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# Determination of thiamine and its phosphate esters in rat tissues analyzed as thiochromes on a RP-amide C16 column

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**Abstract**

A new reversed-phase chromatographic method is described for the separation and quantification of thiamine (T), thiamine monophosphate (TMP) and diphosphate (TDP) in rat tissues. Sample extraction with perchloric acid ($HClO_4$) was found more suitable than extraction with trichloroacetic acid (TCA), as regards convenience and background fluorescence. Derivatization of thiamine vitamers to thiochromes was optimized and complete separation of TDP and TMP thiochromes was obtained on a RP-amide C16 column in isocratic elution, with T thiochrome eluting in less than 10 min. The precision and the accuracy of the HPLC procedure were assessed: ranging from 0.5 to 7.7% for intra-day and from 2.0 to 9.4% for inter-day precision, a recovery average of 101% was determined (range 90–111%). Mean values of recovery for TDP, TMP or T were 91, 96 and 90% for liver extracts, respectively. Analysis of vitamers in tissues of rat submitted to 8 days thiamin deficiency, followed by a 14 days repletion, showed a significant reduction of TPP after 8 days of depletion in liver (−67%), brains (−50%), kidneys (−60%), followed by a complete recovery upon repletion.


*Keywords:* Thiamine; Thiochromes; RP-amide C16 column

## 1. Introduction

Thiamine deficiency is uncommon in western countries, but some conditions may promote the emergence of deficiencies such as unbalanced diets (rich in refined carbohydrates, or containing thiaminases), drastic food processing (high temperature, light, bleaching with sulfites), alcoholism or use of some drugs (diuretics, digoxin) [1–3]. This point is all the more critical since deficiency can develop within a relatively short time span (a few weeks in humans), which certainly reflects the fact that the thiamine stores in the body are quite limited (around 30 mg).

Vitamin B1 is found as free thiamine (T), as well as various phosphorylated forms such as thiamine monophosphate (TMP), thiamine diphosphate (TDP) and thiamine triphosphate (TTP). Dietary Vitamin B1 is extensively converted to free T in the digestive tract and absorbed, a large part of this vitamin circulates in plasma as free T but the tissular pool is largely represented by phosphorylated vitamers (especially TDP, the active coenzyme form) [4].

TDP is involved in several key reactions of metabolism: transketolase (pentose pathway), three mitochondrial enzyme complexes (pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and branched chain α-ketoacid dehydrogenase) and peroxisomal 2-hydroxyphytanoyl-CoA lyase (involved in α-oxidation of 3-methyl-branched fatty acids such as phytanic acid) [5]. Several metabolic features are involved in the pathogenesis of Vitamin B1 deficiency, such as failure of energy production, especially in the nervous system (Wernicke–Korsakoff syndrome) which exhibits lactate acidosis and excessive release of excitotoxic amino acids such as glutamate [6].

Vitamin B1 status has been frequently estimated by measuring erythrocytes transketolase activity, but this technique suffers several drawbacks: (i) difficult to standardize through

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inter-laboratory assays, (ii) detects only thiamine deficiency and then reflects uniquely the erythrocytes TPP levels and (iii) does not distinguish between the various vitamers. Alternatively, the quantification of Vitamin B1 may be effected by various chemical or microbiological procedures [7,8]. Nevertheless, recent advances in HPLC techniques allow the determination of thiamine in foods and biological fluids more rapidly, sensitively and reproducibly than the above methods and HPLC methods offer the possibility to distinguish between the various vitamers.

In most cases, thiamine and its phosphoric esters are analyzed after oxidation to a strongly fluorescent derivative (thiochrome) using potassium hexacyanoferrate in alkaline conditions, and separated on C18 columns and detected by fluorometry (λext 366 nm/λem about 435 nm). A problem with reverse phase conditions on C18 columns is to get separation of the thiochromes of polar phosphorylated vitamers from injection peak (including various contaminants), sufficient resolution between these polar thiochromes and a reasonably short retention for thiamine. Recently, ion-pairing or normal columns have been proposed, but with inherent problems in terms of mobile phase preparation (gradient elution), column stabilization, column life time and fluorescence background. Vinas et al. [9] concluded that isocratic or gradient elution of thiamine derivatives with ODS column were not satisfactory. Alternatively, separation on a more polar columns (such as NH2−) has been evaluated, with good separation of TMP and TDP thiochromes but extraction of sample is time consuming and the most interesting vitamer (TDP) exhibits a relatively long retention time [10]. Vinas et al. [9] have recently shown that a new type of column packing (RP-amide C16) constituted a good alternative to ion pairing or polar columns to analyze underivatized thiamine vitamers; however, this technique was used with UV detection (234 nm/266 nm) and was therefore of limited sensitivity and prone to considerable interferences in biological fluids such as urine. Since all the above approaches were not satisfactory for our experimental needs (quantification of different vitamers at very low concentrations in plasma and in tissues), it was decided to evaluate the feasibility of analysing phosphorylated and non-phorylated thiamine as thiochromes (for maximal sensitivity of detection by fluorimetry) on a RP-amide C16 column, after adaptating chromatographic conditions. Furthermore, the feasibility of simplifying the extraction procedure from biological matrixes was also investigated.

## 2. Experimental

### 2.1. Chemicals and reagents

Standards of T, TMP and TDP, potassium hexacyanoferrate ($K_3[Fe(CN)_6]$) and sodium chloride (NaCl) were purchased from Sigma (L′Isle d′Abeau, France). Potassium dihydrogenophosphate ($KH_2PO_4$) and hydrochloric acid (HCl) were obtained from Fluka (Buchs, Switzerland). Methanol HPLC grade were from Riedel-deHaën (Seezle, Germany). Perchloric acid ($HClO_4$) was procured from Carlo Erba (Val de Reuil, France) and potassium carbonate ($K_2CO_3$) from Normapur (Lyon, France).

Stock solutions of 10 mM T, TMP and TDP were prepared in 0.01 M HCl and stored at 4 °C. Dilution of standards was effected extemporarily with milli-Q water.

### 2.2. Extraction of thiamine and its esters from tissues and derivatization

One gram of tissue (liver, brain, kidney or heart) was homogenized in 7 ml of $HClO_4$ 0.4 M using a Polyton disintegrator (Bioblock, Switzerland). The suspension was then centrifuged at 8000 g for 10 min and aliquots of the supernatant were immediately used for derivatization.

Potassium hexacyanoferrate (20 μl; 30.4 mM in NaOH 15%) was added to 200 μl of perchloric extract, mixed by vortex during 10 s and left to stand exactly during 60 s and then 5 μl of NaOH (15%) was added. The linearity of the standards (T, TMP, TDP) was assayed at points 0.31, 0.63, 1.25, 2.5, 5, 10 and 50 μmol/l. This range of concentration corresponded to the concentration found in the different samples. To verify extraction recovery, the same standard solution were analyzed with and without extraction.

### 2.3. Chromatographic conditions

The liquid chromatography system consisted of a Alliance 2690 Separation Module (Waters, Saint Quentin en Yvelines, France) with a Multi λ fluorescence detector (Waters 2475). The column (15 cm × 4 mm) and the precolumn (2 cm × 4 mm) were packed with a RP-amide C16 stationary phase with a particule size of 5 μm (Supelco, USA). The column and the guard column were placed in an oven at 30 °C.

The mobile phase was potassium phosphate buffer (50 mM, pH 6) – methanol (80/20, v/v) delivered at flow rate of 1 ml/min. The injection volume was 20 μl and the duration of the analytical run was 10 min. Fluorescence detection was operated at 366 nm excitation and 435 nm emission.

### 2.4. Animals

Wistar rats ($n = 36$) were housed individually in metabolic cages in a controlled environment. Basal diet and deionized water were supplied ad libitum. Body weight and diet intake were recorded. Rats were depleted in thiamine during 8 days and then repleted for 14 days with a well-balanced diet covering the requirement in thiamine. Before tissue sampling, rats were anaesthetized by sodium pentobarbital intraperitoneal injection (40 mg/kg of body weight). Twelve rats were killed at the start of the experience, after depletion (8 days) and repletion (14 days) period. All samples were immediately snap frozen to stop metabolism and stored at −80 °C before analyses.

### 2.5. Statistics

Data were analyzed using the analysis of variance (ANOVA). The difference in the vitamin tissues contents between different period (depletion, repletion) were evaluated using a Bonferroni post hoc test. The inter-day precision was evaluated by three experiments repeated on the same day for five non-consecutives days.

## 3. Results

### 3.1. Analytical

As shown in Fig. 1, thiochrome derivatives of TDP, TMP and T were well separated and the most polar vitamer (TDP) was relatively well retained with a retention time of 2.5 min, which is sufficient to minimize interferences and overlapping with polar compounds eluted close to the solvent peak. Table 1 shows a good linearity.

The precision and accuracy of the method was evaluated on aqueous standard solutions submitted to the same treatment as tissues, for various concentrations of T, TMP and TDP, ranging from 0.31 to 50 μM (Table 2). The mean values found were very close to the expected value, especially for the highest concentrations. The intra-day variation was generally lower than 5%, except for the lowest concentration. The inter-day variations were also limited, namely less than 10%. The stability of the derivatives has been checked and it appears that the thiochrome of vitamers show no detectable decomposition during 3 h after derivatization.

Using the described method, the concentration of thiamine vitamers was determined in plasma and various rat tissues (Fig. 2). It appears that TDP is the predominant vitamer in all the tissues examined, especially in the liver (28.2 nmol/g wet weight liver). TMP and T are detectable but their concentration is much lower than that of TDP. Nevertheless, the present method allows determining precisely these minor compounds. In plasma, T is the major circulating form and its concentration is relatively low, namely around 400 nM (Fig. 3). Mean values of recovery samples spiked with 20 nM of TDP, TMP and T were 91, 96 and 90% respectively for liver extracts, 98, 98 and 80% for kidney extracts, 92, 93 and 80% for brain extracts and 81, 85 and 83% for heart extracts. Inter-day variation for different tissues (liver and brain) was also evaluated and no significant difference for T, TMP and TDP was found between the means of ten determinations over three non-consecutive days.

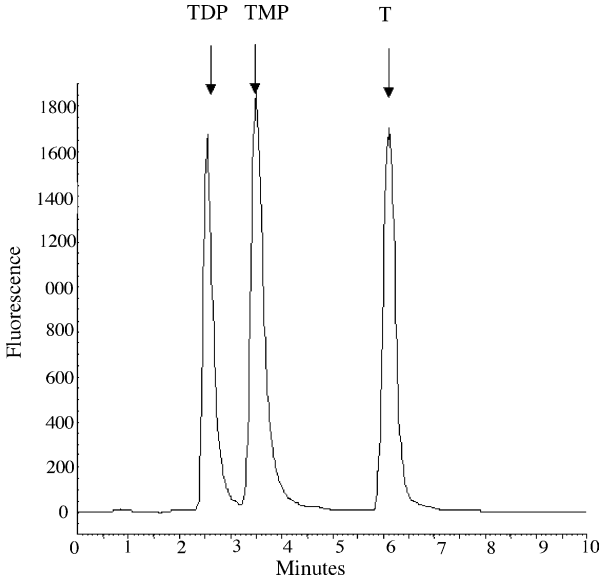


Fig. 1. Standard solutions: 50 μM of TDP (2.5 min), TMP (3.6 min) and T (6.2 min).

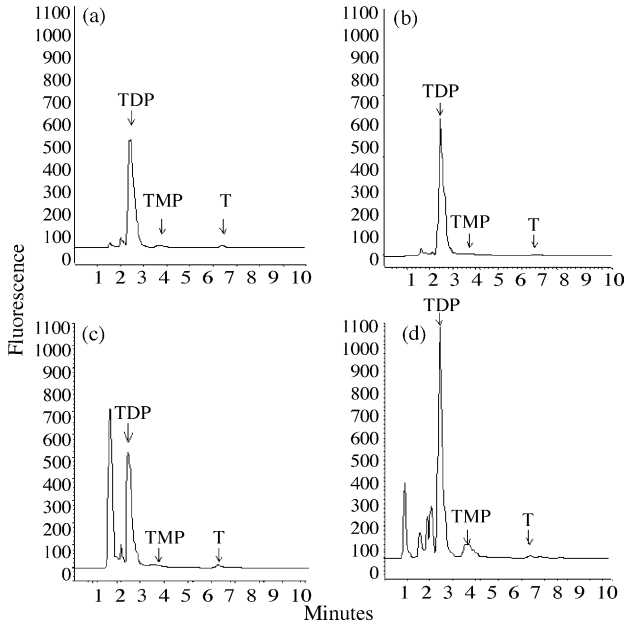


Fig. 2. Chromatograms of thiochromes derivatives of thiamine and thiamine phosphates (in nmol of Vitamin B/g of wet weight tissue). (a) Rat brain: 11.4 nmol of TDP/g, 1.5 nmol of TMP/g and <0.3 nmol of T/g), (b) heart: 19.1 nmol of TDP/g, 0.4 nmol of TMP/g and <0.02 nmol of T/g, (c) kidney: 14.1 nmol of TDP/g, 1.6 of TMP/g and 0.3 nmol of T/g, (d) liver: 28.2 nmol of TDP/g, 2.5 nmol of TMP/g and 0.3 nmol of T/g and order eluted: TPP (2.5 min), TP (3.60 min) and T (6.4 min).

### 3.2. Samples

In order to evaluate the capacity of the present method to detect low tissular concentrations of T vitamers, a group of rats was submitted to a depletion period on a thiamine-free diet for 8 days and a sub-group of these animals was repleted in thiamine for 14 days using a normal diet (insuring 100% of the T requirements). As shown in Table 3, there was a drastic reduction of TDP in the liver after 8 days depletion (down to 10.1 nmol/g wet weight liver), followed by a complete recovery upon repletion. The other minor vitamers were also affected by the depletion and, for example, TMP could be detected at a very low tissular level (0.7 nmol/g). In the brain,

Table 1
Parameters of the peak areas linear regression analysis ($n = 25$)

| | Slope | Coefficient of correlation ($r$) | $Y$-intercept | Retention time | $p$-Value (departure from linearity) (ns) |
|---|---|---|---|---|---|
| T | 544953 | 0.998 | 44477 | 6.21 ± 0.20 | 0.543 |
| TMP | 497124 | 0.998 | 44963 | 3.60 ± 0.06 | 0.333 |
| TDP | 495492 | 0.999 | 20702 | 2.57 ± 0.03 | 0.543 |

Table 2
Accuracy and precision (intra-day and inter-day) of the HPLC procedure

| Expected value (μM) | Mean found value ($n = 5$ μM) | Mean deviation, $n = 10$ (%) | Relative standard deviation | |
|---|---|---|---|---|
| | | | Intra-day, $n = 5$ (%) | Inter-day, $n = 25$ (%) |
| T | | | | |
| 0.31 | 0.33 | +6 | 2.8 | 2.7 |
| 0.63 | 0.66 | +5 | 4.8 | 2.9 |
| 1.25 | 1.32 | +6 | 7.4 | 4.3 |
| 2.50 | 2.75 | +10 | 3.6 | 9.4 |
| 5.0 | 5.57 | +11 | 5.3 | 6.4 |
| 10 | 10.53 | +5 | 3.5 | 6.3 |
| 50 | 50 | 0 | 1.2 | 5.0 |
| TMP | | | | |
| 0.31 | 0.28 | −10 | 9.0 | 8.7 |
| 0.63 | 0.60 | −5 | 7.2 | 9.4 |
| 1.25 | 1.24 | −1 | 4.6 | 5.5 |
| 2.50 | 2.51 | 0 | 7.7 | 5.7 |
| 5.0 | 5.08 | +2 | 6.4 | 8.4 |
| 10 | 10.11 | +1 | 2.4 | 2.2 |
| 50 | 50.5 | +1 | 1.1 | 2.0 |
| TDP | | | | |
| 0.31 | 0.28 | −10 | 6.5 | 6.9 |
| 0.63 | 0.60 | −5 | 5.1 | 4.0 |
| 1.25 | 1.26 | +1 | 3.1 | 5.4 |
| 2.50 | 2.55 | +2 | 5.6 | 4.9 |
| 5.0 | 5.14 | +3 | 4.3 | 5.6 |
| 10 | 10.00 | 0 | 3.5 | 4.7 |
| 50 | 50.5 | +1 | 0.5 | 4.9 |

Table 3
Effect of thiamine depletion (8 days) followed by a repletion period (14 days) on the tissue concentrations of thiamine vitamers

| $n = 12$ | nmol/g wet tissue | Control | After depletion period | After repletion period |
|---|---|---|---|---|
| Liver | T | 0.30 ± 0.13 | 0.16 ± 0.05 | 0.30 ± 0.09 |
| | TMP | 2.4 ± 0.6 a | 0.7 ± 0.2 b | 1.9 ± 0.4 a |
| | TDP | 31.1 ± 4 a | 10.1 ± 2.4 b | 34.1 ± 2.0 a |
| Brains | T | 0.33 ± 0.03 | 1.06 ± 0.03 | 0.22 ± 0.08 |
| | TMP | 1.7 ± 0.2 a | 1.5 ± 0.2 ab | 0.9 ± 0.4 b |
| | TDP | 12.6 ± 2.4 a | 6.5 ± 0.8 b | 11.9 ± 2.1 a |
| Kidneys | T | 0.32 ± 0.05 | nd | 0.31 ± 0.07 |
| | TMP | 1.83 ± 0.21 a | 0.04 ± 0.02 b | 2.22 ± 0.18 a |
| | TDP | 15.1 ± 2.7 a | 5.8 ± 1.3 b | 12.6 ± 2.6 a |
| Heart | T | nd | nd | nd |
| | TMP | 1.0 ± 0.6 | nd | 2.0 ± 0.7 |
| | TDP | 22.1 ± 6.2 a | 6.4 ± 1.9 b | 14.4 ± 3.4 c |

The difference in the vitamin tissues contents between different period were evaluated using a Bonferroni post hoc test for liver, brains and kidney and a non-parametric method followed by a Dunn post hoc test for heart. Data not sharing the same letters are significantly different ($p < 0.01$).

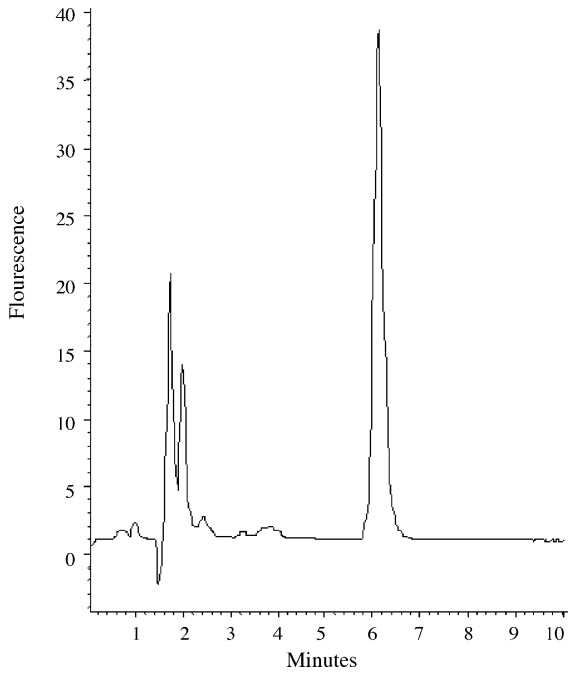


Fig. 3. Chromatograms of thiochromes derivatives of thiamine and thiamine phosphates in plasma (450 nM of T). Example of biological fluids extracts (200 μl plasma in 400 μl of 0.6 M $HClO_4$).

the effect of depletion was lesser than in the liver for TDP (from 12.6 to 6.5 nmol/g) and TMP was not significantly altered. In kidneys, TDP is also the major vitamer (15.1 nmol/g) and it was severely depressed (−60%) after 8 days thiamine depletion and then returned to initial value after repletion. It is noteworthy that, like in liver, TMP was also markedly lowered upon depletion.

## 4. Discussion

Most of the proposed methods of determination of thiamine in biological samples are time-consuming, especially the extraction step, and quantification of the various phosphorylated vitamers is still relatively difficult. The aim of the present approach was thus to attempt to reduce the extraction time as well as to get rapid separations of the different vitamers, in isocratic conditions as far as possible.

### 4.1. Extraction conditions

TCA extraction has been extensively used to deproteinize biological samples. This procedure is very effective but frequently requires subsequent elimination of TCA by diethyl ether, with practical and safety problems [11,12]. Furthermore, TCA removal is not absolutely complete and it turned out that, in our chromatographic conditions, the fluorescence background was markedly elevated thus affecting the sensitivity of the system. Perchloric acid deproteinization has been extensively used in metabolic studies, for example before enzymatic analysis. Furthermore, perchlorate anion precipitation and medium neutralization can be easily achieved upon addition of KOH or $K_2CO_3$. With the present procedure, we observed that the stability of thiamine and thiamine phosphates before derivatization was altered by treatment of perchloric extract with $K_2CO_3$, which reflects the fact that thiamine is unstable under alkaline conditions. Therefore, direct derivatization on the perchloric extract without K neutralization was chosen, and stabilization of thiochromes was performed using NaOH in carefully standardized conditions [13]. Under such conditions, the extraction and derivatization procedure was noticeably simplified and shortened.

### 4.2. Derivatization

Many researchers have studied how to optimize the oxidation reaction and have recommended using certain levels (or range of levels) of oxidizing agent. We have checked that an optimal oxidation level existed between 10 and 2400 μg of $K_3[Fe(CN)_6]$/μg of thiamine, a range slightly wider than those proposed by [13] but comparable to those proposed by various other authors [10,14–16].

It must be kept in mind that most of the investigations in the domain have focused on an only one vitamer (generally T) and, for tissues studies, we had to optimize the oxidation conditions for all the vitamers, in a same sample.

Post-column derivatization has been proposed for T determinations with conventional columns [17–20] or more recently with an RP-amide C16 column [21], since it is a method of choice when the stability of fluorescent derivatives is poor. This was evaluated, using a C18 column and a Waters post-column reaction module and external pump. It turned out that there was a substantial broadening of the peaks, hence a reduced separation and a markedly elevated fluorescence background. Furthermore, the use of concentrated saline solution (15% NaOH) for post-column derivatization is a source of crystallization in the reactor and the detector. Finally, this system was abandoned due to the above drawbacks and the fact that is was possible to obtain a reasonable stability of the thiochromes derivates of T and T phosphates by pre-column derivatization.

### 4.3. Separation on RP-amide C16 column

It is relatively easy to get satisfactory conditions of analysis for thiamine alone, using a C18 column. In contrast, in tissues, several vitamers are present with different degrees of phosphorylation, hence of polarity. Using C18 columns for thiochromes derivates of thiamine and thiamine phosphates separation, the most polar thiochrome derivates (TDP) is very rapidly eluted and the resolution between TDP and TMP is poor. The RP-amide C16 column shows the same pattern of elution as a C18 column but with later elution (and better resolution) of polar thiochrome derivates, together with a relatively short-time elution of thiamine thiochrome. This good resolution of thiochrome esters with RP-amide C16 column

is obtained in isocratic conditions, whereas comparable resolutions would be hardly achieved on a C18 columns, and only using gradient conditions, with inherent drawbacks (several buffers, gradient reproducibility, time of reequilibration). It must be noted that the choice of organic solvent is critical since acetonitrile yielded poor separations in isocratic conditions (excessively rapid elution) whereas 20% methanol offered good quality separations, with short runs (10 min). Acetonitrile was effectively used by Vinas et al. [9] on a RP-amide C16 column, using a gradient, but for separation of vitamers in urine samples (UV detection), hence very different conditions. Another interest of methanol is that it is an enhancer of the fluorescence response of thiochromes [22].

### *4.4. Thiamine assessment in rat tissues*

Thiamine status is classically assessed by blood transketolase activity, a reflect of erythrocyte thiamine stores. This technique is widely used in humans, but transposition in laboratory animals is poorly validated. Furthermore, this approach lacks sensitivity and does not inform about vitamers distribution in tissues and their possible interconversion. Data on tissue thiamine and thiamine phosphate concentrations are scarce and frequently under poorly defined nutritional conditions. Liver vitamer concentrations (expressed in equivalent thiamine) in the literature show a great variability, from 5 up to 82 nmol/g tissue [23–26], compared to 31.1 nmol/g liver in the present study. Variations could arise from quantification as total thiamine (or more specifically TPP), thiamine status of the animals or from the analytical procedure (microbiological, chemical or chromatographic). Data on brain T concentrations (TDP) are more consistent, from 6 to 15 nmol/g tissue [8] in the same range as the value (12.6 nmol/g) found in the present study. After an 8-day depletion period, the most active vitamer (TDP) was severely depressed in the liver (−67%) but to a lesser extent in brain and kidneys (−50 and −60%, respectively) whereas plasma T concentrations were also drastically reduced (−95%) (data not shown). The magnitude of these variations is similar to those reported in the literature, for comparable duration of depletion [8,24], but the present study also provides information on the respective changes of the vitamers. For example, it appears that in the liver T is poorly altered during the depletion/repletion periods in contrast to the TMP and TDP vitamers.

In conclusion, the present method appears particularly suitable for the determination of the different vitamers of thiamine in large series of samples, requiring small quantities of tissues (effective quantity of tissue analyzed: 25–50 mg fresh weight) and using a rapid extraction procedure. We have also established that thiochrome derivatives (for high sensitivity) can be well separated in isocratic conditions, in 10 min, using the recently introduced on the market RP-amide C16 columns in place of classical C18 columns.

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