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Short communication

The analysis of thiamin and its derivatives in whole blood samples under high pH conditions of the mobile phase

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Abstract

In this study a protocol for the analysis of thiamin and thiamin coenzymes in whole blood was developed. Thiamin and its coenzymes are analyzed by reversed phase liquid chromatography (RPLC), precolumn derivatisation with alkaline potassium ferricyanide and fluorescence detection, all at pH 10. Under these relatively high pH conditions the detectability of the analytes and the robustness of the method were substantially improved. The use of a high pH resistant RPLC column was a crucial step in developing this analysis method. Reproducibility, linearity, recovery, detection limit and column robustness were investigated. The within-batch CV was <2.5%, the between-batch CV <4.5%. The method was linear far above the physiological relevant concentration level. Recovery was almost 100% on an average. The limit of quantification was 1 nmol/l. The robustness of the RPLC column proved to be very high. Up to 1500 injections hardly any substantial changes in retention times and efficiency were observed. In summary: Using a high pH resistant RPLC column resulted in a robust, sensitive and precise method for the analysis of total Vitamin B1 and especially of TDP.
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Keywords: Thiamin; Vitamin B1; HPLC; Whole blood analysis; High pH mobile phase

1. Introduction

Thiamin diphosphate (TDP) is the main biologically active form of Vitamin B1 by playing a role as coenzyme in a number of biological reactions involved in carbohydrate metabolism [1]. Beside it the role of some phosphorylated form of thiamin in the nerve conduction process is well established [2]. As in general the amounts of TDP and total Vitamin B1 will hardly differ in fresh blood samples, the concentration of total Vitamin B1 will reflect the Vitamin B1 status of a patient very well. However, for research purposes and in case of inadequate capacity to phosphorylate thiamin to TDP [3,4] it becomes opportune to separate and quantify the different coenzyme forms.

The history of the analysis of thiamin (Vitamin B1) by high-performance liquid chromatography (HPLC) goes back to the early seventies. Chromatographic separation of the different coenzyme forms in food, followed by on-line post column oxidation by reaction with alkaline potassium ferricyanide to fluorescent thiochromes, was first described by Van de Weerdhof et al. [5]. Based on this method Schrijver et al. [6] developed a semi-automated method for the determination of total thiamin in whole blood.

Since, other postcolumn derivatization methods with or without prior hydrolysis of the thiamin coenzymes and using different kinds of mobile phase and oxidizing agent have been developed [7–12]. Apart from these postcolumn

Abbreviations: TDP, thiamin diphosphate; TMP, thiamin monophosphate; QC, quality control; RP, reversed-phase; TEA, triethylamin

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derivatization methods also precolumn oxidation procedures can be used to analyze Vitamin B1 and its derivatives. In these methods the precolumn oxidized coenzymes are chromatographed on columns which, until recently, were only stable up to a relatively low pH (pH 7) of the mobile phase. Therefore, the chromatographic separation of the different thiochromes and their subsequent measurements were performed close to this pH value [13–17].

The limits of quantification reported for these methods and defined as three times the signal-to-noise ratio show great variation from 4 up to 400 fmol on column (4 and 10 fmol for refs. [10,15], respectively, above 30 fmol for the others) and the between CV's range from 3 [15] up to 8.1% [12].

Presently both methods pre- as well as postcolumn oxidation procedures are applied, having their specific advantages and disadvantages. More in detail the use of postcolumn procedures requires post-column derivatization equipment, which needs specific skills and may contribute to increased extra column band broadening. Precolumn oxidation methods usually apply gradient elution procedures. The use of gradient elution also requires a sound knowledge of this technique. Gradient elution equipment, however, has become a more or less standard part of HPLC equipment and is available in nearly all analytical laboratories.

Fluorescence of the thiochromes increases with increasing pH, reaching a maximum above pH 9 [18]. The determination of thiamin at such relatively high pH values offers some distinct advantages. First, measuring the target compounds at maximal signal-to-noise ratios allows the reduction of the amount of sample to be injected on the column. Secondly, detector signals of thiamin and its derivatives significantly depend on the pH of the mobile phase up to a pH value of about 8. Consequently, incidentally small changes, e.g. by not regularly working equipment, in the pH area 7–8 of the mobile phase together with the relatively low responses may negatively influence the reproducibility and robustness of the thiamin analyses. These small changes may not necessarily influence both the sample and calibrator peaks to the same rate. Increase of the sample volume may partly compensate low responses. However, this introduces peak broadening which gives rise to problems with respect to the separation. These problems can easily be circumvented by working at pH values of the eluent above 9.

During the last decade new generations of reversed-phase (RP) columns, which are significantly better resistant towards very high pH values of the eluent, have been introduced [19]. For example Kirkland et al. [20] developed a so-called bidentate bonded RP-phase, that proved to be particularly stable up to pH 11 of the mobile phase. Such types of columns together with an optimized mobile phase composition allow analysis under high pH conditions during an acceptable period of time and at optimal signal-to-noise ratios.

In this study an analysis protocol is described for thiamin and thiamin esters in whole blood under pH 10 conditions. Due to the improved fluorescence properties of thiamin and its derivatives these analysis conditions significantly contributed to an improved detectability and robustness of this method.

2. Materials and methods

2.1. Reagents

All reagents were of analytical grade. Potassium ferricyanide, thiamin diphosphate and triethylamin were purchased from Merck (Darmstadt, Germany). Thiamin and thiamin monophosphate were obtained from Sigma Chemical Company (St. Louis, MO, USA); purity of thiamin and derivatives >99%, perchloric acid from Baker (Deventer, the Netherlands) and methanol from Lamers & Pleuger ('s-Hertogenbosch, the Netherlands). The HPLC column and guard column were purchased from Agilent Technologies (Amstelveen, the Netherlands).

2.2. Preparation of blood samples

Blood samples were collected in EDTA tubes and immediately frozen at −20 °C upon arrival in the laboratory. On the day of analysis samples were thawed in the dark at room temperature. After mixing, 0.5 ml of hemolysed blood was transferred to a polypropylene tube. Subsequently an aliquot of 50 μl of distilled water and 0.5 ml of cold 7.2% perchloric acid were added under vortexing. The distilled water was added to equalize the volumes of the calibrators and the samples. Tubes were placed at −20 °C for 5 min and after vortexing for another 10 min at +4 °C. After vortexing again the samples were centrifuged at 3500 × *g* for 15 min. Subsequently, the supernatants were filtered through a Millipore Millex R-GV 0.22 m/25 mm filter into light protected vials and placed in the cooled autosampler tray of the HPLC system.

2.3. Preparation of calibrators

Different stock solutions of thiamin, TDP and TMP were prepared in 0.01 M aqueous HCl, each at a concentration of 3.0 mmol/l. Concentrations were controlled by measuring the absorption at 248 nm. These stock solutions were diluted 1:100 in distilled water and subsequently 5.0 ml of each diluted stock solution were put together and the mixture was filled up to 100 ml with distilled water. Portions of 1 ml of this working standard (final concentration 1.5 μmol/l) were stable for 2 months when stored at −20 °C in the dark. Pooled EDTA blood samples were diluted 1:1 with distilled water and stored in portions of 8 ml at −80 °C.

Just before each run a portion of pooled blood was thawed in the dark at room temperature. Subsequently 0, 25 and 50 μl of the working standard were added to portions of 0.5 ml of the pooled blood together with 50, 25 and 0 μl of distilled water. Addition of thiamin, TDP and TMP was zero, 75 and

150 nmol/l, respectively. The calibrators were treated in the same way as the blood samples and were run with each batch of seven samples.

2.4. Preparation of quality control samples

Quality control samples (levels 1–3) were prepared by the addition of 0, 40 and 80 nmol/l TDP to pooled and hemolysed EDTA blood samples. The QC hemolysates were aliquoted and were stable for 6 months when stored at −80 °C.

2.5. Chromatographic conditions

HPLC analyses were performed with a liquid chromatograph Model 1100 of Agilent Technologies (Newport, DE, USA) provided with autosampler, cooled sample tray, vacuum degasser, thermostated column compartment and binary pump and a fluorescence detector, all of Agilent Technologies. The analyses of samples and standards were carried out under gradient conditions. Eluent 1 consisted of water–methanol–triethylamin (TEA) and concentrated HCl 94.0:5.2:0.52:0.26 (v/v). This solution was adjusted with concentrated HCl or TEA to pH 10.0. Eluent 2 consisted of water–methanol 30:70 (v/v). The gradient profile was as follows: $t = 0$, 100% eluent 1; $t = 2$ 90% eluent 1 plus 10% eluent 2; $t = 6$ 80% eluent 1 plus 20% eluent 2. The eluent flow was 1.0 ml/min at a temperature of 25 °C. The column used for this analysis was a reversed-phase column: Zorbax Extend C-18; 4.6 mm × 150 mm, $d_p = 3$ μm, protected with a 10 mm long guard column, both from Agilent Technologies, Newport, DE, USA. This column is a bidentate-type column and especially manufactured to operate under high pH eluent conditions.

2.6. Detection

For a fully automated pre-column derivatization of the different coenzyme forms of Vitamin B1, deproteinized blood samples, calibrators and controls were reacted with potassium ferricyanide (5 g/l 50% methanol) and sodium hydroxide (5 M) as follows: 3 μl sodium hydroxide, 3 μl potassium ferricyanide, 25 μl sample, again 3 μl potassium ferricyanide and 3 μl sodium hydroxide were subsequently automatically pipetted by the autosampler, and mixed 10 times. The final pH of the reaction mixture was 10.1 ± 0.1. After 1 min of reaction, samples were injected. Excitation of the target compounds was performed at 367 nm, the resulting fluorescence emission was measured at 435 nm.

2.7. Calculation of results

Chromatographic data were collected and analyzed with the Agilent ChemStation software. Concentrations were quantified by measuring peak heights.

3. Results

3.1. Chromatography

Typical and representative examples of the chromatographic profiles of a calibrator, a patient blood sample and a quality control sample are shown in Fig. 1. All three Vitamin B1 coenzymes are resolved very well. Separation between thiamin diphosphate and thiamin monophosphate is complete. This allows accurate calculations of the concentration of TDP, the biological active coenzyme of Vitamin B1. No interfering substances of significant concentration could be detected at retention times of the different coenzymes.

As can be seen in Fig. 2 above a pH value of approximately 8 of the mobile phase, the retention times of thiamin

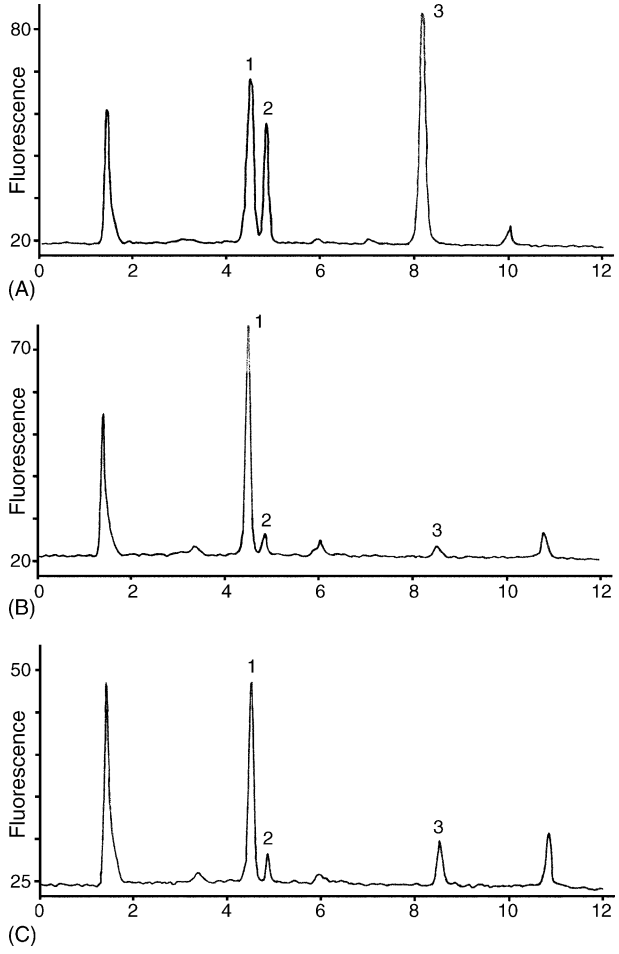

Fig. 1. Examples of the chromatographic profiles of highest calibrator (A), a patient blood (B), and a quality control (C) sample, respectively. The positions of TDP (1), TMP (2) and thiamin (3) are indicated in the figure. Conditions: Precolumn derivatisation. Zorbax Extend C-18 column; $d_p = 3$ μm; 150 mm × 4.6 mm (Agilent Technologies); mobile phase: eluent 1: water, methanol, TEA and hydrochloric acid, 94.0:5.2:0.52:0.26 (v/v), pH 10; eluent 2: water, methanol, 30:70 v.v. For gradient profile see text. Column temperature 25 °C. Detection: fluorescence; excitation and emission wavelengths 367 and 435 nm, respectively.

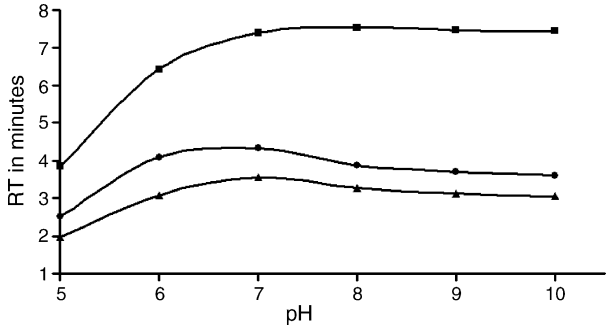

Fig. 2. Plot of retention time of thiamin (■), TDP (●) and TMP (▲) as a function of the pH of the mobile phase.

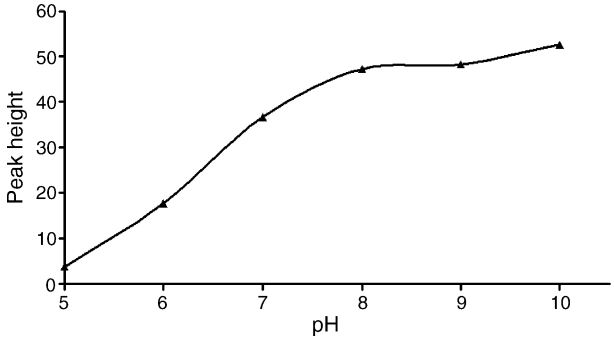

Fig. 3. Plot of the signal-to-noise ratio expressed as peak height for TDP as a function of the pH of the mobile phase.

and its derivatives do not change substantially as a function of pH of the mobile phase. Above this value the retention times reach a plateau and become more or less stable. In addition to that, we observed that at mobile phase pH values larger than 8 peak shapes were also significantly improved. Furthermore, as mentioned earlier, the fluorescence response of thiochromes strongly depends on the actual pH of the mobile phase. In Fig. 3 it can be seen that, compared to the often applied eluent of pH 7, a significant enhancement of almost 30% of the fluorescence signal can be obtained by increasing the pH of the mobile phase to 10. After that signal-to-noise ratios of thiochromes are leveling off. From Fig. 3 it is also clear that measuring above pH 8 the dependency of thiochromes signal-to-noise ratios of incidental changes of mobile phase pH values will substantially decrease. Hence performing the analysis at higher pH values the robustness of the method will be further improved. Therefore, analyzing thiamin and its derivatives at pH 10 of the mobile phase will result in maximal intensity and reproducibility of fluorescence signals. Furthermore, also the repeatabilities of the retention factors of the different thiamins will be improved, resulting in an improved robustness of the assay.

3.2. Column stability

In the analysis protocol described here the applied Zorbax Extend C-18 column proved to be stable under the high pH 10 conditions of the mobile phase. Up to about 1500 injections hardly any substantial changes were observed in the retention times of the sample compounds. In addition, also the efficiency of the column remained nearly unaffected. These findings are in agreement with earlier results obtained by Kirkland et al. [21] on this type of columns. Above that number of injections some peak deterioration may occur indicating that the column quality is decreasing.

Table 1
Precision of Vitamin B1[a], Th, TMP and TDP in whole blood

	Vitamin B1[b]		Thiamin		TMP		TDP	
	Mean[c]	CV (%)	Mean[c]	CV (%)	Mean[c]	CV (%)	Mean[c]	CV (%)
Within-batch ($n = 10$)[a]								
Level 1	95	2.2	10.4	2.6	0.6[d]	79.7	83.8	1.8
Level 2	136	1.6	9.7	3.1	3.7	11.2	122.1	1.2
Level 3	174	1.5	9.4	4.8	7.2	6.4	157.5	2.1
Between-batch ($n = 36$)[e]								
Level 1	102	6.9	11.7	13.9	3.4	56.1	85.9	4.3
Level 2	144	5.9	10.4	13.4	5.8	47.5	126.4	3.2
Level 3	185	4.6	9.7	12.2	8.9	46.4	166.2	4.2
Between-batch ($n = 16$)[f]								
Level 1	100	4.4	11.5	12.2	2.4	38.5	86.3	3.4
Level 2[g]	142	3.3	10.6	14.7	4.4	16.7	126.4	2.5
Level 3[g]	183	3.4	9.8	12.3	7.0	13.3	165.9	3.4

[a] Five samples of each level prepared and injected in duplicate.
[b] Total Vitamin B1, i.e. sum of Thiamin, TMP and TDP.
[c] Expressed as nmol/l.
[d] Under detection limit.
[e] Prepared singular and injected in duplicate for 18 days during a period of 13 weeks by several technicians.
[f] Prepared singular and injected in duplicate for 8 days during a period of 5 weeks by one and the same technician.
[g] Only TDP was added.

Table 2
Recovery of TDP from whole blood analyzed at pH 10

	Calculated from		
	Level 2 – Level 1	Level 3 – Level 1	Level 3 – Level 2
N	22	22	22
Mean (%)	100	98	99
SD	7	6	6
CV (%)	7.5	6.1	5.6

3.3. Analytical validation

3.3.1. Linearity

The assay was linear up to at least 350 nmol/l for each coenzyme. Higher concentrations were not evaluated because they are no longer physiologically relevant. The fluorescence signal at this concentration is far from the maximum that can be detected.

3.3.2. Detection limit

The minimum detectability of thiamin and its derivatives, defined as three times the standard deviation of the blank signal of the eluent, was 1 nmol/l, which corresponds to an absolute amount of 12 fmol on column for each component. This value is necessary to detect TMP at a relevant level according to earlier reported reference values of 0.9–7.3 nmol/l [15].

3.3.3. Precision

To calculate the within-batch precision quality control samples (levels 1–3) were prepared five times and injected in duplicate together with patient blood samples in one normal routine run on the same day. To obtain the between-batch precision the same levels were prepared for 8 days and injected in duplicate during a period of 5 weeks by one and the same technician and for 18 days during a period of 13 weeks by several technicians. The results are presented in Table 1.

3.3.4. Recovery

From the amounts of TDP added to the quality control samples as described before the recoveries were calculated. In Table 2 the different recoveries obtained for the three levels are presented.

4. Discussion

To get optimal signal-to-noise ratios and reproducibility in a HPLC method for the measurement of total thiamin or individual thiamin coenzymes in whole blood the assay conditions have to be optimal. Postcolumn derivatization methods and precolumn derivatization techniques, the latter with chromatography and detection of the thiochromes at sub optimal pH of the mobile phase, are lacking these optimal conditions. Postcolumn derivatization methods have the disadvantage of peak broadening as a result of the additional equipment behind the chromatographic separation for the addition of thiochrome reagent (pump) and for mixing and reacting the thiamin coenzymes with the thiochrome reagent (reaction coil). Therefore, in order to circumvent these problems, precolumn derivatization techniques can be attractive alternatives. These are often performed with an alkaline mobile phase of relatively low pH, below 8. This, in order to avoid a rapid loss of column performance because of the dissolution of silica which may occur at higher pH. In our experience, the different conventional reversed-phase stationary phases indeed showed an unsatisfactory column longevity. These findings are in agreement with the results of other workers ([22], and references therein).

Furthermore, the relatively low fluorescence response of the target components is another drawback of working in between pH 7–8. This, in turn, is not favorable to achieve optimal signal-to-noise ratios. In addition, working at the highest signal-to-noise ratios allows the further reduction of amount of sample injections. Moreover, due to the steepness of the dependency of the fluorescence signal versus the eluent pH the robustness of the method may be negatively influenced working at these pH values.

In order to enable analyses under high pH eluent conditions on silica based RPLC columns especially during the last decennium substantial progress has been made, see, e.g. refs. [19,20] and references therein. In addition to that also relevant knowledge has become available to improve the longevity of RP-columns by selecting proper eluent conditions [23]. For example, replacing the often used phosphate by an organic buffer can substantially lengthen the column longevity and hence the number of injections during a column life cycle [19]. Among the various attempts to synthesize more stable RPLC-columns for high pH eluent conditions the so-called bidentate approach has proven to be a successful concept [24]. In bidentate phases organic ligands, e.g. C-18, are synthesized to the silica by a two-fold attachment. This approach has proven to protect the modified silica backbone from dissolution [20]. The Zorbax Extend C-18 column investigated in the study is based on that bidentate concept mentioned here above.

The use of that high pH resistant column described here resulted in a robust and sensitive method for the analysis of Vitamin B1, which is completely automated and therefore easy to perform. Recovery and linearity are high. The limit of quantification of 12 fmol on column finds itself at the lower end of a huge range of reported limits from 4 [15] up to 400 fmol [6]. The between-batch CV's of total Vitamin B1 and especially of TDP, the biological active coenzyme, are equally low or even better than those of other reported methods [10,12–15,17], even when several technicians perform the assay on a routine basis.

5. Conclusions

The here proposed protocol for the analysis of thiamin and thiamin coenzymes in whole blood has distinct advantages

over existing methods. The separation of the analytes under high pH 10 conditions results in well resolved and more symmetric peaks. In addition under these pH conditions the detectability of thiamin and the coenzymes is significantly increased. The same is true for the robustness of the whole method, which is also improved.

The use of a special high pH resistant reversed phase Extend C18 column of Agilent Technologies allowed for at least 1500 injections, which is substantially more compared to conventional columns. After that, this column could still be used and showed hardly any deterioration or changes in retention factors and efficiency. This high pH stability clearly contributes to the resolution, detectability and robustness of the whole method.

The within-batch CV and the between-batch CV were below 2.5% and 4.5%, respectively. The method is very linear and the recovery was nearly 100%. The limit of quantitation was 1 nmol/l whole blood corresponding to 12 fmol on column for each component.

Summarizing, the use of a high pH resistant Extend C18 RPLC column resulted in a robust, sensitive and precise method for the analysis of thiamin and the coenzymes.

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