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Determination of Thiamine in Human Plasma and Its Pharmacokinetics

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Summary. A sensitive assay for thiamine suitable for clinical use has been developed. It is based on precolumn oxidation of thiamine to thiochrome followed by HPLC-separation and fluorescence detection. The assay is applicable to various biological materials, including human plasma.

The minimum amount detectable was 5 fmol, minimum plasma concentration 0.5 nmol/l and minimum sample volume 0.3 ml plasma. Each chromatographic run took 3 min.

Inter- and intra-assay relative standard deviations (RSD) were 8.3% and 6.3%, respectively, at a stock plasma concentration of 10.8 nmol/l. At 38.8 nmol/l, interassay RSD was reduced to 3.4%. The recovery of 5 nmol/l added thiamine was 102 (SD ± 17)%, that of 30 nmol/l was 94 ± 5%.

Plasma levels in 91 volunteers ranged from 6.6 to 43 nmol/l, showing a log normal distribution with a median of 11.6 nmol/l.

Thiamine kinetics were studied in plasma and urine from 8 men after intravenous and oral doses of 50, 100 and 200 mg thiamine hydrochloride. In all individuals, nonlinear renal elimination kinetics were demonstrated by plotting the fractional amount of thiamine excreted unchanged in urine against the corresponding area under the plasma concentration – time curve.

Key words: thiamine; plasma level, pharmacokinetics, nonlinear renal elimination, assay for clinical use

Thiamine is employed for the treatment of various neurological conditions. For its rational therapeutic use a sensitive assay of plasma levels is necessary, but methods available so far are indirect and nonspecific or insensitive [5, 6, 8, 11]. None of them is sufficiently sensitive or suitable for routinely monitoring plasma levels. The combination of the conventional thiochrome method and HPLC-separation was first established by Roser et al. (1978) [7]. Their tedious method of sample pretreatment, with elution from cation-exchange columns, led to the relatively high detection limit of 90 nmol/l, which is sufficiently for the analysis of thiamine in human urine but not in other body fluids.

HPLC procedures published in recent years have been employed to determine thiamine in whole blood and erythrocytes [2–4] but they have not been sensitive enough to assay the blood plasma level. Although the most recent HPLC procedure [12] is more sensitive than previous techniques, it, too, is not adequate for the very low plasma concentrations which occur in thiamine deficiency. The plasma levels of thiamine, however, should provide the most reliable information on the body content of biologically available thiamine.

The new assay presented here is almost 10 times more sensitive [10] than even the best of those previously published. It is based on an HPLC-separation technique and quantitative spectrofluorometric detection of thiochrome derived from precolumn oxidation of thiamine.

Materials and Methods

Instrumentation

A Perkin Elmer Series 2-pump module as the solvent delivery system and a Rheodyne 7125-injector formed the HPLC-system. The column (4.6 × 120 mm, Knauer, FRG) was filled with Lichrosorb NH_2 (5 µm, Merck, FRG). The eluent used was

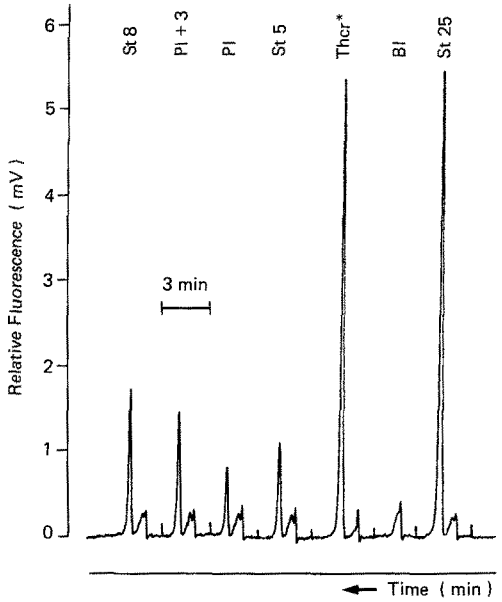


Fig. 1. Representative chromatograms, 20 µl isobutanol extract injected:
Bl : blank,
St 25 : 25 pmol/0.6 ml standard solution,
Pl : 3.7 pmol/0.6 ml plasma,
Pl + 3 : 3.7 + 3 (St) pmol/0.6 ml plasma,
Thcr : 20 µl of thiochrome 17.4 nmol/l in methanol injected, thiochrome yield of thiamine applied to assay procedure: 75%

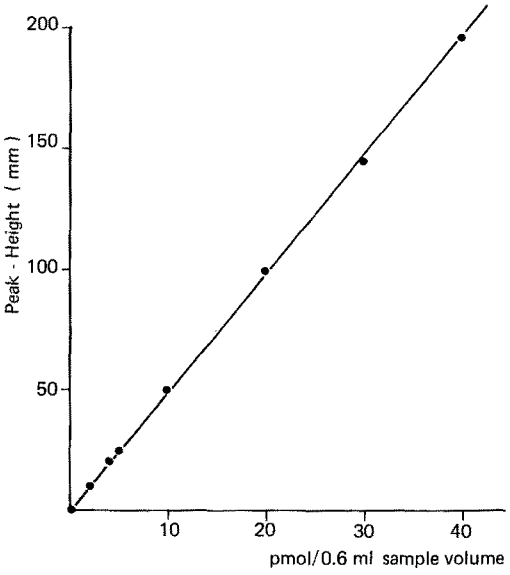


Fig. 2. Calibration curve prepared from standard thiamine solutions

methanol/ether (25:75, v/v) at a flow rate of 2.0 ml/min. A Perkin Elmer 650 - 10 LC spectrofluorometer with an 18 µm flow cell was used for detection. The wave length for excitation was 365 nm and for emission 440 nm; the band width was 20 nm.

Table 1. Precision and Recovery of Thiamine in Plasma

Added TPP [nmol/l]	Determined [nmol/l]			Recovery [%]	
	range	mean	±SD	mean	±SD
–	9.3–11.6	10.6[a, b]	0.7	–	–
–	9.4–11.9	10.6[b]	0.9	–	–
5	14.3–16.7	15.6[a]	1.0	102	17
10	17.9–21.0	19.4	1.2	89	11
15	22.9–26.7	24.7	1.2	94	8
20	26.9–30.4	29.1[a]	1.1	92	5
25	30.8–37.5	34.1[a]	2.0	94	7
30	37.1–40.8	38.8	1.3	94	5

$n = 10$ or [a] $n = 9$; [b] intraassay SD = ±0.7 nmol/l; TPP: Thiamine pyrophosphate

Reagents

Thiamine hydrochloride (TCL; Merck, FRG) and thiamine pyrophosphate (TPP; Sigma, FRG) were used to prepare standard solutions. The solvents for the mobile phase were ether and methanol, both vasol fluorescence grade (Merck, FRG). Thiamine phosphates were hydrolyzed with acid phosphatase EC 31.3.2 (Boehringer, FRG) at pH 4.5 in acetate buffer. Stock solutions containing 2 µmol/ml TCL or TPP were diluted daily before measuring standard curves.

Method

Blood samples taken from a cubital vein were anticoagulated with heparin 50 I.U./ml. After centrifugation, 0.2 N HCl 600 µl and 70% (w/w) perchloric acid 50 µl were added to 600 µl plasma and thoroughly mixed. After 10 minutes at 4 °C, samples were centrifuged, and 9 M potassium acetate 100 µl was added to 1.0 ml supernatant. In this way the potassium perchlorate was precipitated and the solution was adjusted to pH 4.5. To determine total thiamine, the content of thiamine phosphate esters in 400 µl of the weak acid solution was hydrolyzed overnight with 10 µl acid phosphatase (30 I.U., dissolved in 2 N sodium acetate). The free thiamine formed was oxidized to thiochrome by adding 1% mercury(II)chloride 75 µl and a small amount of crystalline sodium chloride to avoid precipitation of mercury(II)oxide. The solution was alkalinized with 7.5 N sodium hydroxide 75 µl and shaken for 5 min. The thiochrome was extracted with isobutanol (p.a., Merck, FRG) 550 µl. To dertermine nonphosphorylated thiamine, a further 400 µl of the deproteinized solution was analyzed without enzymatic hydrolysis of phosphate esters. After oxidation, as the thiochrome phosphate formed was not soluble in iso-

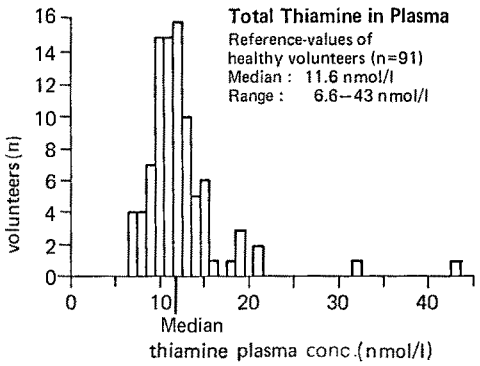


Fig. 3. Frequency distribution (log-normal) of total thiamine in plasma samples from 91 hospital employees; median 11.6 nmol/l; range 6.6–43 nmol/l

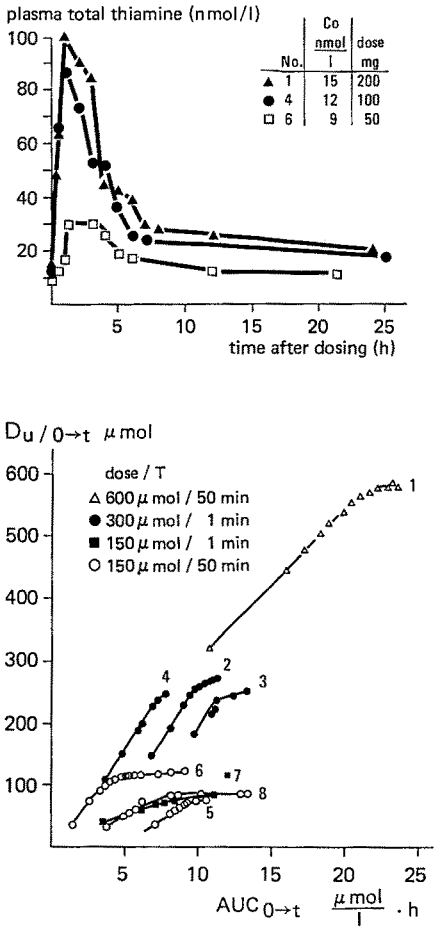


Fig. 4. Amount of thiamine excreted unchanged in urine $D_{u/0\to t}$ plotted against the corresponding area under the plasma concentration-time curve $AUC_{0\to t}$ slope: renal clearance in l/h
a oral and **b** parenteral administration of 50, 100 or 200 mg thiamine hydrochloride
Each curve refers to one numbered patient in Table 2

Table 2. Clearance parameters of thiamine in 8 males

No.	Age [years]	Body weight [kg]	Dose [mg]	Dose [µmol/kg]	$AUC_{0\to\infty}$ [h·µmol/l] i.v.	$AUC_{0\to\infty}$ [h·µmol/l] p.o.	[%] p.o./iv.	Clearance [ml/min/kg] Plasma i.v. Total[a]	Renal Total[a]	Renal (%Plasma)[a]	Initial[a]	γ-Phase	p.o. Total[a]	p.o. (%Plasma)[a]	p.o. Initial[a]	p.o. γ-Phase	Basic[a]	Creat.
1*	27	73	200	8.2	22.1	1.04	4.7	6.2	6.0	(96)	6.5	1.0	4.0	(65)	7.6	1.0	0.9	2.0
2	38	70	100	4.3	11.1	0.28	2.5	6.4	5.3	(82)	9.6	2.4	3.2	(50)	3.4	0.8	0.9	1.9
3	29	75	100	4.0	13.2	0.21	1.6	5.1	4.3	(84)	8.2	1.3	3.0	(60)	5.7	1.6	0.6	1.7
4	42	88	100	3.4	8.1	0.46	5.6	7.0	5.5	(80)	7.5	–	4.2	(60)	6.1	1.5	0.8	2.0
5*	48	58	50	2.6	9.7	0.57	5.9	4.4	2.2	(51)	5.2	0.6	1.0	(24)	2.1	0.5	0.4	1.3
6*	52	70	50	2.1	5.3	0.23	4.4	6.8	5.4	(79)	7.1	0.7	2.5	(36)	2.9	0.6	0.4	1.3
7	28	55	50	2.7	13.7	0.27	2.0	3.2	2.1	(66)	3.3	0.8	1.1	(36)	0.8	0.4	0.5	0.6
8*	40	72	54	2.3	12.8	–	–	3.1	1.6	(51)	1.7	0.2	–	–	–	–	0.1	0.3

[a] ($p<0.1$) correlation to creatinin clearance; i.v.-application for No. 1, 5, 6 and 8 was a 50 min-infusion(*), all other individuals got 1 min bolus injections; p.o.-application for No. 2, 3 and 7 was 1 or 2 50 mg tablets, for No. 1, 4, 5 and 6 one, 2 or 4 tablets were solved in 100 ml water immediately before oral intake

butanol, total and nonphosphorylated thiamine could be assayed separately.

20 µl of the isobutanol extract was injected directly into the HPLC system. The retention time of the thiochrome peak was 2 min. Less than 3 min was required for each chromatographic run. For quantitation, the peak-height method was used. Since the standard were carried throughout the entire assay, a direct comparison of standards and samples could be made.

Results

Linearity, Precision and Accuracy

Representative chromatograms are shown in Fig. 1. Peak-height was linearly related to thiamine concentration over the range 0.5 to 500 nmol/l. The best fit linear regression line was

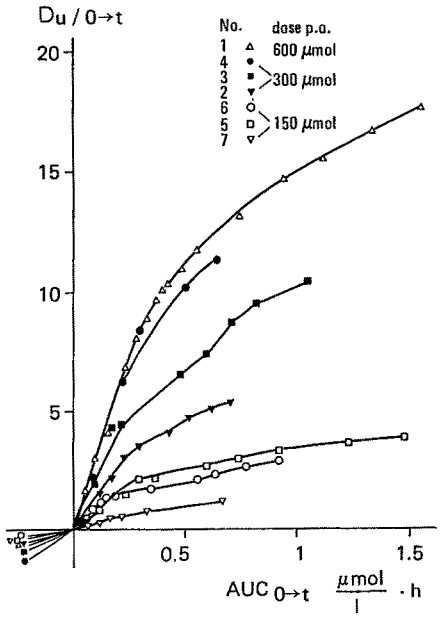
$$y = 4.8810 \times + 0.4928,\ r = 0.9996$$

Y is peak-height in mm and x is the amount of thiamine in pmol/0.6 ml sample (Fig. 2). The slopes of four calibration curves constructed over a period of one month showed a relative standard deviation (RSD) of 2.0%. The lower limit of detection was 0.5 nmol/l plasma.

The precision and accuracy (Table 1) of the method were evaluated by analyzing human plasma samples with added thiamine pyrophosphate and free thiamine in 6 steps from 5 nmol/l to 30 nmol/l. Inter-assay RSDs ranged from ± 8.3% to ± 3.4% ($n = 9$–10 at each concentration step). Mean recovery was 89 to 102%. Intra-assay SD of ± 0.7 nmol/l or RSD of ± 6.7% ($n = 10$) was found for duplicate analyses of the stock plasma (10.6 nmol/l). The method was not impaired by individual plasma factors, as shown by recovery rates in plasma samples from 20 volunteers. The basal concentration ranged from 4 to 19 nmol/l and the mean recovery of added thiamine 10 nmol/l was 9.93 ± 1.45 (SD) nmol/l.

Plasma Levels

Total plasma thiamine levels in 91 hospital employees ranged from 6.6 to 43 nmol/l with a median of 11.6 nmol/l, and were log-normally distributed (Fig. 3). These values are in accordance with the range of 9 to 45 nmol/l reported in 28 subjects by Baker et al. [5] using a microbiological assay with *Ochromonas malhemnis*. To shorten their procedure (several days were required for one assay) Baker et al. [6] changed to *Ochromonas danica* and reported plasma concentrations between 54 and 186 nmol/l (mean 63 nmol/l) in 128 volunteers. The higher range might be due to lack of specificity of the microbiological assay. Myint and Houser [11], using a conventional, nonspecific thiochrome method found total thiamine in serum from 44 volunteers to range from a trace to 61 nmol/l (mean 30 nmol/l).



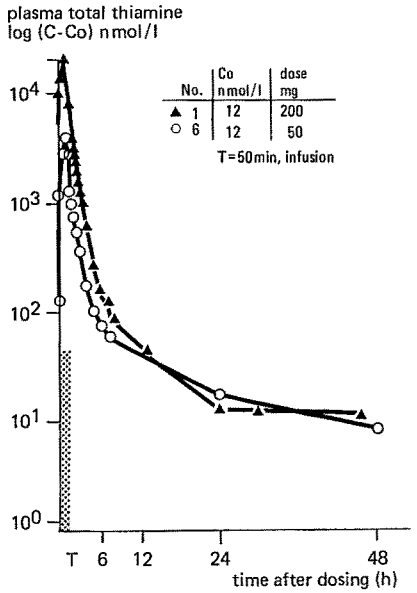


Fig. 5. a Time course of plasma total thiamine in 3 males (Nos. ▲1, ●4, □6; see Table 2) following single oral doses of thiamine hydrochloride 50, 100 or 200 mg, Co: 15, 12 and 9 nmol/l
b Semilogarithmic plot of plasma total thiamine versus time in 2 males (Nos. ▲1 and ○6; see Table 2); i.v. infusion time 50 min, doses 50 and 200 mg thiamine hydrochloride, Co: 12 nmol/l in both volunteers

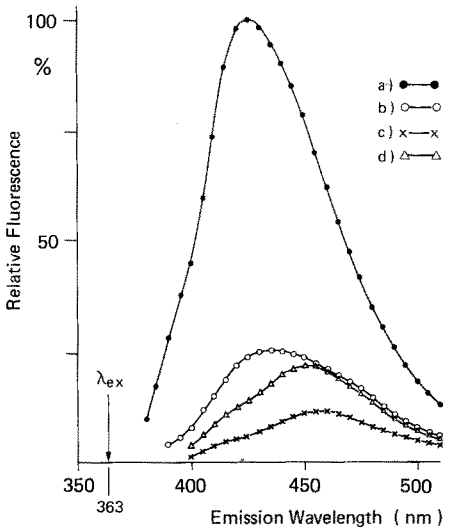


Fig. 6. Fluorescence quenching in thiochrome solutions (11 nmol/l) by various solvents (wavelength and relative intensity of maximum fluorescence in parentheses)
a) methanol (425 nm, 100%)
b) 0.1 n NaOH, water (435 nm, 25%)
c) b + 0.6 m $K_3(Fe(CN)_6)$ (455 nm, 11%)
d) c + 50% methanol (450 nm, 22%)

The thiamine content of whole blood is inhomogeneously distributed between blood cells (15% in leucocytes, 75% in erythrocytes) and plasma (10%); [8]. In diseases like anaemia, inflammations, hypo- or hypervolaemia, changes in the cellular composition of blood lead to variation in thiamine level if whole blood is used instead of plasma. Plasma is the material of choice for clinical monitoring, because it is homogeneous in relation to thiamine concentration.

The method described here can also be applied to whole blood, cerebrospinal fluid, urine and faeces. As concentrations will be higher than in plasma, appropriate dilution and a higher concentration range of calibration standards would be required.

Pharmacokinetics

The plasma kinetics of thiamine have not previously been studied because of the lack of an assay of adequate sensitivity for the measurement of thiamine at low plasma levels. In the present study, nonlinear renal elimination kinetics of thiamine was demonstrated.

The method described proved suitable for study of the kinetics of thiamine in man. 8 males, including 2 healthy volunteers (Table 2, Nos. 1 and 2), 3 patients with polyneuropathy (Nos. 3, 4 and 6), 2 with impaired renal function (Nos. 7 and 8) and one with tumor metastases in various lymph nodes (No. 5) received an i.v. bolus injection or a 50-min infusion and an oral dose of tablets or aqueous solution of 50, 100 or 200 mg thiamine hydrochloride. The time course of total thiamine was determined in plasma and urine.

Venous blood samples were taken after 0, 0.25, 0.5 and 1 h, hourly for the next 6 h, and thereafter at suitable intervals of 6–12 h for from 2 to 10 days. Urine was collected over 30-min intervals during the first 2 h, hourly until 7 h and thereafter in 12-h periods.

Calculation

Total plasma clearance (CL) was determined by

$$CL = D/AUC_{o\to\infty}$$

where $AUC_{o\to\infty}$ is the total area under the plasma concentration-time curve and D the dose. Similarily, the total renal clearance was calculated by

$$CL_R = D_{u/o\to\infty}/AUC_{o\to\infty}$$

where $D_{u/o\to\infty}$ is the total amount of unchanged thiamine found in urine.

The $AUC_{o\to t}$ from the beginning to sample time t was calculated by means of the trapezoidal rule. Residual $AUC_{t\to\infty}$ values for extrapolation of last sample time to infinity were determined by division of the last measured concentration by γ. γ is the time constant of the last phase at low plasma concentrations which showed an almost log-linear decline.

Total clearance values calculated by the above formula are valid only for linear kinetics. In case of nonlinear kinetics, total clearances are only average values.

The plot of the fractional amount of thiamine excreted unchanged in urine versus the corresponding $AUC_{o\to t}$ is shown in Fig. 4. The slope of the curve, representing renal clearance, is steeply increased after administration of therapeutic doses of thiamine.

Whilst at normal plasma levels of thiamine renal thiamine clearance was generally lower than creatinine clearance, its renal clearance was increased during the initially high plasma concentrations to values of the order of the renal plasma flow (see Table 2).

Clearance remained constantly increased to a high level for 6–8 h after administration of thiamine, so linear kinetics may be applied within this time period. After i.v. injection, a linear relation between dose per body weight (D_W) and $AUC_{o\to\infty}$ was observed in individuals with unimpaired renal function (Nos. 1–6):

$$D_W = 6.5\ \text{ml/min/kg} \times AUC_{o\to\infty};\ r = 0.96;\ n = 6$$

The slope of 6.5 ml/min/kg represents an average value for plasma clearance. The relationship

confirms that the kinetics of thiamine was virtually linear. In all individuals, nonlinear relationships were found between the fractional amount of thiamine excreted unchanged in urine and the corresponding area under the plasma concentration-time curve. Thiamine clearance increased from its basal value, well below the glomerular filtration rate (GFR), to values near renal plasma flow (RPF) during the initial phase of high plasma concentration.

This was probably due to greater competition for a tubular carrier system active in renal secretion of thiamine.

Renal clearance dramatically decreased when low plasma concentrations were again reached and, thereafter, plasma levels and renal clearances asymptotically approached their base-line values. All clearances determined are reported in Table 2.

The increase in renal clearance to a constant level close to the RPF during the period of high plasma concentration indicates flow-dependent renal elimination. At a high plasma concentration of thiamine, the intrinsic ability of the kidney to eliminate it is still so great that the renal influx of thiamine becomes rate-limiting.

Returning to the range of the normal, low plasma level, renal clearance was well below GFR. Within this low concentration range, thiamine may be assumed to compete with other substances for the same carrier system. The competition may be one cause for the reduced capacity of the nonlinear elimination process which then becomes rate-limiting.

The significance of the kidney for thiamine elimination can be deduced from the fact that, in individuals with normal renal function (Table 2, Nos. 1–4) 80–96% of an i.v.-dose was excreted unchanged in urine. Moreover, plasma and renal clearance were closely related ($p < 0.01$) to creatinine clearance, except for the γ-phase.

Equations to calculate correctly the time course of plasma level during nonlinear renal elimination are not jet available. Therefore, the best fit of plasma elimination to a triexponential function was used to estimate half-lives from semilogarithmic plots. Median half-lives in the α- and β-phases were 0.15 h (range 0.1–0.5 h) and 1.0 h (range 0.6–1.3 h), respectively, in individuals with unimpaired renal function (Nos. 1–6).

After parenteral administration of thiamine, plasma levels declined rapidly over 2–3 decades. The subsequent fall was slow, with a median half-life of approximately 2 days (range 0.8–9 days). It was possible to follow the plasma kinetics of thiamine to the very low basal concentrations observed in the last "γ"-phase only with the help of the increased sensitivity of the thiamine assay described here.

The changes in clearance after parenteral and oral administration were similar. Therefore, the relationship between the corresponding AUCs can be regarded as a crude measure of bioavailability (Table 2, $AUC_{o\to\infty}$, % p.o./i.v.) which appeared lower after administration of tablets (2%) than of solutions (5%).

The kinetics of thiamine in man have previously been studied by a conventional assay applied to whole blood and urine after i.v.-injection and an oral dose of 50 mg thiamine hydrochloride [14]. Whole blood concentrations showed a triexponential decline after the i.v. injection, but nonlinearity was not mentioned. After oral ingestion of 50 mg tablets, only a minimal increase in total thiamine concentration was detected, which was not clearly distinguishable from the baseline whole blood concentration. However, after oral administration of 50 mg thiamine hydrochloride in the present study, an increase in plasma concentration from the baseline 9 nmol/l to 30 nmol/l was found after 1 h (Fig. 5).

Discussion

The highly sensitive and specific HPLC-procedure reported here is based on the conventional thiochrome method. After extraction and deproteinisation of plasma, enzymatic hydrolysis was followed by alkaline oxidation of free thiamine to thiochrome. The isobutanol extract was separated by HPLC followed by fluorescence detection. No purification step was necessary, because the influence of reducing substances, which are always present in varying amounts in biological fluids, was neutralized by an excess of the oxidizing reagent.

For alkaline oxidation of thiamine to thiochrome, 3 reagents have usually been employed: potassium hexa-cyano-ferrate (III), mercury (II) chloride and bromcyan. In a recent paper [13], it was shown that all 3 reagents were suitable for the thiochrome fluorescent method. Although bromcyan may appear slightly superior, it is an extremely hazardous and rather unstable reagent. The alternative oxidant, potassium hexa-cyano-ferrate (III), in the concentration required to cope with accompanying reducing substances, has the disadvantage of interfering with the fluorescence of thiochrome [7, 11].

Mercury (II) chloride is stable in solution for months and does not interfere with the fluorescence measurement, even when used in excess. Therefore, mercury (II) chloride was considered the most suitable oxidizing reagent.

The HPLC procedure described should be compared with a recently proposed procedure of postcolumn oxidation by hexa-cyanoferrate (III) after sepa-

ration of thiamine by reversed ionpair HPLC [12]. This procedure, however, leads to lower sensitivity, since fluorescence has to be measured in the presence of water and oxidizing reagents, both of which, as known from the literature [1, 9] and confirmed by the curves shown in Fig. 6, greatly quench the fluorescence of thiochrome (almost 90%).

In addition to a significantly shorter retention time, the method of precolumn oxidation by mercury (II) chloride and estimation of thiochrome fluorescence in methanol/ether has a lower detection limit, namely 5 fmol/20 µl instead of 60 fmol/20 µl by postcolumn oxidation. At present, the method described here appears to be the most suitable for diagnosing thiamine deficiency by assay of plasma levels. It is sufficiently sensitive to measure thiamine in the range below 6 nmol/l.

Concerning specificity and accuracy, both pre- and postcolumn oxidation procedures are almost the same, although no information is available on the accuracy of the postcolumn oxidation method in the particularly important range of lower concentrations.

The high sensitivity of the new assay has opened the possibility of research into the plasma kinetics of thiamine, which has been measured for the first time. Nonlinear renal elimination kinetics of thiamine can be concluded from the plasma concentration-time curve and urinary thiamine excretion after oral or parenteral administration of thiamine.

Since the renal clearance of thiamine increased from its basal value beyond the GFR to values near RPF after administration of therapeutic doses, tubular reabsorption and secretion of thiamine are likely to occur. This may be of importance in concomitant drug treatment with other organic bases which could interfere with the handling of thiamine at the renal level. The interaction could lead to increased efficacy or even to a toxic effect if the safety margin were low.

References

1. Ellinger P, Holden M (1944) Quenching effect of electrolytes on the fluorescence intensity of riboflavin and thiochrome. Biochem J 38: 147–150
2. Kimura M, Fuyita T, Itokawa Y (1982) Liquid chromatographic determination of the total thiamin content of blood. Clin Chem 28 [1]: 29–31
3. Schrijver J, Speek A, Klosse J, van Rijn HJM, Schreurs WHP (1982) A reliable semiautomated method for the determination of total thiamine in whole blood by the thiochrome method with high-performance liquid chromatography. Ann Clin Biochem 19: 52–56
4. Warnock LG (1982) The measurement of erythrocyte thiamin pyrophosphate by high-performance liquid chromatography. Anal Biochem 126: 394–397
5. Baker H, Frank O, Pasher I, Sobotka H, Hutner S (1961) Vitamin-levels in blood and serum. Nature 191: 78–82
6. Baker H, Frank O, Fennelly JJ, Leevy CM (1964) A method for assaying thiamine status in man and animals. Am J Clin Nutr 14: 197–201
7. Roser RL, Andrist AH, Harrington WH (1978) Determination of urinary thiamine by high-pressure liquid chromatography utilizing the thiochrome fluorescent method. J Chromatogr 146: 43–53
8. Burch HB, Bessey O, Love RH, Lowry OH (1952) The determination of thiamine and thiamine phosphates in small quantities of blood and blood cells. J Biol Chem 198: 477–490
9. Mickelson O, Yamamoto RS (1958) In: Glick D (ed) Methods of biochemical analysis, Vol. 6. Academic Press, New York, pp 191–257
10. Weber W, Kewitz H (1983) HPLC-procedure for thiamin determination to study kinetics in humans. Arch Pharmacol 622 [Suppl R2]
11. Myint T, Houser HB (1965) The determination of thiamine in small amounts of whole blood and serum by a simplified thiochrome method. Clin Chem 2: 617–623
12. Wielders JPM, Mink CHR JK (1983) Quantitative analysis of total thiamine in human blood, milk and cerebrospinal fluid by reversed phase ion-pair high performance liquid chromatography. J Chromatogr 277: 145–156
13. Dong MH, Green MD, Sauberlich HE (1981) Determination of urinary thiamin by the thiochrome method. Clin Biochem 14 [1]: 16–18
14. Heesen H (1975) Pharmacokinetics in normal persons and obese patients after 14 days of starvation. Inn Med 2: 89–96

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