Determination of Sulbutiamine and its Disulfide Derivatives in Human Plasma by HPLC Using On-Line Post-Column Reactors and Fluorimetric Detection

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Key Words

Column liquid chromatography
Solid-phase extraction
On-line post-column reaction
Sulbutiamine
Thiamine disulfide

Summary

A new direct HPLC procedure for the simultaneous determination of sulbutiamine (Arcalion) and other thiamine disulfides in human plasma has been developed. The method involves an automated solid-phase extraction on octadecylsilyl (C18) cartridges and chromatographic separation of the compounds on an RP Select B column with gradient elution using methanol and phosphate buffer. Detection was by fluorescence of the resulting thiochromes obtained from two on-line post-column reactors. Optimization of post-column reaction parameters has been achieved. This method has been proved to be highly selective for the determination of the thiamine disulfide derivatives and quantitation limits of 5 ng · ml^{-1} were obtained for each compound in human plasma. Linearity was in the range 5 – 200 ng · ml^{-1}. Precision and accuracy were also demonstrated by within-day and between-day assays, and showed the good reliability of the method.

Introduction

Many thiamine disulfide derivatives have been synthesized and developed for their clinical applications in human growth and thiamine deficiency [1]. Arcalion 200 (Sulbutiamine, I) is a thiamine derivative obtained from free thiamine by three structural modifications: i) opening of the thiazole ring, ii) dimerisation to a disulfide compound, iii) esterification of the two alcoholic functions. These modifications enhance the lipophilic properties of sulbutiamine relative to the hydrophilic character of free thiamine [2, 3]. The drug is a psychotropic agent prescribed for the symptomatic treatment of functional asthenias [4]. Effects on long term memory formation [5] and on the waking state [6] have also been shown. In vivo, conversion of thiamine disulfides to free thiamine has been known for many years [1]. Numerous methods for the determination of thiamine and its phosphoric esters have been published [for a review, see 7 and 8], but only a few techniques have been studied for the quantitation of thiamine disulfide derivatives, since these compounds are not directly oxidized to thiochromes. One of the first analytical method developed involved a pre-column reduction of disulfides to thiamine with cysteine followed by chromatographic analysis of the thiaminic residues [9–11]. A polarographic determination of thiamine disulfide in combination with other vitamins has also been described [12–13]. A colorimetric method for the determination of thiamine disulfides, involving derivatization with 2,6-dichloro-*p*-benzoquinone-4-chlorimine has also been published [14]. The disadvantages of these techniques were the poor sensitivity obtained or the non-specificity of the analysis, due to the off-line reduction of disulfides to thiamine derivatives.

This paper describes a new HPLC method for a sensitive simultaneous determination of sulbutiamine (I) and its main derivatives: thiamine disulfide (II) and monoisobutyryl thiamine disulfide (III) in human plasma (see Figure 1). The method involves solid-phase extraction of plasma samples, liquid chromatographic separation of the three disulfides, post-column reduction and oxidation followed by fluorimetric detection of the resulting thiochromes. Optimization of post-column reaction parameters is described and results of analytical validation are presented.

Experimental

Materials and Reagents

Sulbutiamine (isobutyrylthiamine disulfide) and monoisobutyryl thiamine disulfide were obtained from Technologie Servier (Orléans, France). Thiamine di-

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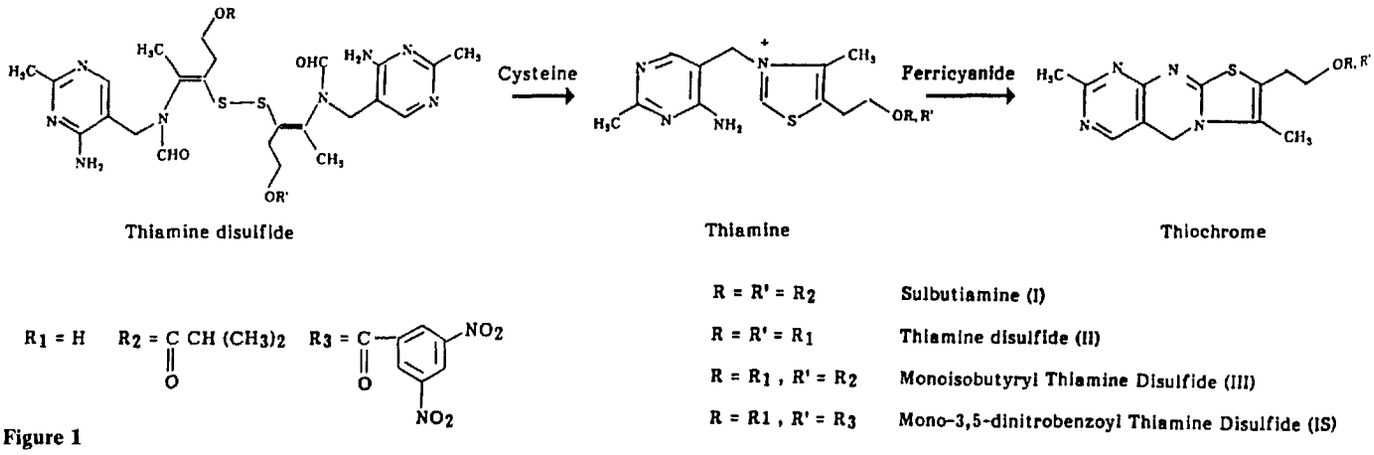

Figure 1
Reduction and oxidation mechanisms of thiamine disulfide derivatives.

sulfide was supplied from Aldrich (Strasbourg, France). Mono-3,5-dinitrobenzoyl thiamine disulfide was obtained from thiamine disulfide by derivatization with 3,5-dinitrobenzoyl chloride [15], Technologie Servier (Orléans, France). L-cysteine hydrochloride was purchased from Sigma (La Verpillière, France). Potassium hexacyanoferrate (III) was obtained from E. Merck (Darmstadt, Germany). All other reagents were of HPLC grade.

Chromatographic System

The chromatographic apparatus consisted of a gradient HPLC system from Beckman (Gagny, France) equipped with a model 126 pump, a model 507 autosampling injector with a 200 µl loop and a cooling module (4 °C). Data acquisition were done on Gold System software from Beckman. Detection was achieved with a programmable fluorescence detector F 2000 from Hitachi (B. Braun Sciencetec, les Ulis, France), with an 18 µl flow cell. The excitation wavelength was set at 365 nm and the emission monitored at 433 nm, with 10 and 20 nm band pass respectively. An RP select B analytical column (125 × 4 mm i.d., particle size 5 µm; Hewlett-Packard, les Ulis, France) was used at a constant temperature of 40 °C. Mobile phase consisted of a gradient of methanol and 0.011 M potassium dihydrogen phosphate buffer at pH 4.5 (Table I). The flow rate was 1 ml · min^{-1}.

Post-column reduction of disulfide compounds was carried out in a coiled polyether ether ketone (PEEK) capillary reactor coil (9 m × 0.5 mm i.d.) heated at 70 °C (SHP99 heating module, Chrompack, les Ulis, France), with an aqueous cysteine solution (0.5 g of cysteine dissolved in 1 l of 0.033 M phosphate buffer, pH 7) which was delivered with a Beckman pump (model 126) at a flow rate of 0.2 ml · min^{-1} and mixed with the eluate stream in a t-piece (0.5 mm hole). Oxidation of thiamine residues was with an aqueous solution of hexacyanoferrate (III) (1.42 g · l^{-1} K_3 [Fe $(CN)_6$], 0.004 M in aqueous 2 M sodium hydroxide) delivered with a Waters pump (M 510) at 0.2 ml · min^{-1}. The reactor consisted of a coiled PEEK capillary (3 m × 0.25 mm i.d.). Both post-column reagents are stable for 48 hours in the dark. A flow diagram of the chromatographic system is given in Figure 2. The reduction recovery was calculated by comparison of peak areas of pure thiamine disulfide and thiamine solutions, injected in to a C1 column (150 × 4.6 mm, 5 µm) purchased from Interchim (Paris, France) and eluted with an isocratic mobile phase of 0.011 M potassium dihydrogen phosphate buffer and methanol (65/35, V/V), at 1 ml · min^{-1}. Post-column reaction systems were the same as described previously.

Table I. Gradient elution profile used for thiamine disulfide separation.

t (min)	Methanol (%)	Phosphate buffer (%)
0	10	90
5	10	90
10	35	65
30	62	38
35	10	90
50	10	90

Sample Preparation

Stock solutions of sulbutiamine and its analogues were prepared in methanol at a concentration of 1 mg · ml^{-1}. A stock solution of the internal standard was prepared in acetonitrile at a concentration of 1 mg · ml^{-1}.

These stock solutions were stored at + 4 °C and kept for four weeks. Standard solutions of the drug and its derivatives were prepared from the corresponding stock solutions by dilution in purified water to a concentration of 100 µg · ml^{-1}, and stored at + 4 °C for one week. Each working day, a standard solution of the internal standard was also prepared by dilution of the stock solution in purified water.

The solid-phase extraction procedure was achieved with an automated Benchmate system from Zymark (Roissy, France), using a non-polar bonded sorbent

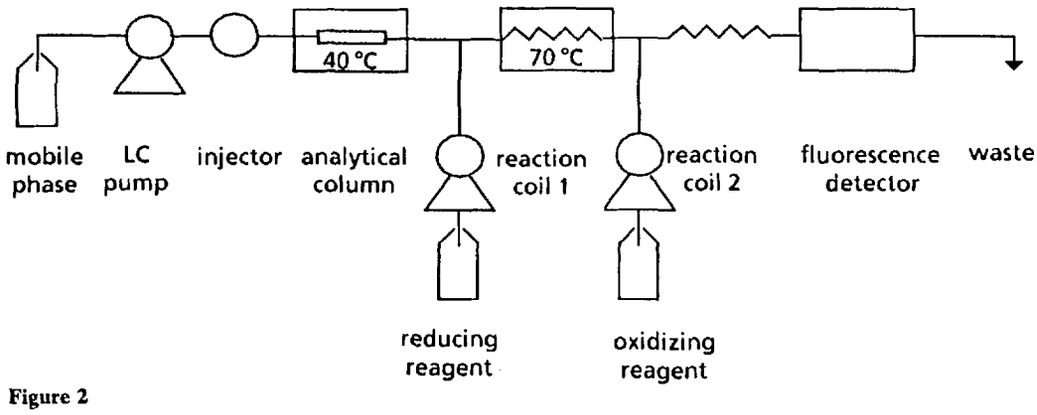

Figure 2
Flow diagram of the chromatographic system.

octadecyl Amprep purchased from Prolabo (Paris, France). All the extraction procedure was protected from direct light to minimize possible degradation of sulbutiamine.

Before extraction, plasma was centrifuged at 1500 g for 10 min. An aliquot of plasma (0.8 ml) was transferred into a 15 ml tube and spiked with 25 µl of the internal standard solution (2 µg · ml^{-1}). After adjustment to pH 1.5 with 200 µl of 1 M hydrochloric acid, the plasma solution was carried through the cartridge which had been previously conditioned with 3 ml of acetonitrile followed by 3 ml of 0.1 M nitric acid. The cartridge was then successively washed with 2 ml of purified water and 2 ml of acetonitrile. Elution was performed with 5 ml of a mixture of aqueous trichloroacetic acid (0.6 %, V/V) and acetonitrile (10/90, V/V). The eluate was then evaporated to dryness under a nitrogen stream, and 300 µl of the chromatographic eluant, 0.011 M phosphate buffer/methanol (90/10 V/V) were added to the extract. 100 µl were injected in the chromatographic system.

Results

On-Line Post-Column Oxidation and Reduction

A double post-column reaction was involved for the detection of disulfide compounds. Thiamine disulfides were first reduced with a cysteine solution to free thiamine derivatives, which were then oxidized to the corresponding thiochromes with ferricyanide (Figure 1).

Oxidation conditions previously described for thiamine oxidation into thiochrome [16–19] in a basic medium were used. Reduction of disulfides to thiol compounds was achieved in a very short time with a cysteine solution in a neutral medium. In fact, a pH of 7 for the cysteine reagent allowed both a high reduction recovery and a quantitative oxidation (Figure 3).

The optimum concentration of cysteine solution was set between 0.5 and 1 g · l^{-1} (Figure 4). Below 0.5 g · l^{-1}, the reduction efficiency decreased and concentrations above 1 g · l^{-1} could lead to an insolubility of both reduction and oxidation reagents, resulting in inhibition of the oxidation.

The effect of temperature in the range 40 °C–70 °C upon the reduction efficiency is shown in Figure 5. A temperature of 70 °C was selected; higher temperatures would have given higher reduction efficiency, but would have induced problems such as degasing in the detector flow cell. Under these conditions, reduction recovery was estimated at 90 %, based on the comparison of responses of thiamine disulfide and free thiamine solutions in the chromatographic system.

As these post-chromatographic on-line reactions gave a highly selective response for thiamine disulfides, an analogue of the disulfide compounds, 3,5-dinitrobenzoyl thiamine disulfide, was chosen as internal standard.

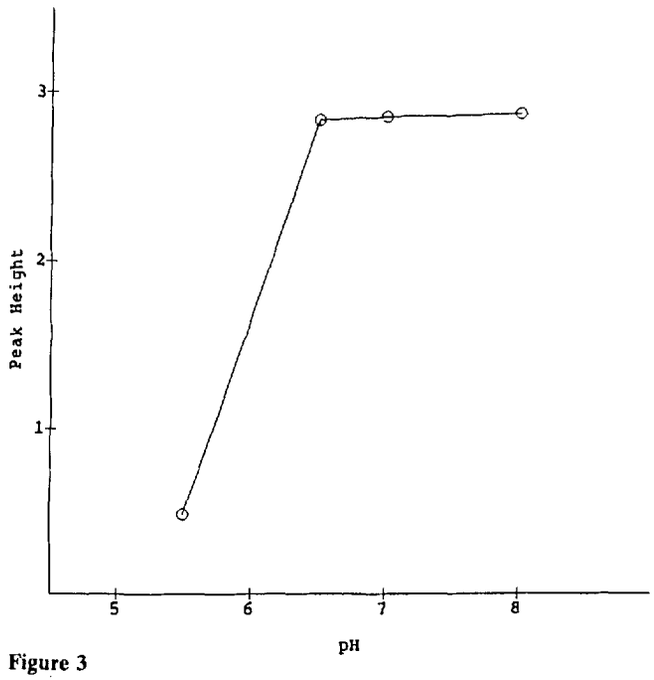

Figure 3
Effect of cysteine reagent pH on the reduction efficiency of thiamine disulfide.

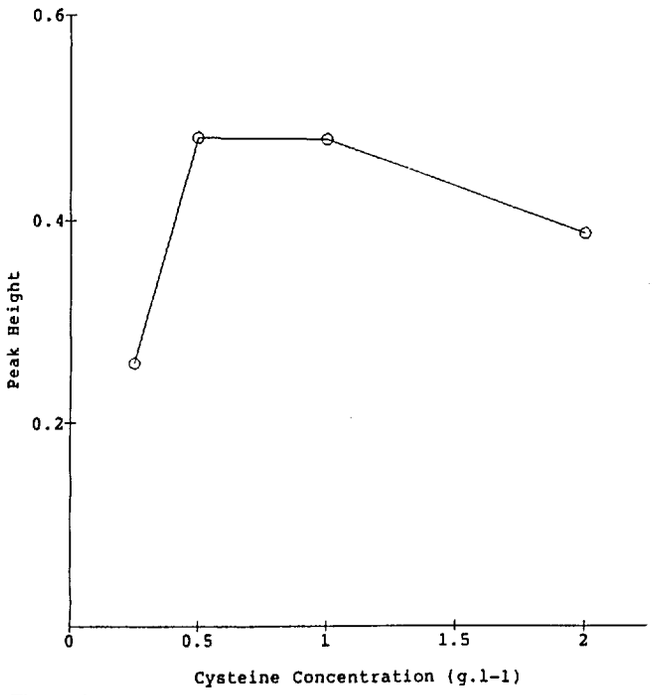

Figure 4

Effect of cysteine concentration on the reduction efficiency of thiamine disulfide.

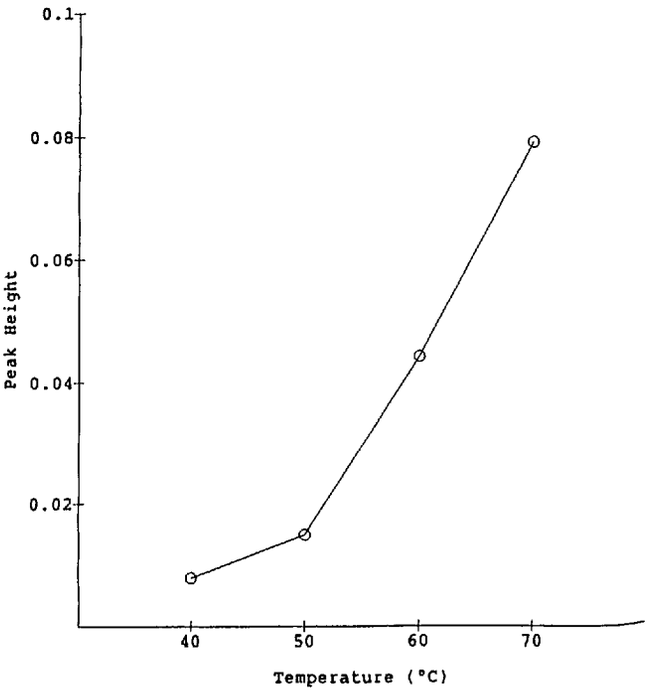

Figure 5

Effect of cysteine reagent temperature on the reduction efficiency of thiamine disulfide.

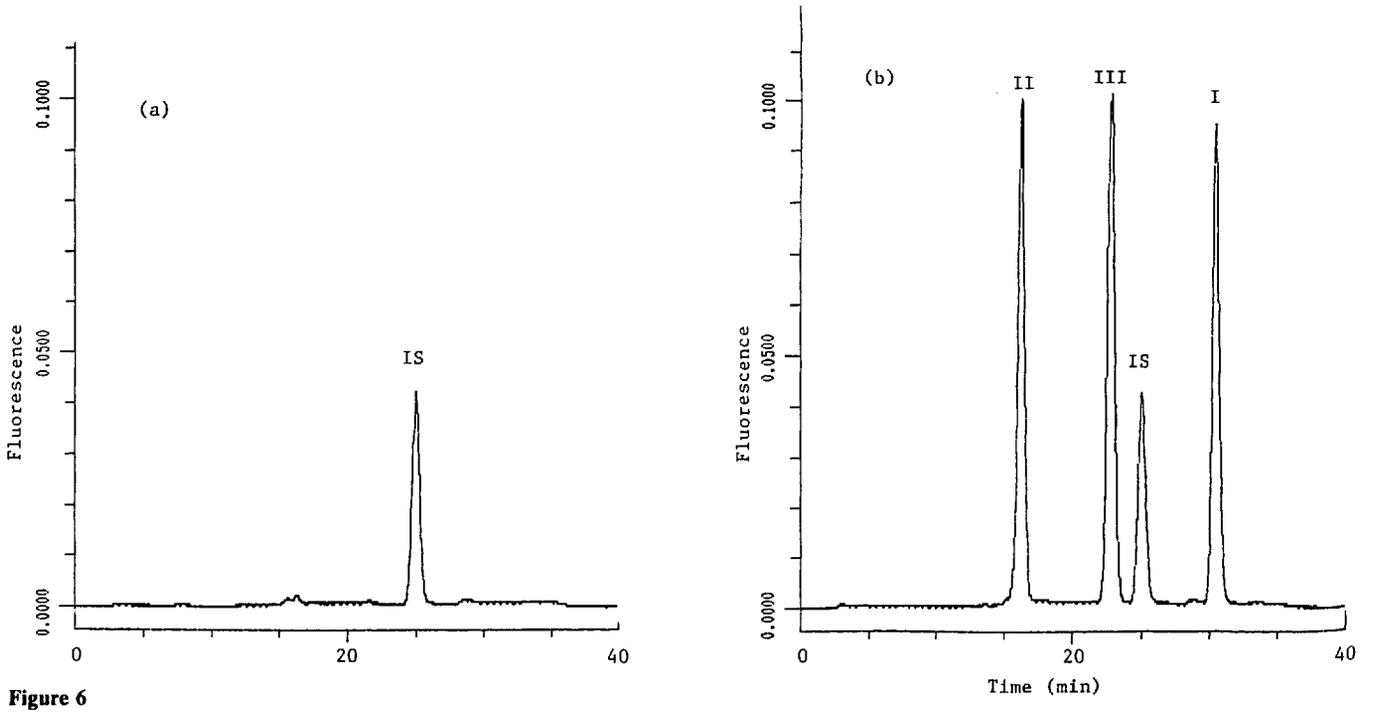

Figure 6

Chromatograms of human plasma extracts obtained from (a) blank plasma spiked with the internal standard (IS) (62.5 ng · ml^{-1}), and (b) plasma spiked with sulbutiamine (I), thiamine disulfide (II), monoisobutyryl thiamine disulfide (III) and the internal standard (IS) (100 ng · ml^{-1} for each compound).

Chromatographic Analysis

Separation of the different thiamine disulfides was achieved on an RP Select B column with a linear gradient of methanol and phosphate buffer. The gradient elution profile was optimized in order to permit an efficient separation of the compounds without affecting the post-column reactions. Thus methanol percentage was maintained below 65 % in order to avoid precipitation of post-column reagents.

Typical chromatograms obtained from plasma samples spiked with the three disulfide derivatives and the internal standard are shown in Figure 6.

The compounds were eluted in the order of their hydrophobic properties. Thiamine disulfide was detected at 16.3 min, monoisobutyryl and mono 3,5-dinitrobenzoyl thiamine disulfides were eluted at 22.8 min and 25.0 min. Sulbutiamine which is more hydrophobic had a retention time of 30.5 min.

Extraction Procedure

Previous methods of determination of thiamine disulfides used plasma deproteinisation with trichloroacetic acid as sample pretreatment [1, 10, 11]. Although this procedure allowed an efficient purification step, the large volume of acid needed limited the sensitivity due to the volume of the extract. A novel extraction procedure has been studied, involving solid-phase extraction on a non-polar C18 bonded sorbent. The three hydrophobic compounds were adsorbed on the C18 cartridge in an acidic medium. The pH of the plasma was thus set at 1.5 with hydrochloric acid. Below this pH, slight degradation of sulbutiamine was observed, and above pH 2, the extraction was less efficient from human plasma. Elution was performed with acidic acetonitrile, that allowed an efficient desorption of the three compounds and of the internal standard. After evaporation of the organic phase, the extract, dissolved in the mobile phase, could be directly injected in the chromatographic system. Figure 6 shows the selectivity of the extraction procedure, with no interfering peaks having been detected in five different human plasmas. An extraction of plasma samples containing free thiamine and thiamine phosphates showed that these compounds did not interfere in the chromatographic separation.

The absolute extraction recovery was greater than 70 % for the three disulfides (Table II), calculated by comparing the slopes of one calibration curve established in human plasma and a standard curve established in a pure water solution. Extraction recovery of the internal standard was 92 %, based on the comparison of responses of six replicates of the compound spiked in human plasma and in pure solution at the concentration level used in the calibration.

Precision, Accuracy and Linearity

Standard curves were constructed by plotting the peak height ratio of each compound to the internal standard versus the concentration added. A weighted least squares regression analysis was used, with a weight proportional to the inverse of the square of the estimated ratio. In this way, linearity was demonstrated over the range 5–200 ng · ml^{-1} in plasma for each disulfide derivative (Figure 7). The mean slope obtained during 5 different batches was respectively of 0.025 (CV = 7 %) for sulbutiamine, 0.033 (CV = 16 %) for thiamine disulfide and 0.030 (CV = 5 %) for monoisobutyryl thiamine disulfide.

Table II. Absolute solid-phase extraction recovery in human plasma.

Compound	Recovery (%)
I	71
II	78
III	72
IS (n = 6)	92

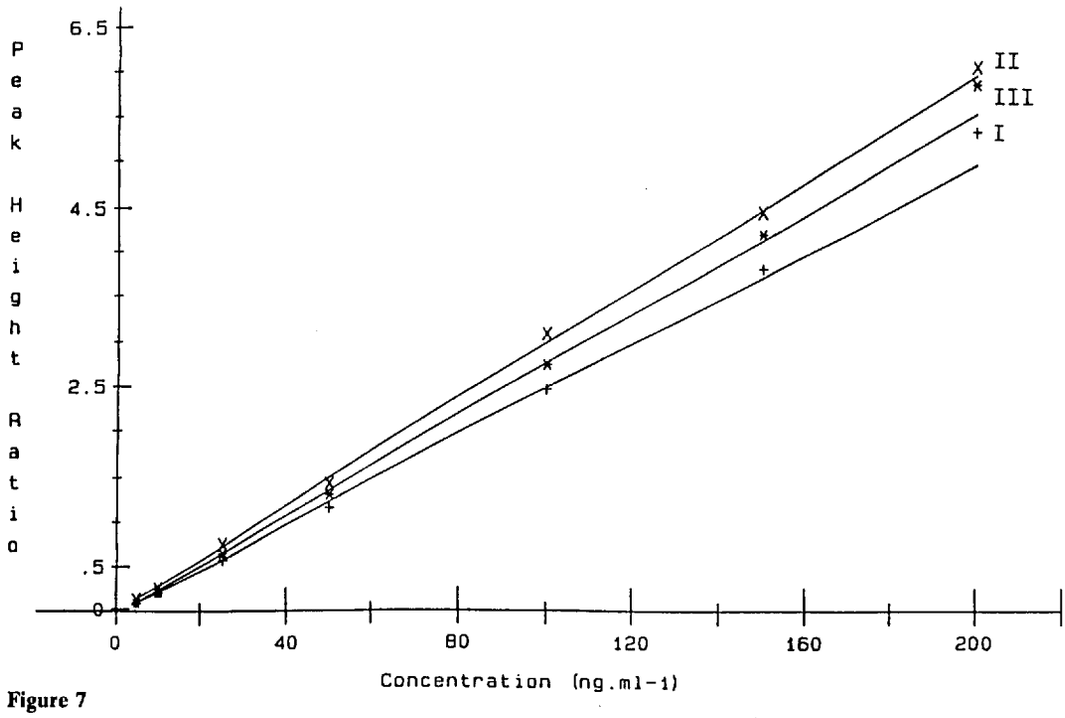

Figure 7

Calibration curves obtained from human plasma in the range 5–200 ng · ml^{-1} for (+) sulbutiamine (I); (×) thiamine disulfide (II) and (*) monoisobutyryl thiamine disulfide (III).

The quantitation limit was set at 5 ng · ml^{-1} for each thiamine disulfide derivative based on a series of four experiments (Table III). The detection limit was estimated at 350 to 450 fmoles depending on the compound, based on a signal to noise ratio of 3.

Precision and accuracy of the method were determined by within-day and between-day assays. The coefficients of variation (CV) were calculated from analysis of different control samples for each assay (Table IV).

Stability of Disulfide Compounds

The stability of the compounds was assessed under different storage conditions. The three compounds proved to be stable in human plasma after one freeze and thaw cycle, and during the solid-phase extraction procedure for at least 28 hours at room temperature (Figure 8). Moreover, the stability of plasma extracts containing the disulfide derivatives was not affected during 24 hours at + 4 °C in the autosampler.

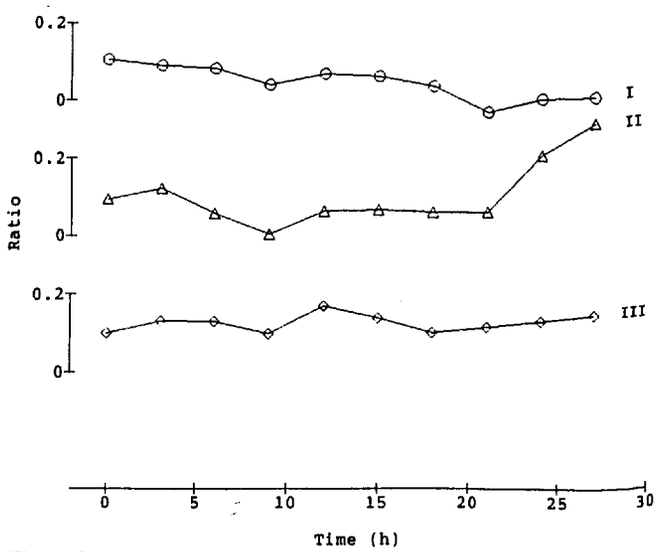

Figure 8

Stability of thiamine disulfides derivatives in human plasma during the extraction procedure (28 h, ambient temperature; (o) sulbutiamine (I); (Δ) thiamine disulfide (II) (◊) monoisobutyryl thiamine disulfide (III).

Conclusion

A novel and direct liquid chromatographic method for simultaneous determination of thiamine disulfide compounds has been developed. This technique, involving on-line post-column reduction and oxidation, allows a highly selective detection of thiamine disulfides. Moreover, this technique is highly sensitive since the quantitation limit is 5 ng · ml^{-1} in human plasma for sulbutiamine and its derivatives. Precision and accuracy of the method have been demonstrated with intra and inter-assays. The efficiency of the solid-phase extraction procedure allows quantitation in human plasma over the concentration range 5–200 ng · ml^{-1}. The stability of each disulfide compound has been observed under different storage conditions.

This method can be applied to the determination of sulbutiamine and its main disulfide metabolites in human plasma after administration of Arcalion.

Table III. Quantitation limits obtained for each disulfide derivative.

Compound	Concentration (ng · ml^{-1})		CV (%)
	added	found (n = 4)	
I	5.0	5.9	6.0
II	5.0	6.1	1.0
III	5.0	5.6	7.0

References

[1] *H. Nogami, J. Hasegawa, S. Nakatsuka, K. Noda,* Chem. Pharm. Bull. **17**, 219 (1969).

[2] *L. Bettendorff, L. Weekers, P. Wins, E. Schoffeniels,* Biochem. Pharmacol. **40**, 2560 (1990).

[3] *R. Du Boitesselin,* Gazette Médicale **95**, 21 (1988).

[4] *L. Crocq, J. Fondarai, C. Nathan,* Psychol. Med. **10**, 2103 (1978).

Table IV. Within-assay and inter-assay reproducibility in human plasma.

Compound	Intra-assay			Inter-assay	
	concentration added (ng · ml^{-1})	concentration found (n = 7)	CV (%)	concentration found (n = 5)	CV (%)
I	10.0	10.6	6	10.2	8
	50	48	4	47	5
	100	102	2	102	7
II	10.0	9.9	8	10.1	6
	50	51	5	46	6
	100	118	5	96	4
III	10.0	10.2	5	10.0	6
	50	51	3	47	4
	100	104	5	99	6

[5] *J. Micheau, T. P. Durkin, C. Destrade, Y. Rolland, R. Jaffard,* Pharmacol. Biochem. Behav. **23**, 195 (1985).
[6] *E. Balzamo, G. Vuillon-Cacciuttolo,* Rev. EEG Neurophysiol. **12**, 373 (1982).
[7] *J. W. I. Brunnekreeft, H. Eidhof, J. Gerrits,* J. Chromatogr. **491**, 89 (1989).
[8] *L. Bettendorff,* J. Chromatogr. **566**, 397 (1991).
[9] *V. Ventura, E. Giacolone, G. Sciorelli,* Int. Z. Vitamforsch. **36**, 286 (1966).
[10] *N. Itada,* J. Vitam. **5**, 61 (1959).
[11] *R. Rindi, V. Perri,* Int. Z. Vitamforsch. **32**, 398 (1962).
[12] *Y. Asahi,* J. Vitam. **4**, 118 (1958).
[13] *T. Vergara, D. Marin, J. Vera,* Anal. Chimica Acta **120**, 347 (1980).
[14] *M. V. S. Krishnan, S. N. Mahajan, G. R. Rao,* Analyst **101**, 601 (1976).
[15] *J. Lehrfeld,* J. Chromatogr. **120**, 141 (1976).
[16] *J. P. M. Wielders, C. J. K. Mink,* J. Chromatogr. **277**, 145 (1983).
[17] *B. L. Lee, H. Y. Ong, C. N. Ong,* J. Chromatogr. **567**, 71 (1991).
[18] *S. Sander, A. Hahn, J. Stein, G. Rehner,* J. Chromatogr. **558**, 115 (1991).
[19] *J. Schrijver, A. J. Speek, J. A. Klosse, H. J. M. Van Rijn, W. H. P. Schreurs,* Ann. Clin. Biochem. **19**, 52 (1982).

Received: Sep 14, 1992
Accepted: Oct 14, 1992