Validation of a HPLC Method for Quantification of Thiamine and Its Phosphate Esters in Rat Brain Tissue

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Abstract

The present data show a fast and efficient biological sample processing method for the extraction of thiamine (vitamin B1) and its mono-(TMP) and di-(TDP) phosphate esters from hippocampus, thalamus and prefrontal cortex (PFC) and blood sample of the rodents. In addition, using the hippocampus and standards of these three compounds we validated an isocratic fluorescence HPLC procedure for a simultaneous detection of them in a single chromatogram within a total run time of about 12 min. Reproducibility for TDP, TMP and B1 was 2.66%, 4.50% and 7.43% (intraday) and 37.54%, 25.39% and 25.87% (interday), respectively. Recovery assays were between 96.0% and 101.7%. The calibration curves were linear and the concentrations of the three compounds, all in nanomolar range, were determined in the brain areas and in the blood samples. When compared to the current methods in the literature, this new method provides information on essential variables, such as linearity range and limit of detection, reproducibility and stability of thiamine, TMP and TDP in rat brain samples. The present data on sample processing and B1 and its phosphate ester level determinations are the first to be validated using hippocampus samples of rats.

Keywords

1. Introduction

Vitamin B1 consists of free thiamine, a pyrimidyl substituted thiazole, and its phosphate esters thiamine monophosphate (TMP), thymidine diphosphate

(TDP) and thiamine triphosphate [1]. TMP is considered as a substrate to generate the active form (TDP or TPP) by the action of thiamine pyrophosphokinase and to produce B1 by thiamine phosphatase [2] [3] [4] [5]. Decreased levels of B1 and its phosphorylated forms can be induced by inadequate intake and/or absorption of thiamine, associated or not with alcohol abuse [6] [7] [8].

In humans, thiamine deficiency (TD) causes brain dysfunctions known as Wernicke's Encephalopathy and Wernicke-Korsakoff Syndrome (WKS), respectively considered by some authors as the acute and chronic stages of the same disorder. Symptoms observed in WKS include oculomotor disturbance, ataxia and confusion (acute symptoms), and amnesia and confabulation (chronic symptoms) [9] [10]. Rodent models of TD have been developed to examine the pathogenesis of these disorders [11]. A plenty of studies have been carried out using these models to understand the neurobiological mechanisms related to the behavioral deficits usually associated with TD. However, until now several points remain obscure.

Our group [12]-[18] and other authors [19]-[28] demonstrated that TD causes several changes in intermediary metabolism including neurotransmitter turnover and focal brain acidosis. The pathophysiology underlying TD is multifactorial in nature, involving a broad cascade of events that ultimately result in focal neuronal cell death similar to the pathological mechanisms inherent to neurodegenerative diseases [29] [30] [31] [32].

TD in rodents is a relevant model to study cellular and molecular mechanisms that lead to a gradual progression of selective neuronal loss that resembles clinical neurodegenerative diseases [33] [34]. The measurement of thiamine and its phosphorylated forms in rodent brain samples is important for studying neurodegenerative mechanisms, and also it may provide an additional tool for several kinds of studies, such as those concerned with drug design, elucidation of central nervous system function and dysfunction associated with different sorts of brain insults involving changes in the level of thiamine and its phosphate ester.

Therefore, the quantification of thiamine and its phosphate esters in rodent brain areas has been of great interest and there are continual attempts to accurately measure their concentrations under physiological and pathological conditions. A multitude of methods for the determination of thiamine levels through microbiological and biochemical techniques such as chromatography (e.g. high-performance liquid chromatography—HPLC), spectrophotometry and fluorescence by ultraviolet exposure were described [35] [36] [37]. Transketolase enzyme activity measurement is an indirect assay also used to quantify thiamine concentration in biological samples [38] [39] [40]. For quantifying thiamine and its phosphate esters, several authors have been using potassium ferricyanide sample treatment in alkaline conditions followed by separation on C18 columns and detection by fluorimeter [1] [41] [42] [43] [44]. However, none of these methods was validated for quantifying thiamine and its phosphate esters in rat brain samples. In the present study we used a similar method described by these

authors adding some modification in mobile phase and sample processing. Furthermore, we added important information to the previous data, carrying out the validation of these quantitative procedures and showing the reference values of B1 and its phosphate esters in samples from blood and from three brain areas of male Wistar rats.

2. Materials and Methods

2.1. Chemicals and Reagents

HPLC grade methanol was obtained from J. T. Baker (Phillipsburg, NJ, USA). Trichloroacetic acid (TCA), potassium ferricyanide ($K_3Fe(CN)_6$), thiamine hydrochloride (vitamin B1), thiamine pyrophosphate (TDP), thiamine monophosphate chloride (TMP) and hydrochloric acid were purchased by Sigma-Aldrich (St. Louis, MO, USA). Diethyl ether was obtained from Cromoline (Diadema, SP, Brazil), disodium hydrogen phosphate ($Na_2HPO_4 \cdot 7H_2O$), sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$), sodium hydroxide and triethylamine (TEA) were obtained from Reagen (Colombo, PN, Brazil). Ethylenediaminetetraacetic acid (EDTA) was purchased from Vetec (Duque de Caxias, RJ, Brazil). All reagents were of the highest purity available. For all solutions in which water had to be added, Milli-Q deionized water was used.

2.2. HPLC System and Analysis Conditions

The HPLC system was a Shimadzu chromatograph (LC-10AD, Tokyo, Japan) with a fluorescence detector (FLD-Shimadzu spectrofluorometric detector RF-551, Tokyo, Japan), a 200 μL loop (Rheodyne 7725-I, CA, USA) and a LC-10 AD PUMP. This system was equipped with a 5 μm particle size analytical column (Purospher Star RP-18 end capped −250 mm × 4.6 mm, ID-Merck, Darmstadt E.R., Germany) and a pre-packed column (Purospher Star RP-18 −4 × 4 mm-Merck, Darmstadt E.R., Germany). An integrator (Shimadzu C-R7Ae plus) was used to analyze the chromatographic data.

The mobile phase consisted of an equimolar buffer solution of 0.14 M $NaH_2PO_4 \cdot H_2O$ and $Na_2HPO_4 \cdot 7H_2O$ and methanol (70:30, v/v), pH 7.0, plus 0.1% TEA. Before the chromatographic analyses, the phase was filtered through 0.45 μm membrane filters (Millipore Durapore) and vacuum degassed prior to use. Chromatographic analyses were performed at 25°C ± 2°C and the compounds were eluted isocratically over 15 min runtime at a flowrate of 1 mL/min. The fluorescence detector was set at an excitation wavelength of 367 nm and an emission wavelength of 435 nm, high sensitivity and range of 1.5. Both peak height and area are proportional to concentration.

2.3. Calibrators

One milligram per milliliter stock solutions of TDP, TMP and B1 were prepared in 0.1 M hydrochloric acid and aliquoted out for keeping at −20°C. Standard solutions (100, 325, 550, 775, 1000 ng/mL for TDP; 50, 100, 175, 250 ng/mL for TMP and 2, 10, 50, 80, 100, 150 ng/mL for B1) were prepared daily by dilutions

of the stock solutions, aliquoted out and stored at 4˚C until derivatization and analysis. 150 μL of standards were derivatized by addition of 150 μL of freshly prepared potassium ferricyanide in 15% (w/v) sodium hydroxide. A blank sample was used containing 150 μL of water and 150 μL of potassium ferricyanide solution.

2.4. Animals

Sixteen male Wistar rats weighing 250 - 300 g were collected from the Centro de Bioterismo da Universidade Federal de Minas Gerais (CEBIO-ICB-UFMG vivarium). The rats were housed in plastic cages in groups of four, maintained on a 12:12 h light-dark cycle and fed *ad libitum*. Animals were killed by decapitation in random order, blood samples were collected, their brains were removed and the tissues were separated and processed as described in Section 2.5. The present study was approved by the Ethics Committee for Care and Use of Laboratory Animals of the Universidade Federal de Minas Gerais (CEUA-UFMG protocol number: 107/2010) and the care and use of animals were done according to the National Institutes of Health Guide for Care and Use of Laboratory Animals [45].

2.5. Sample Processing and Extraction of Thiamine and Its Esters

As mentioned above, the brains were rapidly removed from the cranium and prefrontal cortex, thalamus and hippocampus were dissected out on an ice-cold plate. The tissues were weighed and placed in 1.5 mL microcentrifuge tubes, and stored at −80˚C until analysis. Samples of 500 μL of blood were collected in tubes containing EDTA 6% (w/v) and stored at −20˚C until the day of the assay. All assays were done within one week. On the day of the assay the samples were thawed, homogenized and the thiamine and its phosphate esters extracted. The assays were then carried out in the supernatant extract within one minute after derivatization. All these methods are described below.

The brain tissue and blood samples were processed as described in [1] with few modifications in the quantities of some reagents as detailed below. The tissues were homogenized in 15 vol. of TCA 10% (85:15, v/v) and blood samples were homogenized in TCA 10% (1:1 v/v) for protein precipitation. Samples were vortexed vigorously for 1 minute, left standing on ice for 15 min and vortexed vigorously for 1 minute more. Immediately after, the samples were centrifuged (10,000 ×g for 15 min at 4˚C) and aliquoted until derivatization for thiamine and its phosphate esters analysis. Each supernatant was filtered (Millipore 0.45 μm, 13 mm) and transferred into a falcon tube and washed twice with 500 μL of diethyl ether saturated with water (5:1 v/w) to remove TCA. Aliquots of the solution (100 μL) were transferred to a corresponding tube and methanol (31 μL) was added. The samples were left standing on ice until derivatization. Briefly, 150 μL of supernatant mixture plus methanol were derivatized by adding 150 μL of freshly prepared potassium ferricyanide in 15% (w/v) sodium hydroxide. After the samples were vortexed and analyzed after 1min at room temperature. The

derivatization of the thiamine and its phosphate esters in under alkaline conditions with potassium ferricyanide allows the formation of fluorescent thiochromes derivative [1].

2.6. Assay Validation

The assay validations were carried out with hippocampus samples of male Wistar rats and standards of thiamine and its phosphate esters.

Linearity and detection limit: The linearity of the detector response to standard solutions of TDP, TMP and B1 were determined. Calibration curves obtained from standard solutions of 100, 325, 550, 775 and 1000 ng/mL of TDP; 10, 50, 100, 175 and 250 ng/mL of TMP, and 10, 50, 80, 100 and 150 ng/mL of B1 were done.

Recovery: For evaluating the recovery of TDP, TMP and B1, a mixture of these compounds was added to samples of hippocampus at the moment of homogenization, at final concentrations of 100, 250, 400, 550 and 700 ng/mL of TDP; 25, 75, 125, 175 and 250 ng/mL of TMP and 20, 40, 60, 80 and 100 ng/mL of B1. Recovery was calculated as: [(final concentration-initial concentration)/ added concentration].

Stability: Experiments were done to determine the stability of thiamine and its phosphate esters in two kinds of samples/conditions: 1) hippocampus homogenate and 2) supernatant extract after derivatization process. In the first case, homogenized hippocampus samples were stored at −80°C and assayed 7, 15 and 30 days after homogenization. In the latter, the stability of the thiochrome derivatives in the supernatant extract was assessed 1, 30, 60, 120 and 240 minutes after potassium ferricyanide oxidation. For the two conditions, the data obtained were expressed as nmol/g of tissue for each compound separately or by adding the three obtained values in a single representative total value (TDP + TMP + P = Total, nmol/g of tissue).

Experimental variation: Intraday reproducibility was calculated from 10 consecutive injections of hippocampus samples and interday reproducibility was obtained by comparing the means of 10 replicates over two consecutive days.

3. Results and Discussion

3.1. Assay Validation

Hippocampus samples from male Wistar rat brains were used in the assays to validate the method. Mean elution times for TDP, TMP and B1 were 3.82, 4.28 and 11.67 min, respectively. Thus, in a total run time of less than 12 min the thiamine and its phosphorylated forms were eluted from the HPLC system. **Figure 1(a)** shows the chromatographic profile of standard solutions of TDP, TMP and B1, and **Figure 1(b)** shows the chromatographic profile for these parameters extracted from hippocampus sample. There was no peak in the chromatographic profile obtained with blank samples, indicating that the potassium ferricyanide oxidation does not interfere with the assay.
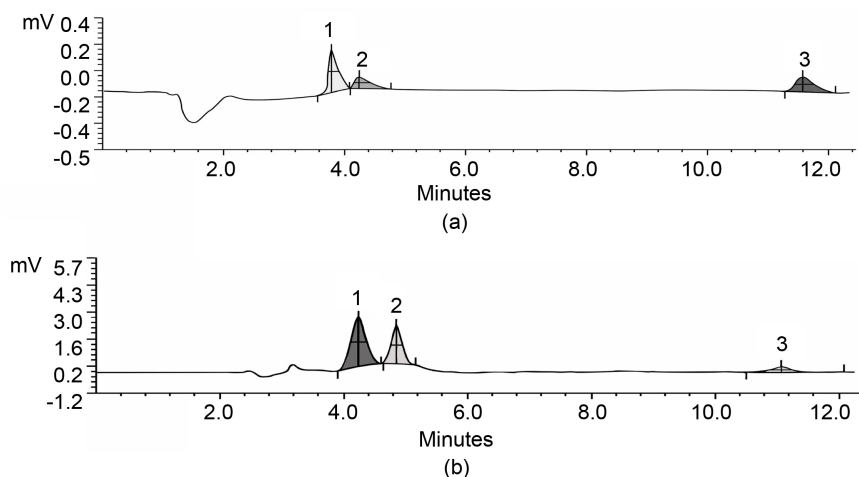
Figure 1. Representative chromatograms obtained using 100 ng/mL of standard solutions (a) and hippocampus supernatant extract (b). The peaks identified with the numbers 1, 2 and 3 are TDP, TMP and B1, respectively. Peak's height represents the fluorescence detector response, in millivolts (mV).

The linearity of the detector response to standard solutions of TDP, TMP and B1 were determined by calibration curves and the obtained data are shown in **Figures 2(a)-(c)**, respectively. The result of each concentration is the average of triplicate assays. Linear correlation between the peak areas and concentrations was demonstrated for the three parameters, with r-values ranging from 0.993 to 0.996. Both peak height and area are proportional to concentration.

The accuracy of the method was estimated by recovery assays. **Table 1** shows the recovery percentage for TDP, 101.7% ± 0.8%, TMP, 96.04% ± 2.7%, and B1, 97.3% ± 1.43% from hippocampus samples. The obtained accuracy indicates that the methods for sample processing and chromatographic analysis shown in the present study can be used for quantitative analyses of these compounds in the rat brain tissue. The values of reproducibility and accuracy obtained here are in accordance with the data described by other authors who used different chromatographic conditions [37] [46].

Figure 3 shows the data obtained in the experiments carried out to investigate the stability of thiamine and its phosphate esters in two different conditions. (a) shows data of the stability of the compounds in hippocampus homogenate and (b) in supernatant extract after the derivatization process. As mentioned in the Methods section, in the first case (**Figure 3(a)**), homogenized hippocampus samples were stored at −80˚C and assayed 7, 15 and 30 days after homogenization. In the second condition (**Figure 3(b)**), the stability of the thiochrome derivatives in the supernatant extract was assessed 1, 30, 60, 120 and 240 minutes after potassium ferricyanide oxidation. The data obtained for the two conditions were expressed as nmol/g of tissue for each compound separately or by adding the three obtained values in a single representative total value (TDP + TMP + P = Total, nmol/g of tissue).

The data show that after the derivatization process the brain extract samples have to be analyzed in less than 30 min. In the present study, all samples were
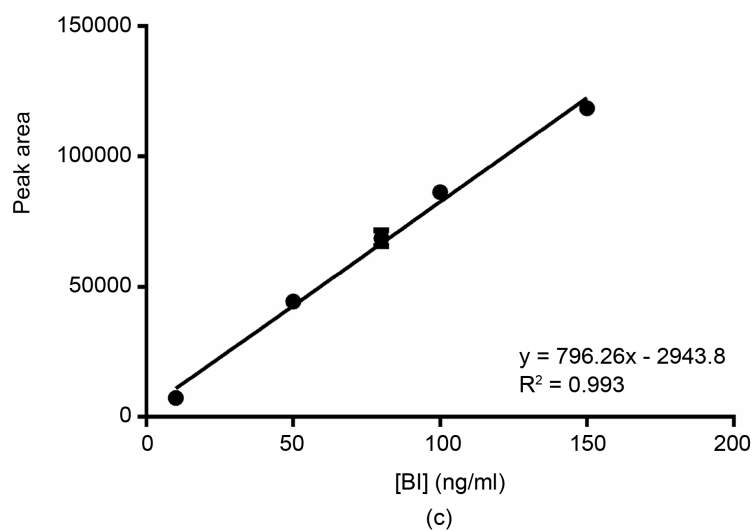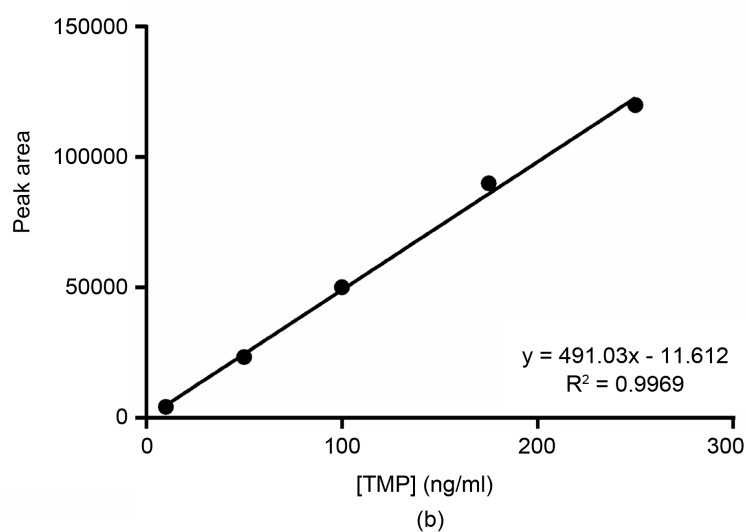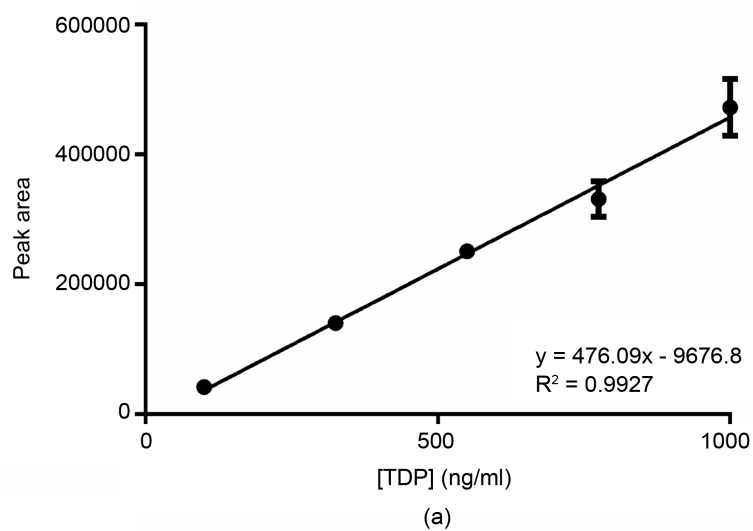
Figure 2. Standard calibration curves for TDP, TMP and B1: linear response over the concentrations for TDP, TMP and B1. Peak areas are shown in (a)-(c), respectively. Both peak height and area are proportional to concentration. Results are expressed as the mean of three assays for each concentration.
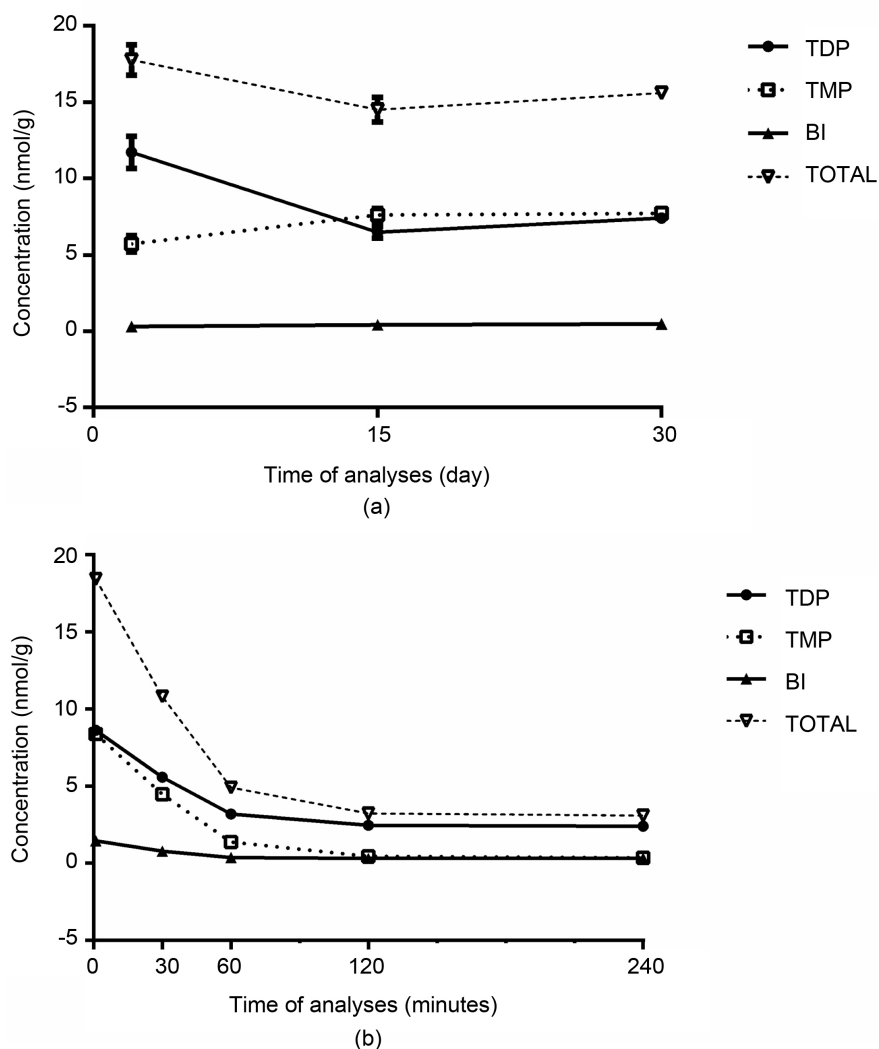
(a)

(b)

Figure 3. Stability of separated analytes TDP, TMP, B1 and values obtained with the Total (TDP + TMP + B1). Panel A shows the concentrations of TDP, TMP, BI and TOTAL in the same sample of homogenized hippocampus, maintained at −80°C and analyzed after 7, 15 and 30 days. Panel B shows the stability in hippocampus supernatant extract derivatized with potassium ferricyanide. Results are expressed as mean ± S.E.M.

Table 1. Recovery of TDP, TMP and B1 from hippocampus samples at final concentration. [TDP]: 100 - 700 ng/mL, [TMP]: 25 - 250 ng/mL, [B1]: 20 - 100 ng/mL.

Concentration added (ng/mL)			Concentration found (ng/mL)			% Recovery		
TDP	TMP	B1	TDP	TMP	B1	TDP	TMP	B1
0	0	0	167.25	17.63	1.03	-	-	-
100	25	20	271.8	40.38	21.17	104.55	90.98	100.67
250	75	40	417.76	87.52	38.18	100.2	93.18	92.86
400	125	60	579.19	135.03	59.92	102.99	93.92	98.14
550	175	80	721.9	188.39	82.34	100.85	97.57	101.63
700	250	100	867.14	279	97.34	99.99	104.55	96.3

injected into the chromatographic system within 1 min. Comparing to data obtained by other study, 30 min can be considered a lower stability [41]. They found that TDP, TMP and B1 kept in a cell culture after derivatization were stable until 3 h, at room temperature. After this period, they detected TDP hydrolysis to TMP. One possible explanation for the difference between these results could be not only the kind of samples, cells and tissues, but also light effects on the compound stability. The derivatized samples were stored in the dark at room temperature [41]. Further systematic studies have to be done to test this hypothesis. Despite the relative low stability it is important to consider that the use of potassium ferricyanide as a derivatizing agent has many advantages, among which are an adequate sensitivity and being safer than other derivatizing agents, like cyanogen bromide and mercuric chloride [44]. As described by other authors [47], 5M phosphoric acid can also be added to derivatized samples before injecting into the HPLC. In some samples we detected that it can improve the resolution of the phosphate esters (data not shown).

The experimental variation of the analyses is shown in Table 2. Intraday reproducibility was calculated from 10 consecutive injections of derivatized supernatant obtained from the hippocampus, yielding relative standard deviations (R.S.D.s) of 2.66%, 4.50% and 7.43% for TDP, TMP and B1, respectively. Interday reproducibility was obtained by comparing the mean of 10 replicates over two consecutive days. The obtained interday R.S.D.s were 37.54%, 25.39% and 25.87% for TDP, TMP and B1, respectively. The data show that the intraday reproducibility is high with small R.S.D. for each of the three assessed parameters. On the other hand, the interday reproducibility was low showing high R.S.D.s. These data indicate that after derivatization the analyses have to be done on the same day.

3.2. Determination of TDP, TMP and B1 Concentrations in Three Different Rat Brain Regions and Blood Samples

Following the validation of the method using hippocampus sample, as described above, we also determined the levels of TDP, TMP and B1 in the thalamus, PFC and blood samples from male Wistar rats. Table 3 shows the concentrations of B1 and its phosphorylated forms expressed in nmol/g of tissue or in nmol/L of

Table 2. Intraday and interday reproducibility of TDP, TMP and B1 concentrations. Intraday values were obtained by comparing results of 10 consecutive injections of derivatized supernatant obtained from hippocampus sample. Interday values were obtained by the mean of 10 replicates over two consecutive days.

	Relative Standard Deviations (%)	
	Intraday	Interday
TDP	2.66	37.54
TMP	4.50	25.39
B1	7.43	25.87

Table 3. TDP, TMP and B1 levels in the hippocampus, thalamus, prefrontal cortex and blood samples from male Wistar rats. Results are expressed as mean ± S.E.M.

Sample		Retention time (min)	Concentration (nmol/g of tissue or nmol/L blood)
Hippocampus	TDP	3.89 ± 0.023	8.78 ± 0.66
	TMP	4.68 ± 0.81	7.38 ± 0.41
	B1	11.79 ± 0.070	0.55 ± 0.03
Prefrontal Cortex	TDP	3.83 ± 0.004	11.19 ± 0.53
	TMP	4.28 ± 0.005	6.02 ± 0.66
	B1	11.67 ± 0.021	0.62 ± 0.05
Thalamus	TDP	3.71 ± 0.010	9.63 ± 0.37
	TMP	4.17 ± 0.014	3.91 ± 0.50
	B1	11.25 ± 0.042	1.33 ± 0.91
Blood samples	TDP	3.78 ± 0.016	1127.11 ± 54.87
	TMP	4.28 ± 0.027	718.02 ± 89.97
	B1	11.51 ± 0.095	187.80 ± 72.89

blood. In addition, it also shows the retention time, in minutes, for each of the three assessed variables. The results are expressed as the average of two independent experiments. In each experiment the measurements were done in triplicate for hippocampus, PFC and blood. The measurements for thalamus were done in duplicate.

Extraction procedures of B1 and its phosphate esters and HPLC quantifications after derivatization under alkaline conditions to form fluorescent thiochrome derivatives were previously reported by other authors who used biological samples obtained from different kinds of tissues [42] [43] [44] [46] [48] [49] [50] [52] and cell culture [41]. However, these authors did not validate the method used for the specific brain samples assessed and also none of them measured these parameters in the hippocampus and prefrontal cortex as shown in the present study. The majority of them used homogenates of the whole brain, cortical hemispheres, cerebral cortex and cerebellum as well as samples from liver, kidney, heart and urine. Similarly, to the present study, the phosphate ester parameters were measured in the thalamus samples [43], but they did not assess the vitamin B1 levels nor did they use a validated method for measurement of these compounds in brain tissue.

The TDP concentrations shown here for each of the three rat brain areas are within the same range, from 6 to 15 nmol/g of brain tissue, described by other

authors [46] [48], who used distinct kinds of samples compared to that used in the present study. The values (nmol/g of tissue) found here were 8.8, 9.6, and 11.2 in the hippocampus, thalamus and prefrontal cortex, respectively.

The methods described here are also useful for measuring levels of B1 and its phosphate esters in rat blood. The average concentration found for the total thiamine (TDP + TMP + B1) in rat blood was 203.3 ± 8.7 μg/L (n = 8). This value is similar to that described by others authors: 283.1 ± 0.75 μg/L (n = 7) [52] and 243.4 ± 12.1 μg/L (n = 7) [53].

3.3. Limitations

This study has limitations, including the stability of thiamine and its phosphate esters, and the amount of the sample.

Thiamine and its phosphate esters are temperature and light-sensitive and their stability is low. Therefore, samples should be storage in −80°C for 1 week and the analysis should be carry out in the same day and until one minute after the derivatization.

Since the thalamic sample obtained is very small, it only allowed for a duplicate run rather than the standard triplicate.

Due to our difficulty in obtaining a standard for thiamine triphosphate (ThTP), we were unable to perform analysis for the compound.

4. Conclusion

In short, when compared to the diversity of methods in the literature, the present data are the first to represent the validation of an efficient HPLC method for determining the vitamin B1concentrations and those of its phosphorylated forms in hippocampus from rats. It is known that rats are an important and widely used experimental model to study the molecular mechanism of neurodegeneration and behavioral deficits induced by thiamine deficiency. In addition, the present work is the first to show validated results of the concentrations of these parameters in the hippocampus as well as their levels in the thalamus, PFC and blood samples. Moreover, it provides information on essential variables, such as linearity range and limit of detection, reproducibility and stability of thiamine, TMP and TDP in rat brain samples.

Acknowledgements

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