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Short communication

High-performance liquid chromatographic determination of thiamine diphosphate in erythrocytes using internal standard methodology

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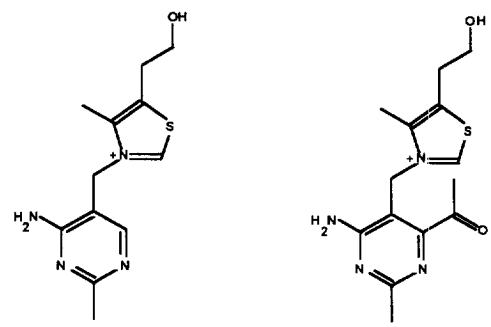
Abstract

A high-performance liquid chromatography method for the determination of thiamine diphosphate (vitamin B_1) in erythrocytes is presented. The method is robust, accurate and reproducible and due to the use of acetylaneurine as an internal standard, offers advantages over previous methods. © 1997 Elsevier Science B.V.

Keywords: Thiamine diphosphate; Vitamins

1. Introduction

Thiamine (vitamin B_1) is a pyrimidyl substituted thiazole [3-(4-amino-2-methyl-pyrimidyl-5-methyl)-4-methyl-(β-hydroxyethyl)-thiazole] (Fig. 1). The principal biologically active form of thiamine, thiamine diphosphate (TDP) is a coenzyme in three important enzyme complexes, pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and transketolase. Hence it plays a vital role in carbohydrate metabolism [1]. The total thiamine pool in the average adult is about 30 mg [2] and an intake of 0.5 mg per 1000 kcal per day is needed to maintain this pool [3]. Due to its relatively short storage time, marginal deficiency can occur within 10 days and



(a) Thiamine (b) Acetylaneurine

Fig. 1. Chemical structures of (a) thiamine and (b) acetylaneurine.

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more severe deficiency within 21 days if intake is stopped [2].

The two classical conditions resulting from severe thiamine deficiency are beriberi and Wernicke's encephalopathy (known as Wernicke–Korsakoff syndrome in the USA). Thiamine deficiency is rare in western countries and is mainly seen in chronic alcoholics [4], the elderly [5], chronic vomiting including hyperemesis gravidarum [6,7], diarrhoea [1], marked anorexia [1] and total parenteral nutrition [8]. Marginal deficiency may also be seen in patients with congestive heart failure treated with diuretics [9–11]. Recently it has been suggested that patients with Alzheimer's disease may be thiamine deficient [12–15].

It appears that no conditions are directly attributable to thiamine excess and that thiamine administration is safe except in extremely rare cases of anaphylaxis from intravenous thiamine [16].

Thiamine status in man may be assessed by a number of methods, including the functional erythrocyte transketolase activity measurement coupled with in vitro activation by exogenous TDP (the TDP effect) [1,2], the non-specific manual thiochrome method [17], or microbiological methods [17]. However it is now generally accepted that the assessment of thiamine status is best achieved by the direct measurement of thiamine levels in blood using HPLC techniques [18–20]. Since eighty percent of the total thiamine content of whole blood is present in the erythrocytes [2], erythrocyte measurements are used. Ninety-five percent of the thiamine in whole blood is present as TDP [21].

This paper presents for the first time a HPLC method for erythrocyte TDP which employs acetylaneurine as an internal standard. The method uses precolumn derivitization via oxidation to thiochrome, isocratic elution, and fluorescent detection.

2. Experimental

2.1. Instrumentation

HPLC analysis was performed using a Rheodyne (Cotati, CA, USA) Model 7125 injection valve with a 50-μl fixed sample loop, a Waters (Waters-Millipore, Milford, MS, USA) Model 510 pump, Waters Model 470 scanning fluorimetric detector and Waters Model 745B data module. The detector was set at excitation 375 nm and emission 430 nm with gain ×1000.

A Phase Separation (Clwyd, UK) Spherisorb 5μm NH_2 125×4-mm steel column with a Phase Separation 5-cm guard column was used for the separation.

2.2. Reagents

HPLC grade methanol was obtained from Labscan Analytical Sciences (Stillorgan, Ireland). Acetylaneurine HCl was purchased from ICN Biochemicals (Thame, UK). All other chemicals, including cocarboxylase (thiamine pyrophosphate chloride TDP) were procured from Sigma (St. Louis, MO, USA).

The mobile phase was methanol:potassium phosphate buffer (pH 7.5, 0.1 *M*) in a ratio of 45:55 v/v which was delivered at a flow-rate of 1.0 ml/min leading to a pressure of 138 bar. Fresh mobile phase was used each time and prior to use was filtered through a 0.22-μm Millipore GV filter and degassed under vacuum.

A 30.4 m*M* solution of potassium ferricyanide and a 0.8 m*M* solution of sodium hydroxide were used as the oxidising agent for the precolumn derivitization to thiochrome.

2.3. Sample preparation

Standard stock solutions of TDP and acetylaneurine (both 40 μ*M*) were prepared in 0.1 *M* hydrochloric acid and stored in the dark at 4°C. Under these conditions the solutions were stable for a minimum of two months (i.e. no additional peaks or shoulders were formed and there was no fall in peak magnitude). Similar stability of TDP stock solutions in 0.1 *M* HCl at 4°C are reported by Baines [18] and Wielders et al. [22]. Herve et al. [21] and Tallaksen et al. [23] stored TDP standards at −20°C in 0.01 *M* HCl for up to three months. However, Lee et al. [24] reported severe hydrolysis of thiamine phosphate esters upon storage in 10% perchloric acid at −20°C, resulting in a 50% fall in TDP after 1 day; when subsequently stored in 0.1 *M* HCl, a 20% fall was noted after 5 days at 4°C. Warnock [25] reported

instability after 3 days but did not say how the standards were stored.

Working solutions were prepared fresh for each run by diluting the stock solutions with deionized water. Immediately after preparation the working solutions were added to methanol, see below.

Venous blood samples were collected into lithium heparin or potassium EDTA tubes and haemolysates formed as described by Baines [18]. To 1 ml of haemolysate, 30 μl of a 4.0 μ*M* solution of acetylaneurine internal standard was added and the mixture thoroughly shaken. Two millilitres of methanol were then slowly added and, following mixing, left standing for 30 min. After centrifugation (10 min, 2000 *g*), 50 μl of freshly prepared potassium ferricyanide (30.4 m*M*) and 50 μl of sodium hydroxide (0.8 *M*) solution were added to 1 ml of the clear supernatant in order to form the thiochrome derivatives. The resulting yellow solution was then filtered through a disposable 0.45-μm Durapore PDVF filter (Millipore) and 50 μl were injected onto the column. Standards equivalent to 50, 100, 200 and 400 n*M* were prepared in a similar fashion.

3. Results and discussion

Acetylaneurine [3-(6-acetyl-4-amino-2-methyl-pyrimidyl-5-methyl)-4-methyl-(β-hydroxyethyl)-thiazole] is a close structural relation of thiamine, the difference being an additional acetyl group on carbon-6 of the pyrimidine ring of acetylaneurine (Fig. 1). Consequently, it has similar chemical, physical and chromatographic properties to thiamine. It has proved an ideal internal standard in that it is detected with similar sensitivity as TDP, it does not occur naturally in the human body and is chemically stable in the sample and throughout the isolation procedure. It elutes near the compound of interest and is well resolved from other peaks. Sander et al. used chloroethylthiamine as an internal standard [26], however their method employed gradient elution. Amprolium has also been used as an internal standard [27] but it is not ideal due to structural differences. Typical chromatograms obtained using our method are shown in Fig. 2.

The retention times of the thiochrome derivatives of acetylaneurine and TDP were 3.0 and 4.0 min respectively. No additional peak could be detected as long as 20 min after the elution of the TDP derivative.

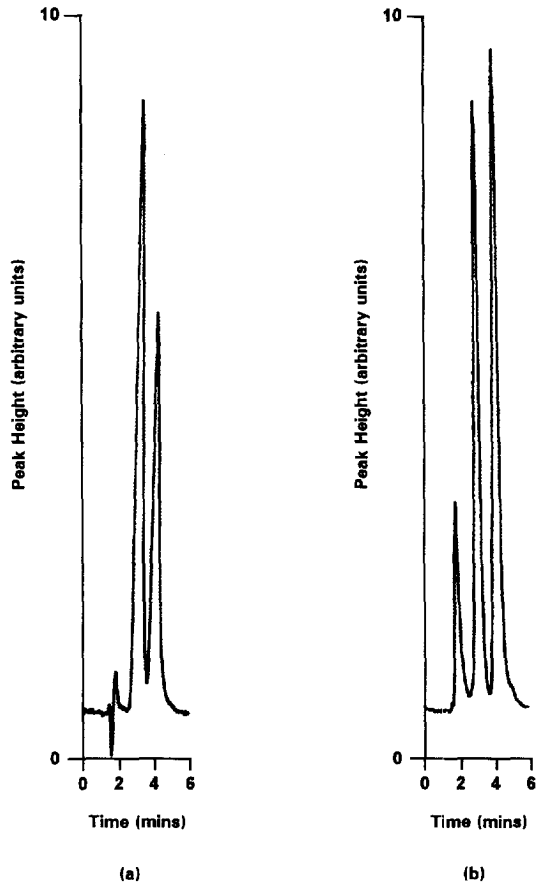

Fig. 2. Chromatograms of (a) 100 n*M* thiamine standard and (b) haemolysate prepared from a healthy control subject. Acetylaneurine internal standard and TDP peaks elute after 3 and 4 min respectively.

Using standard solutions the assay was determined to be linear up to at least 1600 n*M* of TDP, a level far in excess of that found in any samples subsequently analysed ($n=10$, $r^2=0.9964$, intercept=0.1226 S.E. 0.1188, slope=0.0063, S.E. 0.0001).

Using standard solutions the detection limits of TDP and acetylaneurine were found to be less than 2 n*M* (signal-to-noise ratio=3).

The intra-assay imprecision coefficient of variation (C.V.) was 2.5% ($n=12$) on a pooled haemolysate sample with a mean TDP level of 203.8 n*M*. The inter-assay imprecision C.V. was 3.5% ($n=12$) on a

pooled haemolysate sample, stored at −20°C, with a mean TDP level of 216.8 n*M*. These values compare favourably with those of Baines [18], intra and inter-assay C.V. 2.9% ($n=10$) and 5.7% ($n=19$) respectively, Herve et al. [21] intra- and inter-assay C.V. 8.8% ($n=20$) and 11.5% ($n=20$) respectively and Tallaksen et al. [23] intra and inter-assay C.V. 3.5% ($n=20$) and 3.8% ($n=20$) respectively.

Recovery tests were carried out by adding 50 μl of the appropriate dilution of TDP stock to 1 ml of a pooled haemolysate. At additions of 100 n*M* and 200 n*M*, the recovery was 97.6% (S.D. 3.9, $n=5$) and 86.6% (S.D. 3.4, $n=5$) respectively.

Reference values were measured on non-fasting samples taken from a population of healthy laboratory staff (29 male, 16 female; median age 38 years, range 20–60). The reference range obtained was 174.2(68.1) n*M* [mean (2S.D.)]. This range was similar to that found by Baines [18] 225.3(60.5) n*M*, Warnock [25] 268.0(58.6) n*M*, Floridi et al. [28] 233(120) n*M* and Herve et al. [21] 176(56) n*M* but less than that found by Tallaksen et al. [23] 325.0(71.9) n*M*.

4. Conclusion

The method we report has been in use in our laboratory for the past six months with no problems. Column performance has remained unchanged throughout this time. The method is robust, accurate and reproducible and due to the use of acetylaneurine as internal standard, offers advantages over other methods for the determination of TDP in erythrocytes. Since the thiochrome derivatives are stable for at least 24 h, the method may be automated.

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