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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.

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