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Journal of Pharmaceutical and Biomedical Analysis 38 (2005) 66–71
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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# Separation of thiamin and its phosphate esters by capillary zone electrophoresis and its application to the analysis of water-soluble vitamins

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Received 10 September 2003; received in revised form 25 November 2004; accepted 27 November 2004
Available online 13 January 2005

**Abstract**

A capillary zone electrophoresis method for the separation and determination of thiamin and its phosphate esters (free thiamin, thiamin monophosphate, and thiamin pyrophosphate) was developed and optimized. The efficiency achieved with boric acid–sodium tetraborate decahydrate buffer (pH 8.24; 65–8 mM) at an applied potential of 30 kV gave the detection limit (S/N = 3) and the limit of quantitative measurement (S/N = 10) of thiamin and its phosphate esters ranging from $10^{-4}$ to $6 \times 10^{-4}$ mM and from $6 \times 10^{-4}$ to $1.2 \times 10^{-3}$ mM, respectively. The effects of pH on separation and migration times of thiamin and its phosphate esters are described. The method was validated and applied to the quantitative determination of thiamin in commercial tablets containing both a massive and a normal dose of thiamin.
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*Keywords:* Thiamin esters; Separation; Capillary zone electrophoresis

## 1. Introduction

Thiamin, commonly known as vitamin $B_1$, is a molecule of biological importance that belongs to a group of compounds known as B-complex vitamins (water-soluble vitamins). In food and the human body, thiamin exists in the form of free thiamin (Th) and its phosphorylated forms, thiamin monophosphate (ThP), thiamin pyrophosphate (ThPP) and thiamin triphosphate (ThPPP) (see Fig. 1) [1,2]. The ThPP form is the only metabolically active coenzyme of a class of enzymes that catalyze acyl group transfer reactions [3–6], and its deficiency in humans (which is usually caused by a poor diet or alcoholism) can result in an ultimately fatal condition known as beriberi [7,8]. ThPPP and ThP, along with Th, also play an important role in neurological systems and are known to affect physiological responses in nerve tissue [5,9]. Therefore, the rapid analysis of thiamin to assess the vitamin status is of great importance.

Currently, the most commonly used techniques for the determination of thiamin or its phosphate esters in foods, animal feed, pharmaceutical preparations, and clinical applications are based on the *thiochrome procedure* and on the microbiological method [1,2,6,10–17]. In the former approach, thiamin is oxidized in alkaline media by agents such as $Fe(CN)_6{}^{-3}$, $HgCl_2$, and CNBr to blue fluorescing thiochrome [16], which can be readily monitored spectrophotometrically by chemical assays or chromatographic techniques. High performance liquid chromatography (HPLC) both with ultraviolet (UV) and with fluorescence detection are the two main chromatographic methods that have emerged as excellent analytical techniques for the determination of thiamin and its phosphate esters [6,16,17]. Due to limited sensitivities, UV-based methods have been found to be more suitable for preparative rather than clinical applications; whereas fluorescence-based methods, in which the thiochrome procedure is configured in a pre- or post-column derivatization

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doi:10.1016/j.jpba.2004.11.061

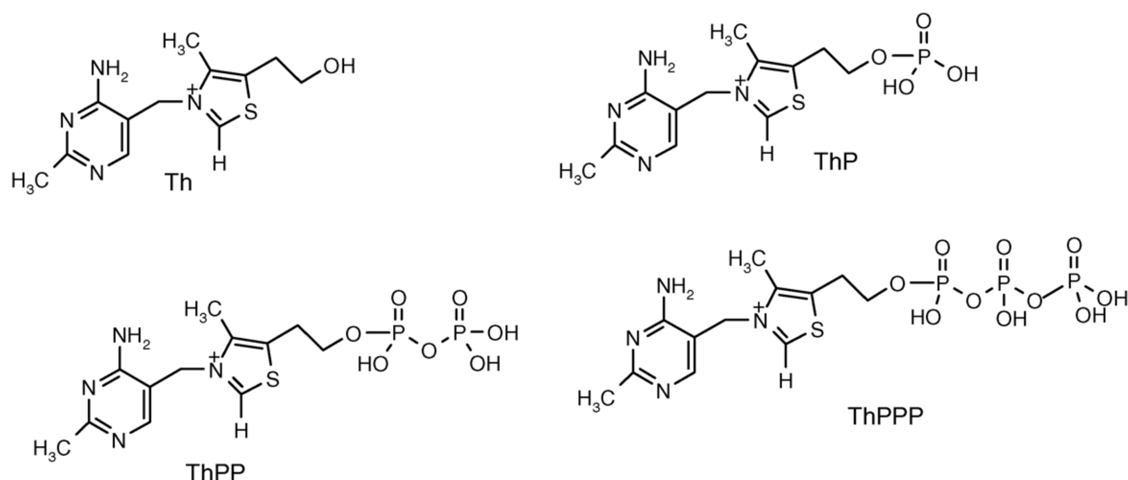


Fig. 1. Structures of the four most common thiamin esters: free thiamin (Th), thiamin monophosphate (ThP), thiamin pyrophosphate (ThPP), and thiamin triphosphate (ThPPP).

of thiamin and its phosphate esters, constitute the basis of many improved HPLC methods for the determination of low levels of thiamin in tissues or blood [1,2,6,10–17].

In recent reports, a number of capillary electrophoresis (CE) techniques such as capillary zone electrophoresis (CZE) [18–25], micellar electrokinetic chromatography (MEKC) [18,19,26–31], microemulsion electrokinetic chromatography (MEEKC) [32], and in-capillary enzyme reaction methods [33] have emerged as potential alternative techniques for the determination and monitoring of thiamin in samples. A careful examination of these reports revealed that the CE-based methods for thiamin analysis utilized to date relate solely to the Th form in foods, biological samples, and pharmaceuticals in conjunction with other water-soluble vitamins. To our knowledge, no extensive CZE methods have come forth for the simultaneous separation and quantification of thiamin esters in samples. The fact that numerous method configurations on the simultaneous HPLC analysis of Th, ThP, and ThPP were proposed and assessed in previous literature [1,2,6,10–17] confirms the importance of this study and the need for efficient analysis methods in thiamin related fields. In this investigation, a CZE method for the separation and quantification of thiamin was optimized and expanded to include the analysis of its phosphate esters (see Fig. 1), providing an attractive alternative to the advanced chromatographic techniques. The method was carefully validated and applied to the quantitative determination of thiamin in commercial tablets.

## 2. Experimental

### 2.1. Apparatus for CZE

All CZE analyses were performed using a computer controlled P/ACE MDQ capillary electrophoresis system (Beckman Instruments, Fullerton, CA) with a UV detector set at 200 nm. A Pentium 300 MHZ IBM computer with P/ACE Station Software (Version 2.3) for Windows 95 was used for data acquisition, and the Beckman CE Expert (software) was used to estimate the injection volume. Separation was carried out in untreated fused-silica capillaries of 50.1 cm to the detector (60.4 cm total length) with i.d. of 75 μm, obtained from Polymicro Technologies (Phoenix, AZ). New capillaries were conditioned by rinsing them for 5 min with methanol (20 psi), 2 min with deionized water, 5 min with 1 M HCl, 2 min with deionized water, 10 min with 0.1 M NaOH, 2 min with deionized water, 5 min with running buffer. A sample was injected for 10 s at 0.5 psi (≈50 nl) and separated with an applied potential of 30 kV while maintaining the capillary temperature at 25 °C. After the separation process, the capillary was rinsed for 30 s with 0.1 M NaOH, 90 s with running buffer, and 5 min with deionized water prior to the next analysis. With standard samples, the capillary was reconditioned only once every two dozen runs and rinsed with deionized water between runs.

### 2.2. Chemicals and reagents

All solutions were prepared with distilled water filtered through a Milli-Q system (Millipore, Bedford, MA). Thiamin derivatives and other standards such as niacin, riboflavin, pyridoxine, and folic acid of analytical grade were obtained from Sigma-Aldrich (St. Louis, MO) and used without any further purification. The vitamin supplements used were Time Released Balanced B-150 (CVS), Energy Enhanced Multivitamin Dietary Supplement Active (One-A-Day), Children's Multivitamin Supplement (Flintstones), and Poly.Vi.Sol Multivitamin Supplement Drops for Infants and Toddlers (Enfamil), obtained from local drug stores. Boric acid, sodium tetraborate decahydrate, sodium acetate, sodium citrate, sodium phosphate, glacial acetic acid, hydrochloric acid, and sodium hydroxide were obtained from Fisher Scientific.

### 2.3. CZE analysis of free thiamin in commercial supplements

A calibration curve was obtained by plotting the ratio of average peak areas ($n = 4$) of free thiamin standard over thiamin monophosphate, the internal standard, ([Th, peak area]/[ThP, peak area]) to that of their corresponding concentrations ([Th, mM]/[ThP, mM]), that ranged from 0.01 to 1.25 mM. The concentration of the internal standard (ThP) added to all samples was kept constant (0.5 mM), and the analyte response was measured relative to that of the internal standard. The equation of the line obtained from this plot was then used in the quantitative determination of free thiamin in commercial supplements. Samples of approximately 56, 500, and 1000 mg of crushed CVS B-150, One-A-Day, and Flintstones tablets, respectively, were dissolved and sonicated for about 4 min in a 25 ml boric acid–sodium tetraborate decahydrate buffer (pH 8.24; 65–8 mM) then filtered with a 0.2 μm nylon filter (Whatman 6870-2502) before being injected into the CZE instrument. Enfamil, the liquid multivitamin, was prepared by dissolving 1 ml of the vitamin supplement in a 10 ml volumetric flask with boric acid–sodium tetraborate decahydrate buffer (pH 8.24; 65–8 mM). The buffer was prepared by dissolving 0.40 g of boric acid and 0.30 g of sodium tetraborate decahydrate in 100 ml of distilled water. When thiamin standards were dissolved in the boric acid–sodium tetraborate decahydrate buffer (pH 8.24), the pH of the solutions (pH*) dropped to approximately 7.29. All samples were freshly prepared, analyzed daily, and stored in a refrigerator (maintained at 8 °C) when necessary.

## 3. Results and discussion

### 3.1. Method optimization

A high-resolution separation of free thiamin and its phosphate esters was achieved using CZE with a UV-based detector (200 nm) as an alternative technique to HPLC. The efficiency of this method was obtained at optimum conditions of pH and applied voltage of 8.24 and 30 kV (current generated ranged from 30 to 40 μA), respectively. Fig. 2A shows an electropherogram of thiamin esters displaying the best (shortest) overall baseline resolution analysis time with the order of migration being Th, ThP, and ThPP. ThPPP was not analyzed in this investigation because of its unavailability, however, it is anticipated that the method will be applicable to its analysis and that its migration time would be observed as being longer than that of ThPP.

The pH condition of background electrolytes (BGE), which have been known to affect both the electroosmotic flow (EOF) and the effective mobility [22,34], can also affect the stability of analytes. The fact that thiamin and its phosphate esters undergo numerous structural changes when the pH is altered [7,35,36] makes the choice of a running buffer critical. With the accepted p$K_a$ of thiamin ranging from 17 to 19 [37], no structural change was anticipated during the CZE analysis given that our running buffer had a pH of 8.24 (pH* = 7.29). At a lower pH range (3.8–5.0), the EOF was greatly reduced, resulting in longer separation times (35–9 min)[38]. By increasing the pH to 8.24 (in boric acid–sodium tetraborate decahydrate buffer), the EOF was substantially increased, and the analysis time obtained was reduced to 6 min (see Fig. 2A).

When Mrestani and Neubert investigated the determination of thiamin in biological media by CZE in 10 mM phosphate buffer at an applied potential of 30 kV, thiamin (Th) migrated out between 3.5 and 4.5 min [24]. Despite the small discrepancy in migration times of Th displayed in electropherograms (R.S.D. = 4%) [24], their results and ours indicated that the use of a higher applied potential (30 kV) did not alter the Th peak shape and no band broadening was observed. Other reports that used applied potentials ranging from 12 to 24 kV, gave Th migration times that varied from 5 to 32 min [29,30,31,33].

### 3.2. Method validation

Standard calibration curves were constructed for Th, ThP, and ThPP to determine their respective sensitivities, linearities (r), linearity ranges, limits of detection (LOD), and limits

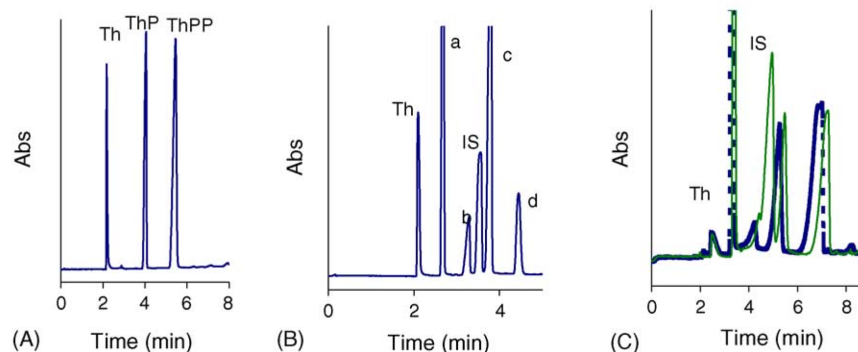


Fig. 2. Electropherograms of (A) a thiamin esters mixture at 2 mM each (Th, ThP, and ThPP, each at 2 mM), (B) CVS-150 (a: niacin; b: riboflavin; c: pyridoxine; d: folic acid), (C) One-A-Day (IS was omitted in the dashed-line), in boric acid–sodium tetraborate decahydrate (pH 8.24; 65–8 mM) buffer with an applied potential of 30 kV.

Table 1
Analytical characteristics ($n = 3$)

| | Intercept ($10^3$) ± Sb ($10^3$) | Slope ($10^5$) ± Sa ($10^4$) | Correlation coefficient ($r$) | LOD ($10^{-4}$) (mM) | LOQ ($10^{-4}$) (mM) | Linear range (mM) |
|---|---|---|---|---|---|---|
| ThPP | 9.17 ± 2.58 | 5.32 ± 3.21 | 0.9996 | 1 | 6 | 0.01 ≥ 2.25 |
| ThP | 5.69 ± 0.31 | 4.77 ± 2.94 | 0.9997 | 1 | 4 | 0.01 ≥ 2.25 |
| Th | 3.49 ± 1.78 | 3.00 ± 2.21 | 0.9989 | 2 | 7 | 0.05–1.25 |
| Th[a] | $(4 \pm 1) \times 10^{-3}$ | $(6.02 \pm 2) \times 10^{-3}$ | 0.9986 | 6 | 12 | 0.05 ≥ 1.25 |

[a] Values for Th with ThP as an internal standard ($n = 4$). The intercept and slope are not multiplied by quantities in parentheses of columns labels.

of quantification (LOQ). The relationship between the concentration and peak area is given in Table 1, where "$a$", "$b$", and "$r$" are the coefficients of the regression equation "$y = ax + b$". $x$ and $y$ are the concentrations and peak areas of Th, ThP, and ThPP, respectively. The sensitivity of this method obtained from slopes of calibration curves favored ThP and ThPP over Th. The LOD and the LOQ of Th, ThP, and ThPP determined using signal-to-noise ratios of 3:1 and 10:1 ranged from $10^{-4}$ to $6 \times 10^{-4}$ mM and from $6 \times 10^{-4}$ to $1.2 \times 10^{-3}$ mM, respectively (see Table 1). The obtained LODs, particularly those of Th and Tha, are comparable to those reported in the literature ($1.5 \times 10^{-4}$ to $8 \times 10^{-3}$ mM) [20,24,33]. The linearity range of Th, determined by using a calibration curve, was approximately 0.05–1.25 mM. In the presence of the internal standard (ThP), the range can be extended to higher concentrations (2.00 mM) with small changes in the coefficient of linearity ($r$). The precision of migration times and peak areas of Th, ThP, and ThPP standards were carefully evaluated for reproducibility within a day, and on different days. The R.S.D. values obtained for both migration times and peak areas were <2% and <6%, respectively. When ThP was used as an internal standard for Th, the R.S.D. value for the Th peak area was reduced to <2.6%. The use of the internal standard greatly improved the precision of the peak areas, particularly in the analysis of real samples [19,26,29,30]. The results corresponding to the LOD and LOQ of Th suggest that the CZE method is sufficiently viable for preparative applications of pharmaceutical samples with a wide range of thiamin concentrations.

### 3.3. *Application to quantitative determination of thiamin in commercial tablets*

The CZE method was optimized for free thiamin (Th), thiamin monophosphate (ThP) and thiamin pyrophosphate (ThPP) and is anticipated to be applicable to thiamin triphosphate (ThPPP) as well. Due to the fact that Th is the only thiamin form of supplement found in commercial tablets, the application of the CZE method to this class of compounds is demonstrated here in greater detail using the Th form.

Commercial thiamin supplement tablets containing other water-soluble vitamins were analyzed to validate the method. The selection of commercial tablets was made in such a way that the method could be applied to samples containing a massive dose of thiamin such as CVS-150 (150 mg of Th per 1.38 g of serving), as well as to samples with a normal (RDA = 1.5 mg of Th per serving for adults) or lower dose (Enfamil, with 0.5 mg of Th per 1 ml of serving). The results from a CVS-150 tablet (illustratively shown in Fig. 2B) and those from other related studies [19,21,23] indicated that thiamin (first peak) is easily detectable in the midst of other analytes (niacin, riboflavin, pyridoxine, and folic acid). The identity of the thiamin peak in all commercial tablets was confirmed by spiking the samples with a thiamin standard and by successively adding to a blank solution an amount of each vitamin standard (niacin, riboflavin, pyridoxine, and folic acid) then recording the resulting electropherograms. Most studies found in literature on the use of CE techniques in the analysis of thiamin [18,20,29,31–33] did not address the variation of Th content in pharmaceutical samples analyzed, nor did they confirm the applicability of their methods to samples of either higher or lower Th contents.

The actual thiamin amounts reported on container labels are presented in Table 2 in terms of weight percentage (wt.%) of thiamin per serving. Although the chemical composition of tablets is constant, it is worth noting that the mass per serving varied from one tablet to another (see average values in Table 2). In all cases, average serving masses were determined (for 45–50 tablets) and used to adjust the label values reported in Table 2. The amounts of free thiamin in selected commercial tablets determined experimentally are presented in Table 2. It was also observed that tablet samples or matrix components appeared to be adhering to the capillary walls, causing irreproducibility in both migration times and peak areas from run-to-run [30,39]. By reconditioning the capillary as described in Section 2 prior to commercial-tablet sample analyses, an improvement in migration times and peak area precision was obtained (R.S.D. is <2% and <4%, respectively). Delgado-Zamarreno et al. proposed the use of both centrifugation and filtration of samples in order to minimize the adsorption [30], but no attempt was made to centrifuge samples in this investigation. Except for the One-A-Day supplement, these results are in agreement with the weight percentages of thiamin reported on labels. The migration time of the internal reference (ThP) used appeared to be overlapping with some of the active ingredients in the One-A-Day tablet, thereby making the peak integration of ThP uncertain (see Fig. 2C). One-A-day can be analyzed accurately by reconstructing the calibration curve with suitable internal standards whose migration times can be

Table 2
Recoveries and contents of thiamin in selected commercial tablets ($n = 4$)

| Compound | Label[a] wt.% mass per serving (g) | CZE values weight % | Recovery (%) |
|---|---|---|---|
| Th standard | – | – | 99.1 ± 2.5 |
| ThP standard | – | – | 94.2 ± 3.9 |
| ThPP standard | – | – | 99.9 ± 3.4 |
| CVS-150 | 10.85 ± 0.14 (1.38 ± 0.02) | 9.78 ± 0.17 | 89.7 ± 0.4 |
| One-A-Day[b] | 0.29 ± 0.01 (1.55 ± 0.04) | 0.44 ± 0.05 | 128 ± 5 |
| Flintstones | 0.12 ± 0.001 (0.89 ± 0.01) | 0.10 ± 0.01 | 97.3 ± 0.1 |
| Enfamil | 0.04 (1.25 ± 0.03) | 0.05 ± 0.01 | 112 ± 4 |

[a] wt.% of thiamin per mass (values in parentheses expressed in grams) of serving.
[b] Values for this pill were obtained without using an internal reference.

observed at longer times than those of ThP and other active ingredients.

### 3.4. Recoveries

A recovery study was performed on both real (tablets) and standard thiamin samples to establish the effect of the sample matrix on the quantification of thiamin. The percent recoveries of Th determined over a concentration range of 0.01–1.25 mM varied from 93 to 109%. However, the results appeared to be more consistent at a higher concentration range (0.25–1.25 mM Th), thus yielding an average percent recovery of 99% (R.S.D. = 2.5%). To determine the percent recoveries of Th in commercial tablets, known amounts of standard thiamin (≈0.85 mg) were added to finely ground tablets prior to the extraction procedure as described in Section 2. The percent recoveries as shown in Table 2, were determined over a concentration range of 0.45–0.85 mM and found to be dependent on the sample matrix. Additionally, no attempt was made to investigate the effect of thiamin time release on the method before and after the filtration process. However, the fact that the percent recoveries of solid samples (CVS-150 and Flintstones) were slightly lower than that of the Enfamil (a liquid sample), suggested that the release of thiamin into solution might have had a small effect on the method.

## 4. Conclusion

A high-resolution separation of free thiamin and its phosphate esters was achieved using CZE with a UV-based detector as an alternative technique to HPLC. The efficiency of this method was obtained at optimum conditions of pH and applied voltage of 8.24 and 30 kV, respectively. The method was validated and proved to be suitable for preparative applications of pharmaceutical samples with a wide range of thiamin concentrations.

## Acknowledgements

We are grateful to Baxter Oncology for supporting this work.

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