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QUANTITATIVE ANALYSIS OF TOTAL THIAMINE IN HUMAN BLOOD, MILK AND CEREBROSPINAL FLUID BY REVERSED-PHASE ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Whole blood hemolysed by freezing, serum, cerebrospinal fluid, and milk of human origin were deproteinized by perchloric acid. Thiamine pyrophosphate and thiamine monophosphate were hydrolysed to thiamine by acid phosphatase. Chromatography was performed on C_{18}-coated silica using an *n*-octanesulfonate containing mobile phase methanol—aqueous citrate buffer pH 4.0 (45:55, v/v). In a post-column reaction $K_3Fe(CN)_6$ is used to oxidise thiamine to thiochrome, which is detected by fluorometry. Two ml blood is needed. The minimum detectable amount is 60 femtomol of thiamine. The intra-assay coefficient of variation (C.V.) is 2.3% and the inter-assay C.V. is 3.9%. The recovery of added thiamine pyrophosphate to blood samples was 98.7%. The reference range was found to be 88—157 nmol/l whole blood. Examples of the analysis of cerebrospinal fluid, serum and milk are given.

INTRODUCTION

The wide activity of thiamine (vitamin B_1) in carbohydrate metabolism is well known. Its main biologically active form, thiamine pyrophosphate (ThDP), is involved in the decarboxylation of α-keto acids and in the hexose-monophosphate shunt. Clinical aspects of thiamine deficiency have been extensively described (refs. 1—3, for example), such as in Wernicke's disease and in Korsakoff's psychosis. Alcohol abuse is accompanied by thiamine deficiency. Recently, attention was drawn to the occurrence of very high thiamine levels in postmortem serum of victims of sudden infant death syndrome [4], whereas the thiamine content of their erythrocytes, the erythrocyte transketolase activity and its stimulation by ThDP showed no abnormalities [4, 5].

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For diagnostic purposes, the direct quantification of total thiamine in whole blood, serum or erythrocytes is now advised. Determination of the ThDP-dependent transketolase activity may be useful to obtain extra information or whenever the direct analysis of total thiamine is not possible. Vitamin B_1 analyses have been performed by microbiological assays or by manual fluorometric methods (refs. 6—8, for example).

Recently the use of high-performance liquid chromatography (HPLC) for thiamine analysis in food, pharmaceutics, blood, etc., has been described (refs. 9—15, for example).

Most publications about thiamine analyses by HPLC deal with artificial mixtures, vitamin products or foods containing rather large amounts of thiamine. Only the method presented by Schrijver et al. [10] and the very similar but limited study of Kimura et al. [11] cope with the specific needs of a very low concentration (20—200 nmol/l) of thiamine and the complexity of the sample matrix of whole blood. Both groups converted the various thiamine phosphate esters present in blood to thiamine by enzymatic hydrolysis. Adsorption chromatography was performed using an aqueous mobile phase and either a silica [10] or a polyeter resin [11] stationary phase. Post-column derivatization and fluorometric detection of thiochrome completed the analysis. Especially the rapid deterioration of the silica column in thiamine analysis using adsorption chromatography [12] initiated our study.

We present an improved HPLC method of analysis of total thiamine in biological samples. Compared with the very few publications on this subject, our method is optimized with respect to sensitivity, time of analysis, chromatographic performance, sample treatment and costs. Reference values for total thiamine in blood are discussed.

MATERIALS

Acid phosphatase solution

Lyophilized acid phosphatase (EC 3.1.3.2) from potatoes (type II) was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.; cat. no. P 3752), activity 0.4 units/mg solid. A solution of 10 mg enzyme per ml physiological saline was prepared just prior to use.

Taka-diastase solution

A solution of 200 mg Taka-diastase (Serva, Heidelberg, F.R.G.; cat. no. 35740) per ml of physiological saline was prepared.

Thiamine and ThDP solutions

Thiamine chloride hydrochloride (Merck, Darmstadt, F.R.G.; cat. no. 8181) and ThDP chloride (Boehringer, Mannheim, F.R.G.; cat. no. 133051) stock solutions contained approximately 30 μmol/l thiamine and 20 μmol/l ThDP, respectively, in 0.1 mol/l HCl. These solutions were stable in the dark at 4°C and the concentrations were calculated every month from their absorbance at 248 nm, as proposed by Penttinen [16]. However, after drying our thiamine as prescribed by the USP [17], we found a slightly higher value for the molar absorptivity (13,570 l/mol cm) than Penttinen. We used our own finding for

the determination of the stock concentrations. Two-hundred-fold dilutions with physiological saline of these stock solutions were used as working solutions for the chromatographic analysis.

Internal standard solution

This was an aqueous solution containing approx. 15 μmol/l salicylamide (2-hydroxybenzamide, Sigma Chemical Company; cat. no. S 07050), 0.6 mol/l sodium hydroxide and 1.8 mol/l sodium acetate.

Mobile phase

Methanol- aqueous sodium citrate buffer pH 4.0, 0.05 mol/l (45:55, v/v) was used. Sodium 1-octanesulfonate (Eastman Kodak, Rochester, NY, U.S.A.; cat. no. 10265) was added in a concentration 10 mmol/l of mixture. Degassing of the mobile phase was performed by ultrasonication under vacuum for 10 min. Flushing with a stream of helium gas did not improve the fluorescence intensity obtained during analysis and was considered unnecessary.

Oxidizing reagent

This was an aqueous solution of 2.5 mmol/l $K_3Fe(CN)_6$ and 3.0 mol/l NaOH. No degassing was necessary. The solution was stable for at least one month stored at room temperature and protected from light.

Reagents

HPLC grade methanol was obtained from Rathburn Chemicals (Walkerburn, U.K.). Reagent grade water was delivered by our Milli-Q deionization unit (Millipore, Bedford, MA, U.S.A.). Perchloric acid solutions 3.0 and 1.2 mol/l were obtained by dilution of 70% perchloric acid (Merck; cat. no 519). Reagents not specified were of analytical grade.

METHODS

Sample preparation

Whole human blood. Venous blood samples were collected in evacuated tubes containing either sodium heparinate or potassium ethylenediaminetetraacetate as anticoagulant. Blood samples were stored at −20° C. After thawing and homogenizing the sample, 2 ml were mixed vigorously with 2 ml of 1.2 mol/l cold perchloric acid and kept at 0° C for 15 min. Thereafter this mixture was centrifuged for 20 min at 2000 *g* and 4° C.

One milliliter of clear supernatant was mixed with 0.5 ml of internal standard solution. Finally 0.1 ml of acid phosphatase solution was added. Enzymatic hydrolysis occurred during overnight incubation at 37° C. Final pH is about 5.2. Samples treated this way were either directly analysed or stored at −20° C. Keeping prepared samples for 15 h at room temperature did not noticeably reduce their thiamine content.

Human serum or cerebrospinal fluid (CSF). Samples were stored at −20° C. After thawing and homogenizing, a 2-ml sample was mixed vigorously with 0.5 ml of 3.0 mol/l cold perchloric acid. The further procedure is as described for whole blood.

Human milk or manufactured baby milk. Samples were stored at −20°C and prepared just like whole blood. Centrifugation leads to a floating layer of fat and a clear solution, which is used for analysis.

Chromatographic analysis

The mobile phase was pumped at 1.2 ml/min by a Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.). A 20-μl sample was injected by a Rheodyne valve (Model 7125, Rheodyne, Cotati, CA, U.S.A.). A μBondapak C_{18} analytical column (10-μm particles, column 30 cm × 3.9 mm, Waters Assoc.) and a corresponding guard column were used.

The post-column reactor consisted of a stainless-steel zero-dead-volume tee-piece (bore 0.25 mm, Valco Instruments, Houston, TX, U.S.A.) and a Teflon capillary (0.75 mm I.D., 1.8 mm O.D., 90 cm long) wound to a spiral tubular reactor 6.5 mm O.D. The Teflon coil was shielded for light. Column and post-column reactor were at ambient temperature (21—25°C).

A peristaltic pump delivered the oxidizing reagent at 0.3 ml/min; its pressure pulses were damped by a 1-m piece of silicon tubing between this pump and the tee-piece.

Detection was performed by a spectrofluorometer Model SFM-23LC (Kontron, Zürich, Switzerland) equipped with a 20-μl flow-through cell thermostatted at 25°C. The excitation was set at 367 nm, the emission was detected at 435 nm. The resulting signal was displayed on a Kipp BD 40 recorder (Kipp & Zonen, Delft, The Netherlands). Peak areas and retention times were calculated by a Minigrator computing integrator (Spectra Physics, Mountain View, CA, U.S.A.). A complete run took less than 7 min.

The total thiamine concentrations were calculated by the well-known internal standard method, using salicylamide as internal standard. The efficiency of the enzymatic hydrolysis during the sample preparation was checked for each batch of analyses by processing and analysing the working ThDP solution. A sample of a stock of frozen donor-blood was included in each batch of analyses for external quality-control purposes.

RESULTS AND DISCUSSION

Chromatographic experiences

The stability of thiamine is bad in alkaline solutions and fairly low in neutral aqueous solutions. However, in acid solutions it is very stable. Therefore, and because the pK_{a_1} is 4.8 [18], we selected the pH range 3.5—6.0 as most suitable for an ion-pair reversed-phase retention mechanism. Phosphate was often used as mobile phase constituent in HPLC analysis of thiamine (e.g. refs. 10—14). For our purpose its pK_a values are inappropriate. Moreover, the low solubility in water—methanol or water—ethanol of the Na_3PO_4 formed post column, leads to clogging of the detector. Citrate was found to be very useful as a buffer constituent.

A number of experiments were performed in which the mobile phase composition was optimized. A chromatogram obtained from the analysis of a test mixture is shown in Fig. 1. The separation of ThDP and ThMP (thiamine monophosphate) was even better at lower ionic strength or lower pH of the citrate

buffer. However, such change added one or two extra minutes to the total analysis time, which was considered a waste of time for the analysis of thiamine. We prefer hydrolysis of the ThDP and ThMP to thiamine because of its convenience for quantitation and because the fluorescence of the thiochrome phosphate esters differ from each other [16]. Besides, the analysis of a test mixture containing 0.1 mol/l HCl showed a slow spontaneous dissociation of ThDP to thiamine.

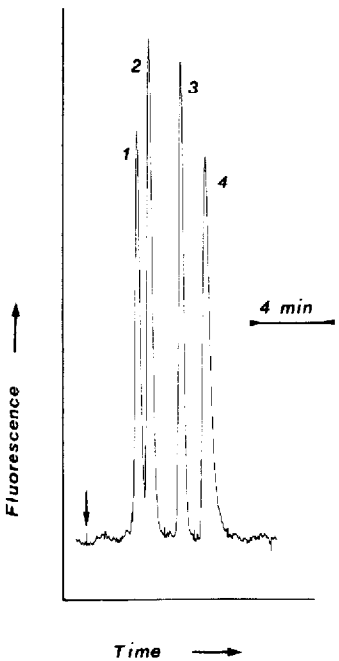

Fig. 1. Chromatogram obtained from a test mixture containing 61 nmol/l ThDP, 64 nmol/l ThMP, 7 μmol/l salicylamide and 82 nmol/l thiamine. For convenience the peaks are named after their parent compounds in the sample injected. Components elute in the order: 1, ThDP (k' = 0.3); 2, ThMP (k' = 0.6) 3, salicylamide (k' = 1.4) and 4, thiamine (k' = 1.9).

However, to demonstrate the suitability of our method to quantify also directly the ThDP content of whole blood, Fig. 2 shows chromatograms of samples before enzymatic hydrolysis (being the faster analysis) and after hydrolysis (being the more accurate analysis). The column was rinsed with methanol—water (70:30, v/v) after the analysis of each batch of samples. Comparing the performance of a new column with a column used four days a week for five months, we found a 24% decrease in the number of theoretical plates and a 2% shift in capacity factor, k', for thiamine. The pump pressure needed was increased from 110 to 160 bar upon aging of the column. Our analysis time (less than 7 min) is much shorter than that reported previously [10, 11]. Hence, our chromatographic system is economical as well as very stable.

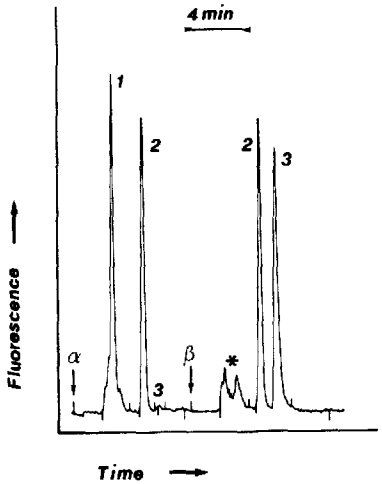

Fig. 2. Chromatograms obtained from a whole-blood sample containing 111 nmol/l total thiamine. The ThDP peak in sample α (without enzymatic hydrolysis) disappears and shows up as a thiamine peak in sample β (including enzymatic hydrolysis). 1, ThDP; 2, salicylamide; 3, thiamine. The asterisk (*) indicates unknown sample components mainly originating from the blood sample. (The new Kontron spectrofluorometer SFM 23B was used for this figure.)

Detection optimalization

The 45% (v/v) methanol in the mobile phase not only serves as a solvent modifier. Its use enhances the fluorescence intensity of thiochrome nearly three-fold and thus improves the signal-to-noise ratio. The observed enhancement of thiochrome fluorescence by the presence of different concentrations of either methanol or ethanol in the solution corresponds closely to Penttinen's results [16].

We tested the suitability of water—methanol (50:50, v/v) and water—ethanol (50:50, v/v) as solvent for the oxidizing reagent. No significant further increase of thiochrome fluorescence was noticed. Besides, methanol but especially ethanol, decreased the $K_3Fe(CN)_6$ stability in the reagent. Therefore, water became the solvent of choice for the oxidizing reagent and methanol was selected as constituent of the mobile phase. Wagner and Folkers [19] discussed the oxidation of thiamine. Important for the formation of thiochrome are the stabilization of a reaction intermediate by methanol and a pH of 11 or even higher. In our post-column reaction the pH is about 13 under the conditions given. The concentration of $K_3Fe(CN)_6$ does influence the thiochrome formation [7], as confirmed by own experiments. By varying both NaOH (1.0 to 3.75 mol/l) and $K_3Fe(CN)_6$ (0.5 to 8.0 mmol/l) concentrations the composition of the oxidizing reagent was optimized for the flow-rates employed.

Rapid mixing experiments using quartz cuvettes showed that thiochrome formation was complete (at least 95% converted) in about 15 sec under conditions equal to our post-column reaction. Obviously, an increased residence time in our post-column reactor leads to peak broadening. We selected a 90-cm length as an optimum for our Teflon capillary, corresponding to a 16-sec residence time.

Sample preparation experiences

Two different sample preparation procedures were tested; the TCA—diastase method as described by Schrijver et al. [10] using Taka-diastase, thiamine and ThDP solutions described under Materials; and a second method, as described in Methods, which will be called the $HClO_4$—phosphatase method. Deproteinization of the sample by trichloroacetic acid (TCA) and hydrolysis of the thiamine esters by Taka-diastase or Clarase have been used by several authors (e.g. refs. 6, 7, 10, 11). In view of several drawbacks that have been reported with TCA [6, 7] we tested denaturation with perchloric acid and found it very useful.

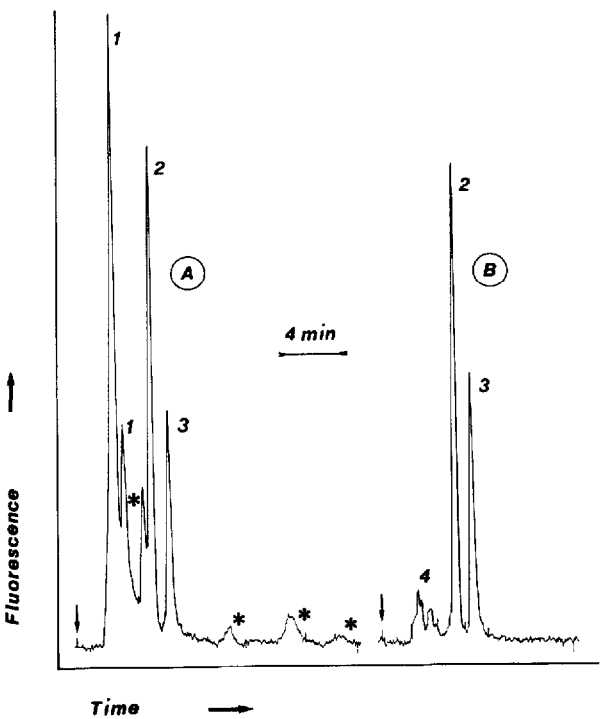

Fig. 3. Chromatograms demonstrating the differences between the TCA—diastase (A) and the $HClO_4$—phosphatase (B) treatments of the same whole-blood sample. Total thiamine contents corresponding to Fig. 3A and 3B were 107 and 126 nmol/l, respectively. 1, unknown sample components mainly from Taka-diastase; 2, salicylamide; 3, thiamine; 4, unknown sample components originating from the blood sample plus the acid phosphatase. Unknown impurities in Fig. 3A are indicated by asterisks (*).

The chromatogram presented in Fig. 3A was obtained from a whole-blood sample stored at −20° C and subjected to the TCA—diastase method. For blood samples stored at 4° C, the occurrence of fluorescent sample components eluting between the bulk of components near $k' = 0$ and the thiamine peak ($k' = 1.9$) was noticed more often. Fig. 3B shows the result of the $HClO_4$—phosphatase procedure for the same sample. The absence of the bulk of components (Fig. 3B) derived from a crude enzyme mixture allows an easy check of the completeness of ThDP and ThMP hydrolysis in the sample

analysed. In one experiment up to 3000 nmol/l ThDP were present in whole-blood samples fortified by ThDP addition. After $HClO_4$—phosphatase treatment no ThDP or ThMP could be found by chromatographic analysis. Reduction of the number and quantity of sample components makes the data handling easier and facilitates the insertion of an internal standard in the chromatogram.

The Taka-diastase preparation used contained a small amount of total thiamine of itself (in the order of 100 ng/g), whereas no total thiamine could be detected in the acid phosphatase.

The thiamine content of samples obtained by TCA—diastase treatment decreased upon freezing and thawing. The thiamine content of the sample in Fig. 3A was 119 nmol/l initially. The $HClO_4$-phosphatase method is clearly superior to the TCA—diastase method.

An example of analyses of whole-blood samples accompanied by a thiamine standard and a blank, is given in Fig. 4. Obviously, the chromatographic part of the assay can be easily automated.

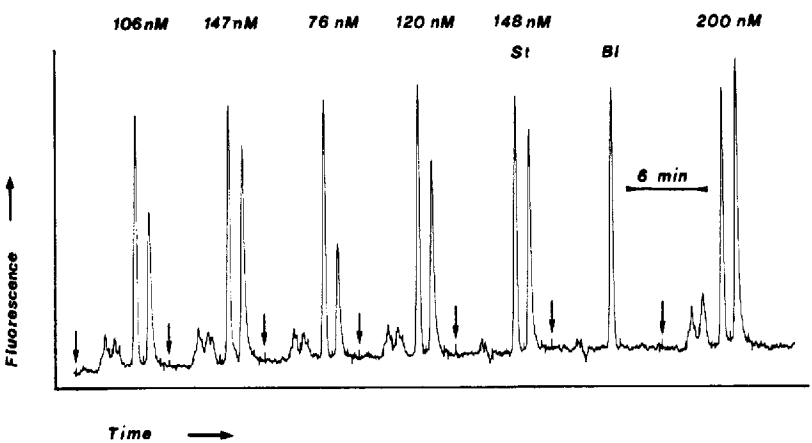

Fig. 4. Chromatograms showing five blood samples together with a thiamine standard (St) and a blank (Bl). Concentrations given are expressed in n*M* total thiamine in the original sample. The times of injection are indicated by arrows.

Thiamine or its esters gradually decomposed during storage of whole blood at 4°C. Five different samples of whole blood were divided and stored separately at 4°C and —20°C. A loss of 12—24% in total thiamine content was found for the 4°C samples and no loss for the frozen samples, after eleven days of storage. Myint and Houser [7] found no significant change in thiamine content upon storage at —20°C of serum and whole blood for eleven and two months, respectively. Hemolysing a blood sample by freezing—thawing increases its handling convenience and reduces the chance for sampling errors caused by sample inhomogeneity. It should be remembered that the total thiamine concentration in erythrocytes and leucocytes is one and two orders of magnitude higher, respectively, than in serum [6, 10].

Two final remarks must be made with regard to the $HClO_4$—phosphatase procedure. Salicylamide could not be included in the perchloric acid solution;

it became partially bound to the mass of denatured blood cells and proteins. No loss of salicylamide occurred when it was dissolved in the acetate solution.

Peak broadening or even splitting up the thiamine peak was noticed occasionally for samples having a pH below 5. If necessary, the pH of incubated samples or standards was adjusted to pH 5.2 by addition of 50 μl of 3 mol/l NaOH.

Precision, recovery and linearity

Donor blood containing disodium citrate as anticoagulant was frozen and thawed. After homogenization it was divided into portions and stored at −20°C in plastic tubes. Samples of such donor blood stocks were used for testing the precision, recovery and linearity of the method.

The intra-assay coefficient of variation, calculated from the analysis of ten samples from the same donor blood stock, was found to be 2.3% at a mean concentration of 74.3 nmol/l. The inter-assay coefficient of variation was found to be 3.9%, based on the analysis of ten donor blood samples in ten batches of analysis.

Because no absolute method was available for comparison, the accuracy of the method was investigated from recovery experiments. Weighed amounts of a 9.1 μmol/l ThDP solution in 0.1 mol/l HCl were mixed with known volumes of approximately 2 ml of donor blood containing 91 nmol/l total thiamine. A perfect linear relationship was found up to 400 nmol/l. Plotting the results of chromatographic analysis as a function of the expected thiamine concentration and using linear regression, we found $f(X) = 1.240 + 0.987X$ for $n = 9$; $r = 0.997$. Hence, the recovery was 98.7% of the ThDP added. At thiamine concentrations of 400–2000 nmol/l, a slight deviation of linearity was observed.

Based on the band width of the noise and considering a signal-to-noise ratio of 3 as the detection limit, the sample injected should contain at least 3 nmol/l thiamine, corresponding to an absolute amount of 60 fmol thiamine injected.

Reference values of total thiamine in blood

Whole-blood samples from 529 patients attending our hospital during two years were analysed elsewhere by the method of Schrijver et al. [10]. Analysing the distribution of the total thiamine content of these samples by a Bhattacharya plot as suggested by Naus et al. [20], we found a mean of 113 nmol/l and 95% confidence limits of 81–144 nmol/l. Taking into account that these samples had been stored at 4°C for up to one week before they were sent away for analysis, we conclude that these 95% limits are equal to the 95–155 nmol/l reference values published [10].

Next, we determined the total thiamine content in whole blood of 56 healthy volunteers (26 males and 30 females) by our own method. By means of the distribution-free method of Rümke and Bezemer [21] and setting the limits of percentiles at 2.5 and 97.5%, a reference range of 88–157 nmol/l was calculated with a reliability of 95%. The mean total thiamine content of our population was 117 nmol/l. The mean values for the 26 men and 30 women were 130 and 105 nmol/l, respectively. Fig. 5 shows the frequency histogram for our 56 volunteers. We believe that its skewness is partly caused by the different hematocrit values for males and females.

Table I summarizes some data on the total thiamine content of human whole blood gathered from the literature. A very good agreement exists between the HPLC analyses themselves and in comparison to the groups of older manual methods.

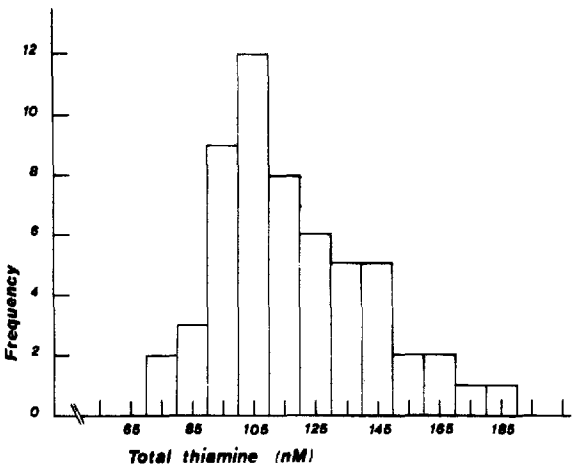

Fig. 5. Frequency histogram of the concentration of total thiamine (n*M*) in whole blood from 56 volunteers.

TABLE I

TOTAL THIAMINE CONCENTRATION IN HUMAN WHOLE BLOOD

Authors	Method	No. of samples	Range found (nmol/l)	Mean (nmol/l)
Burch et al. [6]	Manual—fluorometry	6	128—154	139
Myint and Houser [7]	Manual—fluorometry	44	34—142	87
Schrijver et al. [10]	HPLC—fluorometry	98	70—185	115
Kimura et al. [11]	HPLC—fluorometry	20	not given	137
Wielders and Mink (this study)	HPLC—fluorometry	56	71—185	117

Analysis of serum, CSF and milk

Chromatograms obtained from the assay of serum, CSF and milk are presented in Fig. 6. To our knowledge no HPLC method of thiamine analysis in CSF and (human) milk has been published before. The results obtained for a small number of samples of serum, CSF and milk are presented in Table II, together with data gathered from the literature. Our findings are very similar to those presented by authors using non-HPLC methods. Probably HPLC would be more accurate and less time-consuming than manual or microbiological methods.

Davis et al. [4] suggested a possible relationship between sudden infant death syndrome and the high thiamine content of manufactured baby milk in comparison to breast feeding. Therefore, three different brands of manufactured baby milk were analysed and found to contain 1520, 1450 and 2000 nmol/l total thiamine. The large discrepancy found between natural human milk and

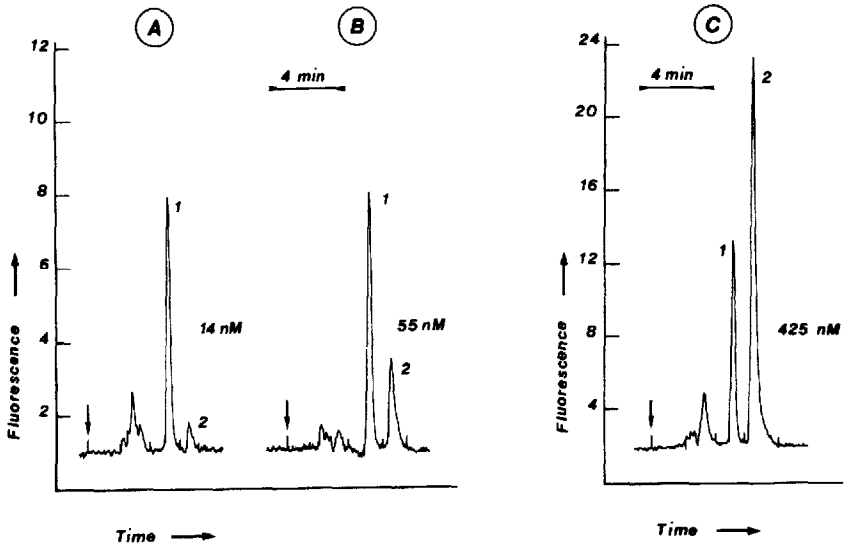

Fig. 6. Examples of typical chromatograms obtained from samples of human serum (A), human CSF (B) and human milk (C). Concentrations are given in nmol/l total thiamine in the original sample. Fluorescence intensity is given in mV recorder response. The salicylamide concentration in Fig. 6C was larger than in Fig. 6A and 6B. 1, salicylamide; 2, thiamine.

TABLE II

TOTAL THIAMINE CONCENTRATION IN HUMAN SERUM/PLASMA, CSF AND MILK

Thiamine or ThMP contents given in μg/l were converted to nmol/l.

Authors	Method	Material	No. of samples	Range found (nmol/l)	Mean (nmol/l)
Davis et al. [4]	Microbiological	Milk	9	163—1100	528
Burch et al. [6]	Manual—fluorometry	Plasma	6	15—30	24
Myint and Houser [7]	Manual—fluorometry	Serum	44	Trace—61	30
Rindi et al. [22]	Manual—fluorometry	CSF	20	not given	56
Wielders and Mink (this study)	HPLC—fluorometry	Milk	4	336—425	378
Wielders and Mink (this study)	HPLC—fluorometry	Serum	4	5—28	14
Wielders and Mink (this study)	HPLC—fluorometry	CSF	4	16—74	49

manufactured baby milk as far as the total thiamine content is concerned confirms Davis' analytical results. Our method will be useful for further study of this subject.

CONCLUSIONS

An optimalized and economical HPLC analysis for the quantification of total thiamine in biological samples at the nmol/l level is presented. The precision is high because of the selection of a purified enzyme preparation, the use of $HClO_4$ instead of TCA for protein precipitation, the insertion of an internal standard, and the fine chromatographic separations achieved. Recently,

Warnock [23] published an HPLC method for ThDP determination in erythrocytes using pre-column derivatization. His method was not able to measure free thiamine in a sample derived from erythrocytes, while ThDP is not stable at neutral pH. Together with Schrijver et al. [10] and Kimura [11], we prefer enzymatic hydrolysis of thiamine esters and on-line post-column derivatization to thiochrome. Our reference values confirm the only published data so far for HPLC analysis of thiamine in human blood [10]. Our method will be a valuable tool for monitoring thiamine content in CSF, serum and milk too.

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