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Simultaneous liquid chromatographic assessment of thiamine, thiamine monophosphate and thiamine diphosphate in human erythrocytes: a study on alcoholics

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Abstract

An isocratic HPLC procedure for the assessment of thiamine (T), thiamine monophosphate (TMP) and thiamine diphosphate (TDP) in human erythrocytes is described. Several aspects of the procedure make it suitable for both clinical and research purposes: limits of detection and quantification of 1 and 2.5 nmol/l, respectively, recovery of 102% on average (range 93–112%), intra- and inter-day precisions within 5 and 9%, respectively, total elution time 15 min. This analytical methodology was applied to a case-control study on erythrocyte samples from 103 healthy subjects and 36 alcohol-dependent patients at risk of thiamine deficiency. Mean control values obtained were: T=89.6±22.7 nmol/l, TMP=4.4±6.6 nmol/l and TDP=222.23±56.3 nmol/l. T and TDP mean values of alcoholics were significantly lower than those of control cases: T=69.4±35.9 nmol/l ($P<0.001$) and TDP=127.4±62.5 nmol/l ($P<10^{-5}$). The diagnostic role of TDP was evaluated and a significant role for thiamine was established in the study of alcohol related problems.

Keywords: Alcohol-related problems; Thiamine

1. Introduction

Vitamin deficiency has become rare in western countries and is typically associated with disease, especially alcoholism, rather than malnutrition.

Vitamin B1 (thiamine, T) and its phosphate esters (thiamine monophosphate, TMP; thiamine diphosphate, TDP and thiamine triphosphate, TTP) play major roles as coenzymes in carbohydrate metabolism and in nerve conduction. Thiamine and TMP were detected mainly in human serum and cerebrospinal fluid, T, TMP and TDP in erythrocytes and TTP in nervous tissue. TTP was detected in blood by Tallaksen et al. [1] and by Herve et al. [2] in very low concentrations.

TDP is a coenzyme for several intra-mitochondrial enzymes involved in carbohydrate and lipid metabolism and participates in the activity of three major enzyme complexes: the pyruvate dehydrogenase complex, the α-ketoglutarate dehydrogenase com-

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plex and transketolase [3,4]. Free thiamine is believed to mediate parasympathetic neural activity and thiamine deficiency leads to impairment of nerve conduction/transmission and may induce peripheral neuropathy and irreversible brain damage, often diagnosable only post mortem [5]. The blood level of vitamin B1 falls dramatically after 2 weeks of deprivation, often depleting liver and muscle stores and impairing functions of enzymes requiring TDP as a coenzyme. Thiamine deficiency and subsequent brain damage may occur in malnourished patients, affected by different diseases. Recently, thiamine deficiency is reported in AIDS and asymptomatic HIV positive patients [6] and a role of impaired thiamine metabolism in Alzheimer disease has been suggested [7]. Additionally, chemotherapy and radiotherapy can also cause vitamin deficiency, although the advisability of thiamine supplementation in malignancy is still under debate [8].

The risk of progressive vitamin deficiency in alcohol abusers is well known, with 30 to 80% of alcoholics in developed countries being diagnosable as thiamine deficient [5]. Furthermore, among alcoholics, malnutrition (primarily due to poor food intake, chronic gastritis and diarrhoea) is quite common and the availability of vitamins by intestinal absorption is compromised. Because of the combination of malnutrition and alcohol, alcoholics are at high risk for thiamine deficiency. Unfortunately, in clinical practice the need for therapeutic administration is often apparent only when thiamine reserves are critically low and the risk of brain damage, such as from Wernicke–Korsakoff Syndrome, is very serious.

Although determination of thiamine and its esters is recommended in alcoholics, since they are closely related to heavy alcohol intake and neurological damage, in clinical practice this is rarely done, because of analytical problems that make difficult reproducibility, comparability and so interpretation of the results.

Several methods for assessing thiamine status by direct measurement of thiamine and its esters in red blood cells or whole blood by high-performance liquid chromatography (HPLC) have been described [1,2,9–13]. The major analytical problems reflect lack of a reference method and of inter-laboratory standardisation. Furthermore, poor comparability is due to the differences in extraction and derivatization procedure for detecting these parameters, to differences in sample matrices used (e.g., red cells or whole blood), to differences in procedures for sample treatment and, even, to differences in the unit used for the results [10]. So, procedures for liquid chromatographic determination of thiamine and thiamine esters, need to be further improved to obtain better comparability of the results and practicability of the procedure.

The current paper proposes a procedure for use in both clinical practice and research for assessment of thiamine status by measurement of T, TMP and TDP. Since about 80% of the total thiamine pool in whole blood is stored in the erythrocytes (mostly as thiamine diphosphate) and erythrocyte concentration of TDP is a reliable indicator of thiamine body stores granted its depletion rate is similar to those of other major organs [5,9], concentrations of T, TMP and TDP were determined in washed red cells. TTP measurements could not be performed since a suitable TTP pure standard was not available to our group. The present work evaluates the HPLC procedure suitable for the simultaneous, isocratic separation of T, TMP and TDP from red cell extracts improving reproducibility, practicability (procedure less time-consuming and safer for the operator) and performance of chromatographic separation; it is the result of the study and elaboration of different experiences, keeping in mind the analytical problems yet to be solved above all as regards sample preparation.

The suitability of this HPLC determination for clinical and research purposes was tested assessing thiamine status of healthy subjects and chronic alcoholics under clinical observation.

2. Materials and methods

2.1. Instrumentation

The HPLC procedure is performed by precolumn, alkaline oxidation of thiamine and its phosphoric esters to thiochrome by potassium hexacyanoferrate $K_3Fe(CN)_6$ and final fluorimetric detection. Levels of thiamine, thiamine monophosphate and thiamine diphosphate were determined under isocratic con-

ditions using HPLC instrumentation by Merck, equipped with the automatic sampler AS-2000A (Merck, Darmstadt, Germany) and a computer with software for storing and processing data. The column was an Ultra Amino 5 μm, 100 Å, 250×4.6 mm with an Ultra Amino 5 μm, 100 Å 10×2.1 mm Guard Cartridges (Restek, Bellefonte, PA, USA). The fluorimetric detector was a Model F 1050 (Merck–Hitachi). A Beckman centrifuge Model J-6B, Vortex VELP Scientifica and pH meter Crison (Micro pH 2001, Modena, Italy) were used for preparation of samples and buffer. Sample extracts were filtered by 1 ml syringes equipped with Minisart RC4 filters 0.45 μm (Sartorius, Gottingen, Germany).

2.2. Chemicals and reagents

Powdered standards of thiamine, thiamine mono- and diphosphate esters, and potassium hexacyanoferrate were procured from Sigma (Milan, Italy). Sodium hydroxide (NaOH), trichloroacetic acid (TCA), hydrochloric acid (HCl), diethyl ether, acetonitrile HPLC grade, potassium dihydrogenphosphate (KH_2PO_4), sodium chloride (NaCl) were obtained from Carlo Erba, Milan, Italy. Ultrapure water was from the laboratory Milli-Q Unit (Millipore, Bedford, MA, USA). Sample processing involved saline to wash red blood cells, aqueous solutions of TCA 2.44 *M* (40%), potassium hexacyanoferrate 30.4 m*M*, and sodium hydroxide 0.8 *M*. Stock solutions 0.01 *M* of each standard were prepared in 0.01 *M* hydrochloric acid and stored at 4 °C. Working standard solutions must be prepared daily by dilution with ultrapure water. Dilutions from 1 to 1000 nmol/l were used to perform analytical tests and derive calibration curves. No standardised reference material for these analytes is currently available.

2.3. Chromatographic conditions

The mobile phase was potassium phosphate buffer (pH 7.5, 85 m*M*)–acetonitrile (65:35, v/v) delivered at a flow-rate of 1 ml min^{-1} and leading to a pressure of 80–85 bar. The mobile phase was freshly prepared and degassed under nitrogen flow before use. The injection volume was 50 μl at room temperature. The duration of the analytical run was 15 min. Fluorescence detection was operated at 375 nm excitation and 430 nm emission.

2.4. Patients

Following informed consent, 36 consecutive patients, 29 males (mean age 45.7±12.2 years) and seven females (mean age 40.7±12.3 years), hospitalised in the Alcohol Liver Disease Unit day-hospital (University ''La Sapienza'', Rome, Italy) served as subjects for the project.

Inclusion criteria

All the patients were alcohol-dependent, according to DSM-IV established criteria [14]. Only one patient was affected by active HCV hepatitis, as demonstrated by blood positive HCV-RNA. Nine were affected by liver cirrhosis (seven Child-Pugh A, two Child-Pugh B), and 26 by liver steatosis. None of the patients had acute alcoholic hepatitis.

Only six patients produced positive blood alcohol levels. The mean lifetime length of alcohol abuse was 25±13.7 years (min. 8–max. 52) with a mean current daily alcohol intake of 12.4±6.47 (min.=4, max.=30) standard drinks each containing about 13 g of pure ethanol. None of the patients had clinical evidence of thiamine deficiency.

None of the subjects had received vitamin supplementation prior to study enrollment.

Exclusion criteria

Patients affected by HIV infection, by neoplastic diseases, by any metabolic disease affecting liver functions. No females could be pregnant or on contraceptive drugs.

Control subjects were randomly selected from a group of employees undergoing routine medical examinations. One hundred and three subjects (mean age 42.9±10.2 years, range 25–62 years), 45 males (mean age 45.8±10.2 years) and 58 females, (mean age 40.2±9 years), participated voluntarily in the study. Written consent was given. Hematologic and biochemical values were within reference ranges and none had a significant medical history nor on a restricted or abnormal diet.

2.5. Preparation of samples

After overnight fasting blood samples (7 ml) were collected in EDTA vacutainers. Samples were stored at 4 °C and processed on the day that blood was drawn. After plasma separation, the red blood cells were washed three times with four volumes of saline in graduated tubes: cells were pelletted at 1000 *g* for 10 min at 10 °C and the buffy coat removed to carefully eliminate TDP-rich leukocytes. Finally, cell suspension was centrifuged at 1400 *g* for 20 min at 10 °C to obtain well packed red blood cells and then the saline was partially removed leaving a 1:1 cell suspension, thus obtaining all the analytical samples with final hematocrit 48–50%. (The centrifugation procedure and the correct dilution of the packed red blood cells are very important for the reproducibility of the final results). The procedure described here has been tested and employed in previous studies on red blood cells [15,16].

Extraction

The 1:1 suspension was well mixed to form a uniform suspension, and 2 ml were immediately transferred to a glass tube for extraction, yielding 1 ml packed-erythrocyte samples.

A 250-μl volume of TCA 40% was added to the 2 ml sample transferred to the glass tube, mixed by Vortex and left for 1 h in the dark at room temperature to complete protein precipitation. Then, the tube was centrifuged at 1900 *g* for 20 min and the liquid phase transferred in a glass tube. TCA was removed by five volumes of water-saturated diethyl ether and the obtained extract recovered and filtered through the 1 ml syringe equipped with MINISART filter. The procedure at this phase can be stopped and the extracted samples stored at −80 °C. In our experience, samples remain stable for at least 2 months.

Derivatization, linearity and recovery

To 1 ml of the extracted, filtered sample, 50 μl of potassium hexacyanoferrate (30.4 m*M*) was added, mixed by vortex and then 50 μl of NaOH (0.8 *M*) added. The derivatized sample (thiocrome) was thereby ready for HPLC fluorimetric determination.

The linearity of the procedure was assayed at points 2.5, 10, 50, 100, 125, 250, 500, 1000 nmol/l for thiamine and TDP. Since its concentration in the erythrocyte sample was much lower, thiamine monophosphate linearity was tested up to 250 nmol/l. Thiamine, thiamine monophosphate and thiamine diphosphate standard solutions were processed both separately and by mixing them to obtain the concentrations above. To verify extraction recovery, the same standard solutions at 5, 50, 100, 125 and 250 and 1000 nmol were analysed with and without previous TCA extraction. Recovery, intra- and inter-day precision was evaluated by replicates of the extracted standard mixtures at 5, 50, 100, 125, 250 and 1000 nmol/l. The inter-day precision was evaluated by two experiments repeated on the same day and every day for 5 non-consecutive days. Recovery from washed erythrocytes spiked before deproteinization was also evaluated. The reproducibility of the whole procedure was verified by replicating the assays of 30 actual blood samples. For sample quantitation, calibration curves were derived daily. Standard mixtures at concentrations 100 and 250 nmol/l were included in all analytical series.

3. Results

3.1. Analytical

The HPLC analytical run produced sharp and well resolved peaks. Fig. 1a–c show chromatograms from blank reagent, standard mixture containing the three substances and sample. The blank appears free from interferences and T, TMP and TDP are eluted in this order at well separated times (3.63±0.03, 6.72±0.16 and 9.89±0.20 min). Resulting data were linear in the 2.5–1000 nmol/l range concentration and the parameters of the peak areas linear regression analysis were the following: T: intercept=38.43, slope=4.19, standard error=73.14, r^2=0.998; TMP intercept=1.92, slope=5.13, standard error=5.70, r^2=0.999; TDP intercept=−19.6, slope=6.31, standard error=49.48,, r^2=1.000.

The detection limit, defined as the lowest concentration of the analytes in the calibrator that could be distinguished from zero, was 1 nmol/l and the limit of quantification, defined as the lowest concentration which can be measured with suitable precision and accuracy, was 2.5 nmol/l. The precision and accuracy of the extraction procedure, evalu-

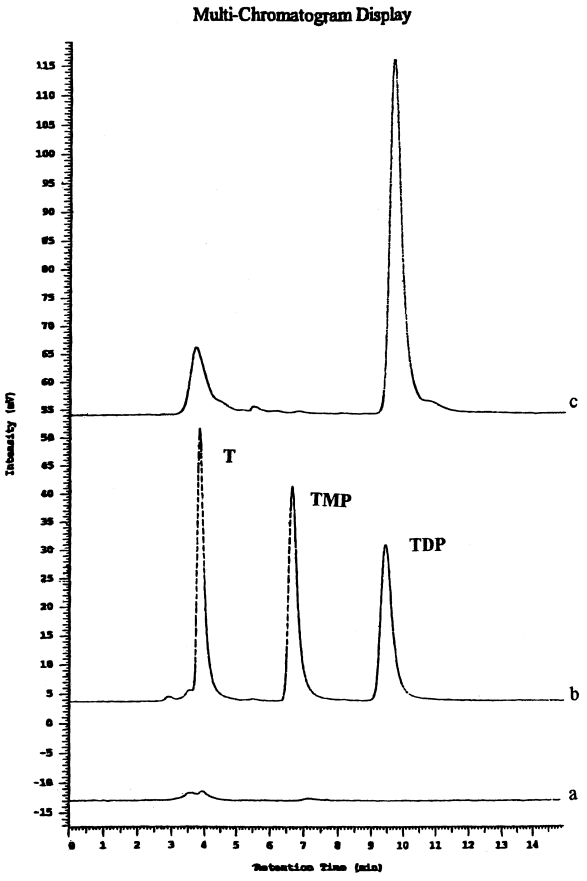

Fig. 1. Chromatograms from (a) blank reagent (redistilled water, potassium hexacyanoferrate and NaOH), (b) standard mixture containing 100 nmol of T, 100 nmol of TMP and 100 nmol of TDP, (c) actual red blood cell sample from a healthy subject (T=49 nmol, TMP<1 nmol and TDP=264 nmol). Order eluted: T (3.63 min), TMP (6.72 min) and finally TDP (9.89 min).

ated by aqueous standard solution in the range 5–1000 nmol/l for T and TMP and 5–250 nmol/l for TMP, provided results displayed in Table 1, where the percentages of recovery for each molecule and the intra-day and inter-day RSDs are reported. In washed erythrocyte samples spiked with 100 and with 10 nmol/l of T, TMP, TDP mean values of recovery were 107, 96, 100%, and 89, 98, 90%, respectively.

The derivatization obtained by addition of a mixture of $K_3Fe(CN)_6$ and NaOH instead of adding them separately to the sample, was excluded since unreliable results were generated.

The imprecision of the entire procedure was evaluated by duplicate determinations of 30 different blood samples. Analysis of variance between the means of the duplicates demonstrated that they were not statistically different. The regression analysis of the replicate pairs clearly suggested linearity ($r=1$ for thiamine, $r=0.98$ for TMP and $r=0.94$ for TDP). According to both the *t*-test for paired data and Mann–Whitney *U*-test results, the distributions were not found to differ ($P>0.20$).

No significant differences were found between blood samples that had been collected both in EDTA and in heparin tubes to verify anticoagulant effect ($P>0.20$).

3.2. Samples

A difference was found in thiamine values between controls and alcoholics with the respective means being 89.6±22.7 nmol/l (min. 44–max. 141 nmol/l) and 69.4±35.9 nmol/l (min. 16–max. 170 nmol/l). The 28% T values in the alcoholics were below the lowest T value in controls. The difference was significant on both the Mann–Whitney *U*-test for medians ($P<0.001$) and the *t*-test ($P<0.001$).

No significant effects were observed for the TMP assay between the mean values in controls (4.5±6.6 nmol/l) and in alcoholics (4.8±5.2 nmol/l); neither the means nor the medians differed, indicating the poor utility of TMP in discriminating the two groups. In contrast, TDP results were particularly interesting since the mean values of the controls 222.2±56.3 nmol/l (min. 94–max. 347 nmol/l) varied substantially from those of the alcoholics 127.4±62.5 nmol/l (min. 54–max. 270 nmol/l); 36% of the alcoholics values were below the lowest control value.

Pronounced differences resulted ($P<0.00005$) on both the Mann–Whitney *U*-test and the *t*-test, thus demonstrating TDP as a discriminating marker between controls and alcoholics. (Table 2). Mean value of TDP in alcoholics resulted not close to median: this seems indicate a skewed distribution of TDP in alcoholics.

On the basis of the results obtained, the following cut-off limits are suggested: T=80 nmol/l (specificity 68%, sensitivity 64%) and TDP=180 nmol/l (specificity 80%, sensitivity 75%). These values

Table 1
Precision and accuracy of the HPLC procedure (TCA-extracted samples)

	Expected value (nmol)	Mean found value (nmol)	RSD (%)		Mean recovery (%) (n=10)
			Intra-day (n=5)	Inter-day (5 days) (n=10)	
Thiamine	5	5.2	4.9	8.9	104
	50	57	3.6	5	114
	100	96	3.0	6.4	96
	125	116	4.3	8	93
	250	241	4.0	8	96
	1000	988	2.7	3.6	99
TMP	5	4.8	2.7	3.9	97
	50	47	3.7	4	94
	100	104	4.9	7.9	104
	125	134	4.2	7	107
	250	280	4.7	7	112
TDP	5	5.1	2.1	5.6	101
	50	52	3.9	5	104
	100	98	4.0	8	98
	125	133	2.4	3	106
	250	249	3.5	8.7	100
	1000	1008	4.3	6.5	101

Table 2
Comparison between control and alcoholics values

	Control		Alcoholics		P
	Mean±SD (nmol)	Median	Mean±SD (nmol)	Median	
Tiamine	89.6±22.7	88	69.4±35.9	73	<0.001
TMP	4.5±6.6	3	4.8±5.2	3	>0.20
TDP	222.2±56.3	225	127.4±62.5	110	$<10^{-5}$

closely correspond to the 25th percentile of control values.

To evaluate the possibility of a gender confound, data from males and females in both groups were evaluated and compared. No significant differences resulted for T, TMP and TDP between males and females ($P>0.20$), within either the control or the alcoholic group (Table 3).

Table 3
Gender-based comparison of control and alcoholics data

	Control			Alcoholics		
	Males=45 (nmol)	Females=58 (nmol)	P	Males=29 (nmol)	Females=7 (nmol)	P
Age	45.8±10.2	40.2±9	0.004*	45.8±12.2	40.7±12.2	0.989
T	89.2±22.2	90.0±18.4	0.842	67.5±29.9	69.5±35.9	0.879
TMP	4.3±6.7	4.6±6.5	0.849	5.4±5.2	4.3±5.2	0.612
TDP	222.4±57.4	222.1±52.8	0.975	126.9±57.1	128.6±5.8	0.965

Significant T values were found between control males and alcoholic males ($P<0.0001$) as well as between control females and alcoholic females ($P<0.05$). (The lower level of significance in the females was probably due to the smaller group sizes). TMP results could not distinguish control and alcoholic males or control and alcoholic females ($P=0.47$ and $P=0.91$, respectively). TDP did, however, highly differ across genders ($P<0.00001$).

The possible impact of age was also investigated, since the control group males were significantly older than the females: 45.8 ± 10.2 vs. 40.2 ± 9 years ($P=0.004$). No statistical differences in correlations between T, TMP and TDP and age ($P>0.20$) were found.

No association was found between thiamine, TDP and years of problem drinking (thiamine $r=0.01$ and TDP $r=0.14$).

4. Discussion

4.1. Methodology

In spite of the relevance of thiamine levels for nerve functions and the extensive number of studies on thiamine deficiency [17–24] and evaluation of thiamine status [25–32], assessment of thiamine status in healthy subjects and in alcoholics has been little investigated in clinical activity. Although chromatographic separation of thiamine compounds is not novel and a variety of methods have been proposed [9], the reported values show large differences, e.g., ranging from 4 nmol/l [2] to 36 nmol/l [23] for blood T concentration in healthy people, while only minor differences were found for TMP (<2 nmol/l) and TDP (>200 nmol/l) [1,2]. The differences, probably related also to sampling choice (whole blood or washed erythrocytes) and preparation (simple technicalities like red cell washing procedure, derivatisation methods, etc.), are relevant and may lead to wide discrepancies among results, as discussed in some recent, authoritative studies [9,21,26].

In our study, the indirect assessment of thiamine by the evaluation of erythrocyte transketolase (ETK) activity was not performed, since most authorities have reported it as a less sensitive indicator of thiamine status in contrast to direct HPLC measurement in erythrocytes. In fact, ETK determination is affected by interpretation problems and limitations, particularly in alcoholics, probably related to the controversial existence of apoTK variants with reduced affinity for TDP, abnormal apoTK synthesis and enzyme inhibition by ethanol and acetaldehyde. Thus, the direct HPLC measurement was assessed to evaluate the thiamine status of controls and alcoholic patients.

For sample preparation, the choice of the anticoagulant was not critical since comparable results were obtained from the sample with different anticoagulants. The most important step was to obtain a cell sample that was fully reproducible in order to achieve comparable results. Previous studies have described poorly or not at all the number or the exact volume of cells used for determination and this makes result comparison and standardization of reference ranges nearly impossible. Thus, centrifugation procedure carefully performed is essential to obtain for all samples the same cell volume (1 ml of packed red blood cells) as the basis for determination. In previous papers, the procedure here proposed produced fully satisfactory results [15,16].

Precolumn derivatization by ferricyanide was chosen since, in our experience, it is more reliable and easier to perform than post column procedures. A number of oxidizing agents have been used for derivatization and potassium ferricyanide is clearly the most popular. Other investigators have used cyanogen bromide but it is dangerous, unstable and difficult to handle. The preparation of $K_3Fe(CN)_6$ solution is simpler, and the solution is stable for a long time (some months) at 4 °C. The concentration of $K_3Fe\ (CN)_6$ and NaOH had a substantial impact on analytic performance. To obtain the better fluorescence signal, several tests established that the optimum concentration for derivatization was 30.4 m*M* for potassium hexacyanoferrate and 0.8 *M* for NaOH. The addition of NaOH after $K_3Fe\ (CN)_6$ was demonstrated to give the most reliable results in respect to the use of a NaOH solution containing hexacyanoferrate. Preference for precolumn versus postcolumn thiochrome formation is a matter of debate: precolumn offers convenience, sharper peaks and better resolution, postcolumn requires additional equipment, but allows longer preservation of column

performance and lifetime. In our study, on the basis of cost/benefit considerations, the precolumn procedure was selected. Despite different internal standard methods having been previously suggested [9,12], in this study sample quantitation was assessed by external standards that were processed in the same way as the sample to control the entire procedure.

4.2. Mobile phase

The buffer molarity is a crucial point because of interaction with acetonitrile. Concentration adopted here (85 m*M*) was chosen since, a molarity higher than 85 m*M* might cause a salt precipitation when mixed with acetonitrile, with the risk of column damage; on the other hand, molarity lower than 82 m*M* could generate less reproducible and less defined chromatographic peaks. Some of these problems have been addressed by other authors [1] and were verified in our study. Moreover, in our study a relevant difference between phosphate potassium buffer and sodium phosphate buffer was found, leading to the choice of potassium buffer to avoid salt precipitation and to obtain good stability with acetonitrile. This mobile phase was proved the most suitable for stability of signal, analysis time, reproducibility of results and separation performance.

For chromatographic separation, an amino column was chosen since the use of C_{18} columns reported by some authors in our experience did not prove most satisfactory. The Ultra Amino column, recently introduced on the market and not used in previous studies, performed well even at the low concentrations employed in this study. Some authors reported the use of gradient [1] to separate thiamine, TMP and TDP. The aim of our study was to obtain a mobile phase suitable for the isocratic separation of the three analytes to avoid problems of column conditioning during the chromatographic run. The ratio of phosphate buffer to acetonitrile (65:35) was shown as best able to satisfy this aim and makes operating conditions easier.

4.3. Thiamine assessment in alcoholics

The methodology assessed by this study was used to evaluate thiamine status in controls and alcoholics by the direct measurement of thiamine, thiamine monophosphate and thiamine diphosphate. In erythrocytes, thiamine was present mainly as TDP both in controls and in alcoholics, in accordance with results obtained by other studies. Furthermore, resultant TDP values were not correlated with age or gender in either alcoholics or in controls (10, 26). Our data clearly indicate the potential of TDP to distinguish chronic alcoholics from controls, in combination with other established markers of alcohol abuse, e.g., MCV, CDT, γGT and mitochondrial AST. Statistical significance was also observed for free thiamine fraction. The discrepancy between our data and previous studies [23] may be related to better performance of the present method, like the choice of carefully washed red cells as substrate, and better selection of populations to study, avoiding possible confounding factors such as inclusion of some at-risk drinkers among controls. Moreover, the highly significant decrease of free thiamine in alcoholics is a further confirmation of the profound deficiency of the total thiamine pool in alcoholics. Even assessment of free thiamine alone could discriminate between alcoholics and non alcoholics. Reduced concentrations of thiamine and thiamine diphosphate were found in the chronic alcohol-dependent patients, not related to the level of liver impairment, without clinical evidence of thiamine deficiency.

The HPLC procedure presented in this study will be employed in further clinical studies, to establish reference values for erythrocyte free thiamine and TDP in a larger series of subjects, and their suitability as markers of alcohol abuse. Moreover, considering our findings, thiamine treatment should likely be recommended for all alcoholics.

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