Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Journal of Pharmaceutical and Biomedical Analysis 37 (2005) 1025–1029
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Determination of thiamine and its phosphorylated forms in human plasma, erythrocytes and urine by HPLC and fluorescence detection: a preliminary study on cancer patients

R. Losa[a,*], M.I. Sierra[a], A. Fernández[a], D. Blanco[b], J.M. Buesa[a]

[a] *Laboratory of Medical Oncology, Instituto Universitario de Oncología del Principado de Asturias (IUOPA), Hospital Central de Asturias, C/ Julian Clavería s/n, 33006 Oviedo, Asturias, Spain*
[b] *Department of Physical and Analytical Chemistry, Chemistry Faculty, University of Oviedo, 33006 Oviedo, Spain*

Received 16 May 2004; received in revised form 23 August 2004; accepted 23 August 2004
Available online 20 October 2004

Abstract

In man, neurotoxicity associated to ifosfamide treatment can be reversed by intravenous thiamine administration. Trying to explain this clinical finding, we decided to study possible changes in thiamine availability and activation in patients exposed to ifosfamide. Free thiamine and its phosphate esters levels were measured in plasma, erythrocytes and urine by an ion-pair HPLC method with pre-column derivatization, which allowed separation of the fluorescent compounds in less than 10 min. The method was validated by linearity, sensitivity and reproducibility studies, whose values met the demands for bioanalytical assays. This method was applied to assess thiamine status in cancer patients exposed to ifosfamide therapy for advanced disease.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Ion-pair HPLC; Fluorescence detection; Thiamine phosphate esters; Urine; Erythrocytes; Plasma; Thiamine

1. Introduction

Ifosfamide (IFOS) is an alkylating agent used in the treatment of solid tumors that, among other untoward side effects, may cause central nervous toxicity. The mechanism of this encephalopathy is not well known, but it could be mediated by chloracetaldehyde, an ifosfamide metabolite considered neurotoxic [1]. However, as data from our institution indicated that this toxicity could be reversed by thiamine administration, we hypothesized that either ifosfamide or its metabolites might interfere with thiamine functions. For this reason we determined total blood thiamine content in patients before and after ifosfamide administration, but the data obtained were not conclusive [2].

Thiamine exerts its biological function upon phosphorylation. Its main active form is thiamine diphosphate (TDP), what makes up 80% of total thiamine present in humans, the rest consisting of thiamine triphosphate (TTP) (10%), thiamine monophosphate (TMP) or free thiamine (T). Erythrocytes transport small amounts of T, TMP and 80% of the TDP present in blood, while plasma contents mainly T and TMP. In urine T can be detected, as well as minor molecules derived from thiamine degradation. TDP acts as a coenzyme for oxidative decarboxylation of α-keto acids, and in the transketolase catalyzed reaction, and reduced TDP availability disturbs carbohydrate metabolism, leading to alterations in cerebral energy metabolism [3]. To further check our hypothesis, and since determination of total blood thiamine levels does not discard changes in the pattern of thiamine activation, it was necessary to set up an analytical method to measure both thiamine and its phosphorylated derivatives in plasma, red blood cells and urine.

HPLC methods for determining the concentration of thiamine and its esters in blood have been reviewed [4]. Due to

* Corresponding author. Tel.: +34 985 10 61 00x36400.
E-mail address: raquel.losa@hca.es (R. Losa).

0731-7085/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2004.08.038

the low levels of these analytes, either pre-column [5–9] or post-column derivatization [10–12] is needed to generate fluorescent compounds (thiochromes) that will provide the necessary sensitivity and specifity. The present paper presents an analytical method based on ion-paired HPLC and pre-column derivatization with potassium ferricyanide that was applied to assess thiamine status in oncologic patients exposed to ifosfamide.

2. Experimental

2.1. Chemicals and reagents

Pure thiamine (T), thiamine monophosphate, thiamine diphosphate (TDP, co-carboxylase) and derivatizing reagent (potassium ferricyanide) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Thiamine triphosphate was obtained from Wako Pure Chemical Industries Ltd. (Chuo-Ku, Osaka 541, Japan). The ionic-pair reagent tetrabutylammonium hydroxide (TBAH), inorganic reagents (analytical grade), and methanol (HPLC grade) were obtained from Merck (Hohenbrunn, Germany). Purified water (Milli-Q system, Millipore, Milford, MA, USA) was used. Human red blood cells, plasma and urine, needed to set-up the experimental conditions, were obtained from healthy volunteers.

2.2. Chromatographic equipment

Chromatographic analyses were performed on the following configuration: a system controller model SCL-10A^{VP}, two pumps LC-10AD^{VP}, an autoinjector Sil-10AD^{VP} (all from Shimadzu, Kyoto, Japan) and a fluorescence detector model F-1050 (Hitachi Ltd., Tokyo, Japan). Data were recorded on a personal computer equipped with a Class VP Chromatographic Data System version 5.1 (Shimadzu, Kyoto, Japan).

2.3. Chromatographic conditions

The chromatographic separation was accomplished on a Microsphere C_{18} analytical column (100 mm × 4.6 mm, 3 μm particle size) with a ChromSep SS guard column (10 mm × 2 mm) (Varian, Middelburg, Netherlands). The separation of the target compounds was performed in gradient mode. Two solutions were used: (A) KH_2PO_4 (0.2 M) plus TBAH (0.3 mM) (pH 7):methanol (88.5:11.5), and (B) methanol:H_2O (70:30). The gradient run conditions were programmed as follows: 10–20% B in 2 min, an isocratic step with 20% B for 8 min and return to initial conditions over 3 min. Prior to use, all eluents were filtered through a membrane filter (pore size: 0.22 μm) and degassed. Elution was performed at room temperature, the injected volume was 50 μL, the flow-rate was 1 mL/min and the compounds were detected at $\lambda_{excitation} = 365$ nm and $\lambda_{emission} = 435$ nm.

2.4. Sample preparation

Blood samples (5 mL) obtained from a peripheral vein were collected in heparin-containing tubes, protected from light and kept at 4 °C. Plasma was obtained after blood centrifugation at 1800 × g for 5 min at 4 °C. After careful removal of the upper layer of the pellet containing mainly white blood cells, packed erythrocytes were washed three times with phosphate buffered saline (PBS), resuspended and counted in a Coulter. Plasma and erythrocytes were stored at −70 °C until analysis. Sample storage time never exceeded 2 months [13,14].

Before derivatization, 7.2% of perchloric acid was added to the samples (1:1, v/v). The mixture was vortexed, placed in an ice bath for 10 min, and the tubes were again vigorously mixed, and placed on ice for a further 5 min to allow protein and nucleic acids precipitation. After centrifugation at 2000 × g for 15 min at 4 °C, the supernatant was collected. All the procedure was performed in light-protected tubes.

The 24 h urine samples were collected in light-protected urine bottles containing 20 mL acetic acid and an aliquot was stored at −25 °C until analysis. Prior to derivatization urine samples were diluted 1:5 (v:v) with Milli-Q water.

2.5. Derivatization procedure

Stock standard solutions of thiamine, TMP, TDP and TTP were prepared at 1500 μg mL^{-1} in HCl 0.1 M and protected from light exposure. Stock solutions at −20 °C were stable for at least 2 months. Working standard solutions were prepared each day by dilution of the corresponding stock solution.

A freshly prepared aqueous solution of 12.14 mM ferricyanide and 3.35 M NaOH was used as derivatizing agent.

Prior to injection in the chromatographic system, the standards were treated with 7.2% perchloric acid in 0.25 M NaOH (1:1) and then derivatized.

For urine sample derivatization, 1 mL of diluted sample was mixed with 100 μL methanol and 210 μL of derivatizing solution. The mixture was vortexed for 30 s and then 245 μL of phosphoric acid 1.43 M was added in order to reach a final pH of 6.9 ± 0.2.

Blood sample derivatization was performed as previously described [15]. Briefly, 1 mL sample (erythrocytes, plasma or standard) was treated with 100 μL methanol; while mixing, 200 μL of derivatizing reagent was added, mixed for 30 s and, finally, 100 μL phosphoric acid (1.43 M) was added. Final pH of samples and standards was 6.9 ± 0.2. This reaction yielded the corresponding thiochrome derivatives: Thc, ThcMP, ThcDP and ThcTP (Fig. 1). The derivatized samples were thereby ready for HPLC fluorimetric determination.

3. Results and discussion

Our approach to set-up a chromatographic method for T, TMP, TDP and TTP determination was based on a previ-

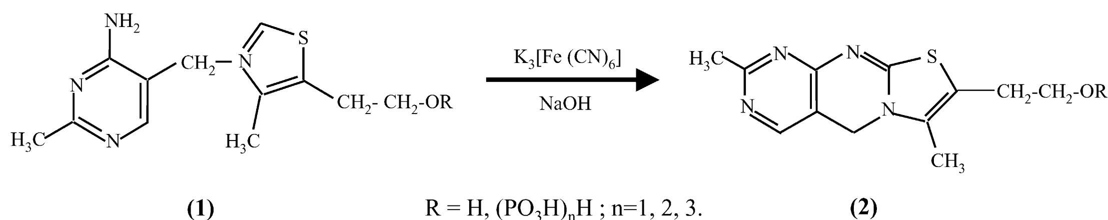

Fig. 1. Scheme of the derivatization reaction. Thiamine and thiamine phosphate esters are oxidized with ferricyanide under alkaline conditions leading to thiochrome formation. (1) Thiamine (R = H) or thiamine phosphate esters ($R = (PO_3H)_nH$), and (2) thiochrome derivatives.

ously reported method [13], which allowed an early elution (2.8 min) of derivatized TTP (ThcTP), and therefore, high sensitivity. This is an important issue to take into account due to the low TTP levels in blood samples [11,12]. To simplify the chromatographic conditions, the composition of the mobile phase reported [15] was changed, by removing the dimethylformamide (DMF) content. Elimination of DME led to longer retention times, but increasing the percentage of solvent B in the initial gradient conditions solved this problem. To further optimize our method, the influence of some experimental variables in the retention times was studied. A minor decrease in methanol to 11% in solution A led to a better resolution between TTP and TDP peaks. Since only minor changes in the capacity factor were observed, this methanol proportion was chosen as the working concentration. Furthermore, the effect of ionic strength and ion-pair reagent were studied in the range from 0.1 to 0.3 M, and from 0.1 to 0.5 mM, respectively. In both cases, only small changes in the capacity factor were found. In view of these results, a concentration of 0.3 mM for ion-pair and 0.2 M phosphate buffer were chosen. The effect of pH was also evaluated over the range from 6 to 7.5, and when its value decreased from 7.5 to 7, a better resolution between TTP and TDP was achieved. Although some fluorescence intensity of the analytes was lost at pH 7, this pH value was selected since it prolonged column life. Under these conditions thiamine thiochrome (Thc) and its phosphate esters eluted for 10 min, the retention times for ThcTP, ThcDP, ThcMP and Thc being 2.6, 3.1, 4.0 and 7.3 min, respectively. Fig. 2 shows chromatograms corresponding to standards, erythrocytes, plasma and urine samples from patients analyzed under the described conditions.

Selection of the derivatizing reagent was based on the easiness to handle, since alkaline ferricyanide offers an adequate sensitivity and it is safer than cyanogen bromide [11] or mercuric chloride, although the fluorescence intensity obtained with the latter doubles that obtained with ferricyanide [16].

Linearity, sensitivity and precision of the method were studied. For this purpose, calibration curves of different concentration of derivatized T, TMP, TDP and TTP versus peak areas were plotted. Data obtained from triplicated determinations of five different concentrations ranging from 1.4 to 20 ng mL^{-1} of TTP, TMP and T (1.4, 5.6, 10, 15 and 20 ng mL^{-1}) and from 17 to 170 ng mL^{-1} of TDP (17, 50, 90, 130, and 170 ng mL^{-1}), were fitted by linear least-square analysis. Good correlation between the peak area and concentration was demonstrated in every case, with r-values ranging from 0.996 to 0.999. The linearity of the calibration curves was checked using two different statistical tests (linearity and proportionality tests). Regression variance analysis was used for the linearity test, obtaining an f-value lower than the tab-

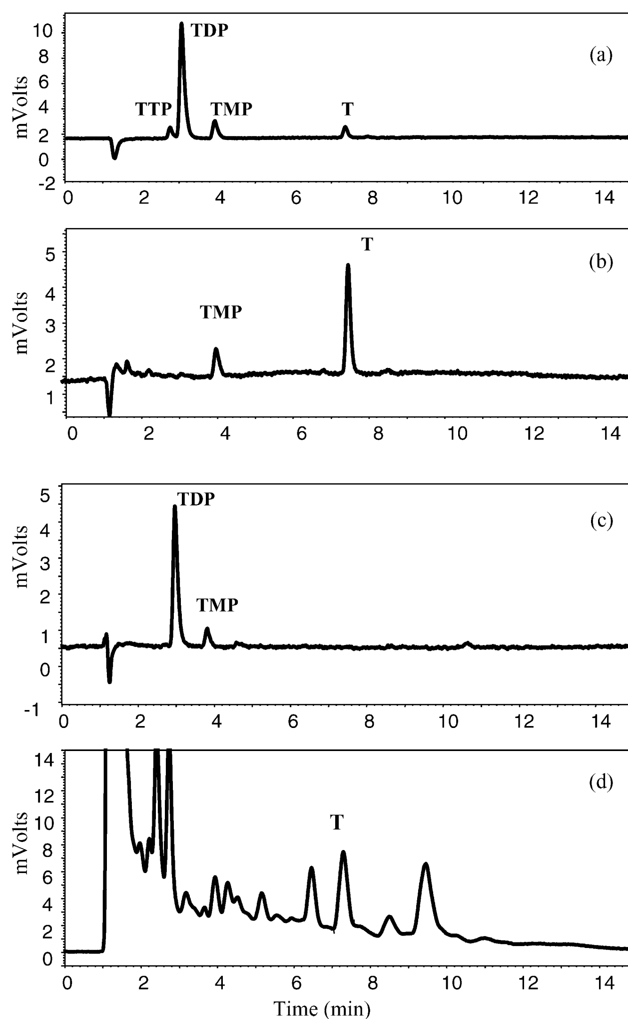

Fig. 2. Chromatograms corresponding to: (a) mixture of standards (50 μg mL^{-1} TDP, 10 μg mL^{-1} TMP and 5 μg mL^{-1} TTP and T), (b) plasma, where only T and TMP are detected, (c) erythrocytes containing TDP and TMP, and (d) urine samples where only T is present. Chromatographic conditions as reported in Section 2.3. Thiamine (T), thiamine monophosphate (TMP), thiamine diphosphate (TDP), thiamine triphosphate (TTP).

Table 1
Sensitivity of the chromatographic method for each analyte

Analyte	Detection limit (ng mL^{-1})	Quantification limit (ng mL^{-1})
TP	0.2	0.6
TDP	0.3	1.0
TMP	0.4	1.2
T	0.2	0.7

ulated at the 0.05 significance level for all the compounds studied. In the proportionality test, the Student *t*-test values calculated for the intercept were always lower than tabulated values for $\alpha = 0.05$, and the Student *t*-test values obtained for the slope were always higher than those tabulated for the same level of significance. Linearity was thus demonstrated.

The method sensitivity was also studied. For this purpose, the detection and quantitation limits were calculated for each analyte. The detection limit, defined as the lowest concentration of the analyte that can be clearly detected above the baseline signal, was estimated as three times the signal to noise ratio [17], and ten times this ratio was the quantitation limit. Table 1 shows the detection and quantification limits obtained for all compounds.

Precision of this method was investigated using human erythrocytes. The instrumental repeatability, calculated as the relative standard deviation (R.S.D.) of the areas, obtained from 10 injections was ≤4.8%. The method repeatability was evaluated by analyses of 10 erythrocyte samples from the same donor, spiked with 5 ng mL^{-1} T, TMP and TTP, and 20 ng mL^{-1} TDP, since erythrocytes always have measurable levels of TDP. All these analyses were performed under identical conditions in a single day. The R.S.D. of the areas obtained from these samples was lower than 6% in all cases. The inter-day reproducibility of the method was evaluated by sample injections in two consecutive days obtaining a R.S.D. < 8% for all the analytes. The day-to-day variation of the elution time and the peak areas of the individual standards for multiples analyses were also calculated, being both highly reproducible in this system (R.S.D. < 2.6 and 12.4%, respectively). Precision of the method for plasma and urine samples were also studied and results are shown in Table 2. All values of precision met the demands for a bionalytical assay: R.S.D. ≤ 20% for amounts close to the quantification limit and R.S.D. ≤ 15% for higher levels [18,19].

Accuracy of the method in the three different matrices (erythrocytes, plasma and urine) was assessed. Blood samples were spiked with three different concentration levels of each analyte, and three replicas of each level were injected in the chromatographic system. The determination of the recovery percentage for thiamine and its phosphate esters was calculated by comparing the absolute response of the treated samples with the absolute response of external standards. The calculated amount of thiochrome derivatives varied between 85 and 112% of the expected values in plasma and erythrocytes (Tables 3 and 4).

Thiamine recovery was studied in urine samples, since this is the only analyte detected in this fluid. Direct thiamine determination from urine was unreliable, with mean recoveries close to 60%, and sample dilution (1:5) was necessary to obtain recovery values of around 90% (range 87.5–94%). Thiamine levels assayed and the results obtained from recovery studies are shown in Table 5. The low detection limit of the method allowed quantification of vitamin levels in diluted urine, even in those patients with the lowest normal values (66–346 ng mL^{-1}) [20].

By applying this chromatographic method, the levels of thiamine and its phosphate esters in plasma and erythrocytes

Table 2
Precision data obtained from plasma and urine (R.S.D. (%))

	Plasma	Urine
Instrument repeatability ($n = 10$)	4.65	4.26
Method repeatability ($n = 10$)	5.50	5.90
Inter-day repeatability (2 days)	7.92	8.70

Table 3
Recovery from erythrocytes spiked with TTP, TDP, TMP and T (%)

Analyte	Sample (ng mL^{-1})	Added (ng mL^{-1})	Recovery ± R.S.D.
TTP	1.0	2	90.8 ± 5.4
		3	89.4 ± 4.5
		4	105.5 ± 14.6
TDP	69.9	40	98.5 ± 7.8
		60	111.1 ± 4.7
		80	102.5 ± 5.2
TMP	6.9	2	89.5 ± 5.4
		3	85.1 ± 4.5
		4	89.0 ± 14.6
T	–	2	111.5 ± 7.6
		3	107.3 ± 5.0
		4	102.0 ± 7.2

Table 4
Recovery from plasma spiked with TTP, TDP, TMP, and T (%)

Analyte	Sample (ng mL^{-1})	Added (ng mL^{-1})	Recovery ± R.S.D.
TTP	–	2	95.0 ± 4.0
		8	91.2 ± 5.1
		16	105.5 ± 1.1
TDP	–	2	102.4 ± 4.4
		8	110.8 ± 2.0
		16	95.5 ± 5.7
TMP	6.1	2	111.2 ± 7.3
		8	110.9 ± 1.9
		16	111.7 ± 2.1
T	9.0	2	85.3 ± 3.4
		8	90.2 ± 3.5
		16	97.8 ± 4.8

Table 5
Recovery from urine samples spiked with thiamine (%)

Analyte	Sample (ng mL^{-1})	Added (ng mL^{-1})	Recovery ± R.S.D.
T	45.5	16.3	89.1 ± 3.3
		32.7	94.2 ± 5.1
		65.5	87.5 ± 1.1

Table 6
Levels of thiamine and its phosphate esters in erythrocytes and plasma from oncologic patients

Sample	Thiamine (ng mL^{-1})	TMP (ng mL^{-1})	TDP (ng mL^{-1})	TTP (ng mL^{-1})
Erythrocytes	–	3.6 ± 1.5	138.03 ± 25.1	1.6 ± 0.32
Plasma	4.2 ± 2.2	3.9 ± 1.7	–	–

Mean ± S.D. in seven patients, except for TTP (two patients).

from seven patients treated with ifosfamide were determined (Table 6). As expected, TDP content was highest in erythrocytes, where low levels of TMP and no thiamine were detected, while TTP trace levels were exclusively found in two patients. Similarly, T and TMP were detected in plasma, and only when inadvertent hemolysis had occurred, was TDP found. Thiamine levels from urine samples ranged from 110 to 190 μg mL^{-1}, figures within the normal range reported by others [20]. So far, the limited number of patients studied does not allow to advance conclusions on the clinical aspects of this study.

4. Conclusion

This paper describes a chromatographic method for the determination of thiamine and thiamine phosphate esters in human biological fluids (plasma, erythrocytes and urine) with minor sample treatment. The validation studies show good recoveries and precision. In addition, the detection limit for all compounds ranged at the low ng mL^{-1} level.

Acknowledgements

The authors are indebted to the program FEDER-FSE of the European Community, Grant FIS 01/0329 from Instituto de Salud Carlos III (Ministerio de Sanidad y Consumo, Madrid, España), and the Instituto Universitario de Oncología del Principado de Asturias (Obra Social CajAstur, Asturias, Spain) for financial support.This study was performed within the frame of Program 9 of the Redes Temáticas de Investigación Cooperativa de Centros de Cancer (C03/10), Instituto de la Salud Carlos III, Spain.We appreciate the collaboration of the nurses from the Medical Oncology Department.

References

[1] T. Kerbusch, J. De Kraker, H.J. Keizer, J.W. van Putten, H.J. Groen, R.L. Jansen, J.H. Schellens, J.H. Beijnen, Clin. Pharmacokinet. 40 (2001) 41–62.
[2] J.M. Buesa, P. García-Teijido, R. Losa, J. Fra, Clin. Cancer Res. 9 (2003) 4636–4637.
[3] V. Tanphaichitr, in: M.E. Shils, J.A. Olson, M. Shike (Eds.), Modern Nutrition in Health and Disease, Lea and Febiger, Philadelphia, 1994, pp. 359–365.
[4] P.L.M. Lynch, I.S. Young, J. Chromatogr. A 881 (2000) 267–284.
[5] L. Bettendorff, M. Peeters, C. Jouan, P. Wins, E. Schoffeniels, Anal. Biochem. 198 (1991) 52–59.
[6] P. Viñas, C. Lopez-Erroz, N. Balsalobre, M. Hernández-Córdoba, J. Chromatogr. B 757 (2001) 301–308.
[7] F.J. Jiménez-Jiménez, J.A. Molina, A. Hernánz, E. Fernández-Vivancos, F. de Bustos, B. Barcenilla, C. Gómez-Escalonilla, M. Zurdo, A. Berbel, C. Villanueva, Neurosci. Lett. 271 (1999) 33–36.
[8] L. Bettendorff, C. Grandfils, C. de Rycker, E. Schoffeniels, J. Chromatogr. 382 (1986) 297–302.
[9] R. Mancianelli, M. Ceccanti, M.S. Guiducci, G.F. Sasso, G. Sebastiani, M.L. Attilia, J.P. Allen, J. Chromatogr. B. 789 (2003) 355–363.
[10] M. Kimura, Y. Itokawa, Clin. Chem. 29 (1983) 2073–2075.
[11] C. Herve, P. Beyne, E. Delacoux, J. Chromatogr. B 653 (1994) 217–220.
[12] C.M.E. Tallaksen, T. Bohmer, H. Bell, J. Chromatogr. 564 (1991) 127–136.
[13] J. Gerrits, H. Eidhof, J.W.I. Brunnekreeft, J. Hessels, Methods Enzymol. 279 (1997) 74–82.
[14] D. Talwar, H. Davidson, J. Cooney, D. JO'Reilly, Clin. Chem. 46 (2000) 704–710.
[15] J.W.I. Brunnekreeft, H. Eidhof, J. Gerrits, J. Chromatogr. Biomed. Appl. 491 (1989) 89–96.
[16] A. Laschi-Loquerie, S. Vallas, J. Viollet, Int. J. Vitam. Nutr. Res. 62 (1992) 248–251.
[17] J.D. Winefordner, G.L. Long, Anal. Chem. A 55 (1983) 712A–724A.
[18] H. Rosing, W.Y. Man, E. Doyle, J. Liq. Chromatogr. Related Technol. 23 (2000) 329–332.
[19] V.P. Shah, K. Midha, S. Dighe, Eur. J. Drug. Metab. Pharmacokinet. 16 (1991) 249–255.
[20] B. Botticher, D. Botticher, Int. J. Vitam. Nutr. Res. 56 (1986) 155–159.