established method was superior in ease of use, high throughput and simplicity by virtue of use of conventional equipment without complicated modification and adjustment. Application of this method to routine assay is expected after due consideration of advantages and disadvantages. This method based on CE, including an analysis technique, the in-capillary enzyme reaction method, will widely extend the area of application in pharmaceutical quality control.

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