



Original Paper

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Evaluation of vitamin B1 status of alcoholic subjects in Abidjan (Côte d'Ivoire)

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ABSTRACT

Given the clinical importance of free thiamine and its esters on the essential human organs, their determination seems necessary to appreciate vitamin B1 status in physiological and pathological states of local populations. The present preliminary study describes a method of analysis of free thiamine and its phosphate esters: thiamine monophosphate and thiamine diphosphate by reverse phase liquid chromatography and fluorimetric detection. Validation tests were applied to verify the reliability of the proposed method. The results indicated that the chromatographic system was suitable for chromatographic analysis of free thiamine and its esters in whole. Thus, the method was applied to whole blood samples of 35 alcoholic patients under alcoholic detoxification cure in Abidjan. The results of the application showed that 100% of the alcoholic subjects presented a total thiamine concentration under the accepted norm of $20\mu\text{g.L}^{-1}$. Before the cure at Day 0, 74,28% of the subjects presented a severe deficiency. After 30 days, all of the subjects still presented a marginal status of vitamin B1. Thiamine supplementation included in the alcoholic detoxification cure seems to improve its status. However, it seems that the latter should be of sufficient duration to ensure the return to an effective normal vitamin B1 status.

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INTRODUCTION

Vitamin B1 is a component, present in the human body as free thiamine (T) and its esterified forms: thiamine monophosphate (TP), thiamine diphosphate (TPP) and thiamine triphosphate (TPPP). The literature reports the presence of T, TP and TPP in the serum and erythrocytes while TPPP is mainly

found in neural tissue (Le Gruse and Watier, 1993). Although TPP, as the main coenzyme in the metabolism of sugars appears as the main biologically active form, the role of other esterified forms has been analysed. Moreover, literature reports that the effects of vitamin B1 deficiency in humans are numerous (general, neurological and

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cardiac...) and of varied severity (Le Gruse and Watier, 1993; Harper, 2006). Chronic alcoholism has been reported to be one the possible causes of thiamine deficiency (Gloria et al., 1997; Ke et al., 2009). Because of the relatively high consumption of alcoholic beverages imported or locally manufactured in Côte d'Ivoire, the aim of this study was to assess the status of vitamin B1 in chronic alcoholism. Thus, a method for the simultaneous determination of all significant compounds of vitamin B1 in whole blood was developed and applied. Several methods of analysis of vitamin B1 in whole blood have already been developed in Côte d'Ivoire since 2001 (Aké et al., 2006). In the present study, we applied the method of determination of free thiamine and its esterified forms to evaluate the status of vitamin B1 of alcoholic subjects during their alcoholic detoxification cure (Ryle and Thompson, 1984; Koffi 1990; Tallakssen et al., 1992a; Tallakssen et al., 1992b; Bogbe, 1999; Agabio, 2005; Aké et al., 2006).

MATERIALS AND METHODS

Subjects

The subjects recruited were alcoholics hospitalized or not at the "Centre d'Accueil de la Croix Bleue" (CACB) which is a centre that receives and takes care of alcoholics through detoxification cures. The subjects were consenting, presenting a period of intoxication of at least five (5) years. They consumed at least 50g of alcohol per day and were not under vitamin B1 or multivitamin treatment. Three samples of five (5) ml of venous blood were withdrawn in subjects fasting, on the day after the recruitment (Day 0) and after 15 days (Day 15) and 30 days (Day 30) in tubes containing heparin lithium. The whole blood samples were then stored at -70 °C until analysis in the laboratory of Nutrition (INSP).

Apparatus, reagents and working solutions

Precision scale- Toledo PB 303-S (Viroflay, France); Thermoregulated water bath- Jouan (Saint Herblain, France);

Centrifuge- Jouan E 82 (Saint Herblain, France); Liquid chromatograph- SHIMADZU (Toussart and Matignon, Les Ullis, France) equipped with a LC-6A pump, a RF-10 A fluorimetric detector and a Chromatopac CR-6A integrator.

The following reagents used were of analytical grade: Distillated water, Hydrochloride acid, Trichloroacetic acid, Orthophosphoric acid, Sodium chloride, Dimethylformamide, Potassium ferricyanide, Potassium phosphate dibasic, Sodium hydroxide. HPLC grade Acetonitrile and Methanol were also used.

Reference products used were: Thiamine hydrochloride (ref. T 4625), Thiamine monophosphate chloride (ref T 1770), Thiamine pyrophosphate (C 8754) (Sigma Aldrich, Saint Louis, USA). Diluted working standard solutions were prepared extemporaneously by appropriate dilution of the mixed stock solution (10^{-6} mol.L⁻¹) with water.

Analytical method

The method used included an extraction step followed by a derivation step and reverse phase liquid chromatography (Aké et al., 2006). Liquid/liquid extraction was applied to a whole blood sample using trichloroacetic acid (1.44 mol.L⁻¹) for the deproteinisation step and diethyl ether for the removal of lipids in the blood. The derivation of the compounds was done using an alkaline solution of potassium ferricyanide. Thiochrome fluorescent derivatives were thus produced in the extract. The latter is subjected to the chromatographic analysis carried out under the following conditions:

- Stationary phase: Lichrospher NH2, (125 x 4,6 mm) (Merck, Darmstadt, Germany);
- Mobile phase: Methanol/Phosphate buffer pH 7,5; 12 :88 (v/v) ;
- Flow: 1.0 mL.min⁻¹;
- Injection volume: 20μ L
- Fluorimetric detection : $\lambda_{exc} = 375$ nm, $\lambda_{em} = 450$ nm.

Validation procedure

Different validation tests were applied to assess the reliability of the proposed method (Caporal et al., 1990; Carr and Wahlich, 1990). Linearity of the method was determined for each compound for six concentrations ranging from 0.67 to 67.5 $\mu\text{g.L}^{-1}$ for T, 0.83 to 83.3 $\mu\text{g.L}^{-1}$ for TP and 0.92 to 92.1 $\mu\text{g.L}^{-1}$ for TPP. Precision was evaluated by examining the repeatability of the chromatographic analysis (repeatability of the measurement of six successive injections of a mixed standard solution at three concentration levels of T, TP and TPP as well as of a whole blood extract). The repeatability of the analytical procedure was also evaluated by performing at least six independent extractions of a whole blood sample. Accuracy was achieved by using the standard addition method. Samples of whole blood were spiked with the mixed standard solution at three levels of concentration of T, TP and TPP. Spiked and unspiked samples of whole blood were then submitted to the whole analytical procedure. The average recovery percentage was then calculated. Limits of detection (LOD) and quantification (LOQ) were evaluated as the lowest concentrations, giving a signal to noise ratio (S/N) respectively equal to 3 and 10.

Statistical methods

Linearity was assessed from a scatter diagram and the regression line was determined using the method of least squares. An analysis of variance (ANOVA) was conducted to test the overall statistical significance and the slope of the regression line. The precision was assessed through the relative standard deviation values (RSD). Results of the application to the determination of vitamin B1 status in alcoholic patients were treated using the software Epi Info version 6.0. Vitamin B1 status in alcoholic patients over 30 days was described using mean \pm standard deviation at three days points. The differences of vitamin B1 concentrations in alcoholic patients between the days were

assessed using paired t test. The distribution of patients in different vitamin B1 status according to the day of analysis was compared using χ^2 test. The level of statistical significance was set at 0.05.

RESULTS AND DISCUSSION

Method and validation procedure

Under the analytical conditions used, T, TP and TPP peak were identified with a retention time of less than eleven (11) min (Figure 1). The total time of analysis is slightly higher than in the previous study by Aké et al. (2006) who had obtained a total time of 10 minutes. Despite the overall analysis time of 90 minutes, the method is easy to apply and can be used for the routine determination of free thiamine and its esters in the evaluation of vitamin B1 status in alcoholics during their treatment. Linearity was settled for T between 0.67 to 67.5 $\mu\text{g.L}^{-1}$, for TP between 0.83 to 83.3 $\mu\text{g.L}^{-1}$, for TPP between 0.92 to 92.1 $\mu\text{g.L}^{-1}$. The determination coefficient r^2 was always better than 0.9993. RSD assessing the repeatability was less than 2% for the chromatographic analysis and less than 5% for the entire procedure. The method was accurate, since the average percentages of recovery obtained for T, TP, TPP were respectively of 93.12%, 93.96% and 92.17%. The limits of detection and quantification were assessed respectively at 67 pg.L^{-1} and 670 pg.L^{-1} for T, 83 and 830 pg.L^{-1} for TP and 92 and 920 pg.L^{-1} TPP. These results demonstrate the reliability of the method and indicate that it is adapted to the analysis of free thiamine and its esters in whole blood (Caporal and al., 1990; Carr and Wahlich, 1990).

Application

Thus, the validated method was then applied to whole blood of 35 consenting alcoholic patients followed up in the CACB of Abidjan. From an epidemiological perspective, we found that the age of the subjects ranged from 25 to 75 years, with an average age of 46 years. The study showed a clear predominance of male subjects (sex ratio

of 4.83), as reported in a previous study by Bogbe (1999). The study showed that 88.57% of subjects were of Ivorian nationality against 11.43% of the subjects that came from surrounding countries. Most alcoholic patients belonged to the Kwa ethnic group probably because the CACB is located in Abidjan, with a low socio-economic level (51.43%). The maximum length of the alcoholic intoxication in the patients was 35 years, with a high proportion of alcoholics who were intoxicated for periods that stand between 16 and 20 years (45.71%). The beginning of alcoholism was mainly found in peri-adolescence in 48.58% of the patients while for 40% of the patients, alcoholism began when they were young adults. Subjects were generally poly-intoxicated, with a high proportion who consumed beer (42.86%) and koutoukou, a traditional manufactured local alcoholic beverage (34.28%), as shown in a previous study (Koffi, 1990).

From a clinical point of view, patients often showed hepato-digestive signs among which abdominal pain (54.28%), nausea and vomiting (22.86%). An important percentage of the subjects (65.71%) showed neuropsychiatric signs (polyneuritis, tremor of the extremities) (Thomson, 2000; Ke et al., 2009). This noted polymorphism could be due to the ravages of alcohol on various organs such as liver and central nervous system (Rooprai et al., 1996; Bogbe, 1999; Sgouros et al., 2004; Harper, 2009; Ke et al., 2009). On the biological level as shown in Tables 1 and 2, it is clear from the data analysis that the average concentrations of free thiamine and its esters are below the standard norm of $20 \mu\text{g}\cdot\text{L}^{-1}$ during the 30 days of the study (Tallakssen et al., 1992a; Tallakssen et al., 1992b; Aké et al., 2006). The average concentrations increased from Day 0 to Day 15 then to Day 30 for T, TP, TPP and total thiamine, so that the paired differences means are negative. Statistical analysis indicated that all these increases were statistically significant ($p < 0.05$). These results clearly indicate that alcoholic patients are deficient in vitamin B1, as reported in Table 2 (74.28% of the patients showed a severe deficiency at Day 0). Moreover, even if they are below the standard

norm, the average content increased significantly during the alcohol detoxification cure that includes multivitamins supplementation, from Day 0 to Day 15 and to Day 30 (Tallakssen et al., 1992b). The proportion of subjects with severe deficiency has significantly decreased from Day 0 (74.28%) to Day 30 (20%) and inversely for those presenting a marginal status from Day 0 (25.72%) to Day 3 (80%) ($\chi^2 = 21,11$; $df = 2$; $p = 2,6 \cdot 10^{-5}$). At Day 15 and Day 30, the number of patients with marginal vitamin B1 status increased from 16 to 28 patients, with still no patient with a normal status in vitamin B1. These results show the relative benefits of vitamin B1 supplementation. However, since no normal status was obtained during the thirty (30) days, it seems more appropriate to continue the supplementation for a period of time, longer than 30 days (Tallakssen et al., 1992b). Moreover, the hepato-digestive (54,28%) and/or neurological (65,71%) signs reported during the present study in patients that showed marginal or severe deficiency status were also seen in other studies (Majumdar et al., 1982; Tallakssen et al., 1992b; Gloria et al., 1997; Harper, 2006). Further investigation should be considered to evaluate a potential decrease of these signs during alcohol detoxification cure.

Conclusion

The method proposed in the present study allows the determination of free thiamine and its esterified forms in blood by reverse phase liquid chromatography after a liquid/liquid partition extraction step. The results of validation tests showed that the method was simple to implement, reliable and could be applied to the determination of total thiamine concentration. Applying the method to thirty five (35) alcoholic subjects following a detoxification cure at the CACB of Abidjan (Côte d'Ivoire) during thirty (30) days showed that 74.28% were vitamin B1 deficient at Day 0. Thirty days later, no normal vitamin B1 status could be observed among the alcoholic subjects. Moreover, only 20% of the subjects were still thiamine deficient. It seems that

Table 1: Free thiamine and its esters values¹ of alcoholic subjects from Day 0 to Day 30

Vitamin B1 ²	Day	Mean ($\mu\text{g}\cdot\text{L}^{-1}$)	Standard deviation ($\mu\text{g}\cdot\text{L}^{-1}$)	Paired differences	
				mean (SD) p*	mean (SD) p**
T	0	1.681	1.261	-1.196 (1.106)	
	15	2.878	1.735	<10 ⁻⁵	
	30	4.048	1.906	-1.170 (0.840) <10 ⁻⁵	
TP	0	0.322	0.263	-0.302 (0.282)	
	15	0.624	0.353	<10 ⁻⁵	
	30	0.843	0.383	-0.219 (0.267) 3x10 ⁻⁵	
TPP	0	3.824	1.460	-0.863 (1.663)	
	15	4.688	1.755	0.00418	
	30	5.931	1.785	-1.244 (1.343) <10 ⁻⁵	
Total Thiamine	0	5.828	2.834	-2.362 (2.670)	
	15	8.190	3.635	10 ⁻⁵	
	30	10,822	3.793	-2.631 (2.050) <10 ⁻⁵	

¹: Tabulated values are Mean \pm Standard Deviation (SD) of 35 determinations;

²: **T**: free thiamine; **TP**: thiamine monophosphate; **TPP**: thiamine diphosphate

* Paired t test D0-D15; ** Paired t test D15-D30; df=34

Table 2: Vitamin B1 status of alcoholic subjects from Day 0 to Day 30

		Day		
		0	15	30
Vitamin B1 Status n alcoholic subjects (%)	Normal*	0 (0%)	0 (0%)	0 (0%)
	Marginal**	9 (25.72%)	16 (45.71%)	28 (80%)
	Severe deficient***	26 (74.28%)	19 (54.29%)	7 (20%)
	Total	35 (100%)	35 (100%)	35 (100%)

$\chi^2 = 21,11$; $df = 2$; $p = 2,6 \cdot 10^{-5}$ for Marginal and Severe deficient status of alcoholic subjects

*Normal status: Total thiamine concentration > 20 $\mu\text{g}\cdot\text{L}^{-1}$;

**Marginal status: 10 $\mu\text{g}\cdot\text{L}^{-1}$ < Total thiamine concentration < 20 $\mu\text{g}\cdot\text{L}^{-1}$;

***Severe deficient status: Total thiamine concentration < 10 $\mu\text{g}\cdot\text{L}^{-1}$;

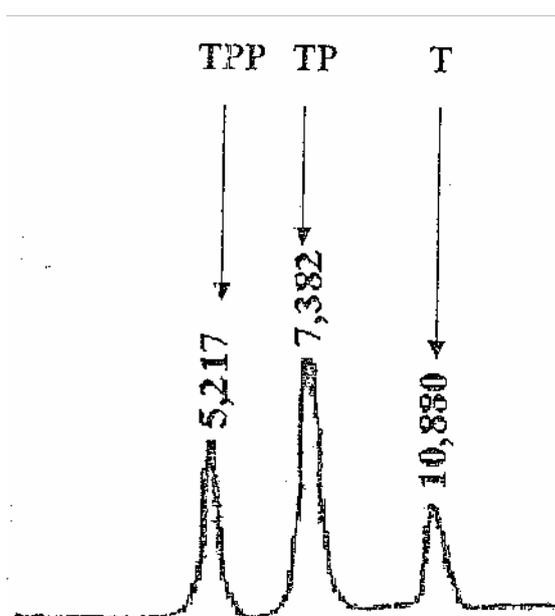


Figure 1: Chromatogram of a blood extract
T : Free Thiamine ; TP : Thiamine monophosphate ;
TPP : Thiamine diphosphate

vitamin B1 supplementation favourably influence the normalization of its blood content and reduced clinical signs as hepatodigestive and neurological, usually observed in alcoholic subjects.

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