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Journal of Chromatography A, 881 (2000) 267–284

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Review

Determination of thiamine by high-performance liquid chromatography

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Abstract

High-performance liquid chromatographic methods for the determination of thiamine (vitamin B_1) in foodstuffs or biological tissues and fluids are outlined and discussed. The methods are often similar and interchangeable, sample extraction and clean up procedures being the major difference. Most of the methods use either ultraviolet or fluorescence detection. Fluorescence detection requires either precolumn or postcolumn oxidation of thiamine to thiochrome. A number of methods are recommended and problems with standardization are emphasized. Crown copyright © 2000 Published by Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Food analysis; Thiamine; Vitamins

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1. Introduction

Thiamine, a pyrimidyl substituted thiazole [3-(4-amino-2-methyl-pyrimidyl-5-methyl)-4-methyl-(β-hydroxyethyl)-thiazole], was first isolated in 1936 by Williams and Cline [1]. Of the 30 mg of thiamine in the average adult approximately 80% is in the form of thiamine diphosphate (pyrophosphate). The remainder consists of triphosphate, monophosphate and free thiamine [2].

Thiamine diphosphate plays a vital role in carbohydrate metabolism, being a coenzyme in three

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PII: S0021-9673(00)00089-3

major enzyme complexes [3]; the pyruvate dehydrogense complex, an assembly of three enzymes, catalyses the oxidative decarboxylation of pyruvate in the formation of acetyl CoA. The α-ketoglutarate dehydrogenase complex, structurally similar to the pyruvate complex, catalyses the oxidative decarboxylation of α-ketoglutarate [3,4]. Transketolase, in tandem with transaldolase, provides an important link between the pentose phosphate pathway and glycolysis [4]. Thiamine diphosphate, thus, plays a vital role in both major carbohydrate pathways. It has also been shown recently that thiamine triphosphate may have an as yet unidentified role in nerve tranduction [5,6]. Thiamine monophosphate and free thiamine are thought to be simple intermediates with as yet no specific role found.

The activity of the transketolase enzyme in erythrocytes is still used as an indicator of thiamine status [3]. Generally, a lower than normal basal enzyme activity coupled with an enhancement of activity after the in vitro addition of excess thiamine diphosphate (known as the thiamine diphosphate effect) suggests thiamine deficiency. It was recently proposed that the ratio of the two be used as an index [7]. The transketolase assay, being an indirect marker of thiamine status, does have a number of limitations. It is difficult to standardize [8] and liver disease can impair both apoenzyme synthesis and phosphorylation [9]. Certain apoenzyme variants have been shown to possess lower affinities leading to lower thiamine diphosphate effects [10]. Prolonged thiamine deficiency may result in lower apoenzyme levels and hence the thiamine diphosphate effect may be blunted [11], so that low values may not accurately reflect true thiamine concentrations but rather low apoenzyme levels [12]. It has been suggested that such difficulties in interpretation may have led to an overestimation of the prevalence of thiamine deficiency in alcoholics [13]. Concerns over low throughput and poor precision, have been partly resolved thanks to recent improvements in the assay [14], but despite these improvements the most appropriate method for the determination of thiamine status is the direct measurement of erythrocyte thiamine by high-performance liquid chromatography (HPLC) [9]. Erythrocyte thiamine is usually assessed since 80% of thiamine is stored there, mostly as thiamine diphosphate, while 10% is stored in leucocytes the rest in plasma. Kuriyama et al. showed that the total thiamine level in blood was significantly lower in beriberi patients than in normal controls [15]. Warnock et al. showed that erythrocyte thiamin diphosphate levels fell before changes in tranketolase activity occurred [16].

The four chief sources of dietary thiamine and their relative contribution to the total daily intake are whole grain cereals and cereal products (50%), vegetables (20%), meat (10%) and milk products (10%) [11]. Polished rice, sugar, alcohol, fat and other refined foods are poor sources [3]. Cooking may result in substantial loss of thiamine. Ayranci and Kaya, investigating the loss of thiamine of up to 50% during the cooking of macaroni, showed it was partly due to thermal breakdown but mostly due to leaching of the vitamin into the cooking water [17]. Cooking losses are greatest following the boiling of rice and green vegetables. For example, spinach may lose up to 90% of its thiamine content, especially if the water used has a high pH and contains high levels of chlorine [18]. Thiamine is mostly preserved after frying [19] and microwaving [18], due to the short transit times. Tea and raw fish contain antithiaminases that can hydrolytically destroy the vitamin in the gut [20].

The average daily intake of thiamine is 0.5 mg. When the daily intake of thiamine is less than 5 mg it is readily absorbed in the small intestine via an active carrier mediated transport system. If larger amounts are consumed passive diffusion occurs [21,22]. Phosphorylation to form thiamine diphosphate occurs in the jejunal mucosa, liver and kidney [21].

Thiamine deficiency is relatively rare in western society and mostly seen in alcoholics due to a combination of poor diet and inhibition of thiamine absorption in the gut by ethanol [23–27]. Ethanol inhibits the carrier mediated active process at low concentrations but not the diffusion at higher concentrations [21]. It is also relatively common with chronic vomiting especially hyperemesis gravidarum [28–31], gastrointestinal disease [3], anorexia [3], total parenteral nutrition [32–39], and in the elderly [40,41]. Rarer associations include patients with chronic febrile infections such as malaria [42], multiple sclerosis [43], epilepsy [44], lymphoblastic leukaemia [45], schizophrenia especially with con-

current alcoholism [46], AIDS [47,48] and Alzheimer's disease [49–54]. Patients with chronic heart failure on long term treatment with diuretics, especially furosemide, have been shown to be susceptible to mild deficiency leading to impairment of cardiac function [55–59]. The mechanism is believed to be urinary loss [60] in combination with reduced thiamine uptake in cardiac cells [58]. Chemotherapy and radiotherapy can also cause deficiency, although thiamine supplementation in malignancy is controversial since thiamine can increase tumour growth [61–64].

Due to its relatively short storage time, marginal thiamine deficiency can occur within 10 days and more severe deficiency within 21 days if intake is stopped [2]. Symptoms of mild thiamine deficiency in adults include poor sleep, malaise, weight loss, irritability and confusion. Newborns, breast fed from deficient mothers, may develop dyspnea and cyanosis. Diarrohea, vomiting, weight loss, and aphonia may follow. A number of rare thiamine responsive inborn errors of metabolism have been described [65–67].

The two classical conditions resulting from severe thiamine deficiency are beriberi and Wernicke's encephalopathy (known as Wernicke–Korsakoff syndrome in the USA). Three forms of beriberi are described. Symptoms of dry Beriberi include poor appetite, fatigue and peripheral neuritis. Patients with wet beriberi have cardiac failure and oedema. Very severe beriberi is often referred to as Shoshin beriberi [68,69]. Patients who develop Wernike's encephalopathy show central nervous system manifestations such as ataxia, double vision, nystagmus and drooping eyelids [70]. They can also show signs of Korsakoff's psychosis, i.e., intelligence disturbance, apathy, recent memory loss and confabulation. Interestingly, the response of individuals to thiamine deficiency is genetically determined. Most patients do not develop a severe form of Wernike's encephalopathy, just some loss of higher function; however all can develop beriberi. Saltwater crocodiles [71] and alpacas [72] may also develop the neurological signs of thiamine deficiency!

The response to thiamine administration in deficient patients is usually rapid, hence clinical improvement post thiamine replacement may be used to make a diagnosis of thiamine deficiency. In such cases routine laboratory measurements are seldom necessary. It may, however, be useful to formally document low concentrations especially for research purposes and in conditions not usually associated with thiamine deficiency. Potential problems with the former approach may occur if a patient fails to respond to thiamine administration. This may be seen when oral forms of the vitamin are given to patients with malabsorption [34] or with accelerated thiamine breakdown in total parenteral nutrition solutions caused by bisulphite containing amino acids [32]. For the same reason, sulphur dioxide, often used in the preservation of ''fresh'' pet food, can cause severe deficiency in animals even with concurrent thiamine administration [73].

Although no conditions are caused by thiamine excess rare anaphylactic reaction to thiamine administration has been reported [74].

The nutritional labelling of food introduced by governments and regulatory bodies around the world has led to an increased demand for cheap, fast, accurate and precise methods for the determination of nutrients and vitamins in food, pharmaceutical preparations, multivitamin products and infant formulas. Many methods for the determination of thiamine in such matrixes have been described, by far the most popular being those employing HPLC. Many techniques for the measurement of thiamine in biological fluids and tissues have also been described. The techniques are often similar and interchangeable, with the initial sample preparation and clean up procedure being the only difference. For the purposes of this review methods have been divided into those measuring thiamine in food, multivitamin preparations and other medical products and those measuring thiamine in biological fluids and tissues.

2. HPLC methods

2.1. Early developments

Chromatographic separation of thiamine compounds is not new. Levin and Wei developed a microassay for thiamine and its phosphate esters after separation by paper chromatography in 1966 [75]. Thin-layer techniques have also been used [76,77]. Practical use of thin-layer chromatography

for the quantitative determination of thiamine in pharmaceutical preparations for quality control purposes was described in the early 1970s [78,79]. Gas chromatography–mass spectrometry analysis of thiamine was also outlined in the early 1970s [80,81].

In 1973 Van de Weerdhof et al. elegantly described the first application of liquid chromatography for the analysis of thiamine [82]. The assay used a 25×0.3 cm glass column containing 20–30 μm particle diameter silica gel. The elution buffer was 0.1 *M* phosphate with 10% ethanol. Postcolumn oxidation of thiamine to thiochrome using potassium hexacyanoferrate [$K_3Fe(CN)_6$] allowing fluorescent detection was used. The method was used to determine thiamine concentrations in a number of food samples. Prior to their application to the column food samples (potatoes, meat and vegetables) were hydrolysed by treatment with sulphuric acid and the enzymes takadiastase and papain; trichloroacetic acid precipitated any protein. This precolumn enzymatic hydrolysis of thiamine phosphate esters to their free form allowed determination of the total thiamine content of the sample. All of the HPLC methods subsequently described are more or less modifications based upon this pioneering method.

The alkaline oxidation of thiamine leading to the formation of the highly fluorescent thiochrome compound used by Van de Weerdhof et al. was first described by Berger et al. in 1935 [83]. Under the conditions described the oxidation reaction is rapid and complete in less than 15 s [84]. The reaction was the basis for what is now described as the manual thiochrome assay for thiamine analysis [85,86]. The manual assay is, however, prone to error since no separation of interfering compounds causing non specific fluorescence occurs, hence the advantage of HPLC. A number of oxidizing reagents may be used for the reaction. Potassium ferricyanide appears to be the most popular. Although more hazardous, others have used cyanogen bromide (BrCN). Mercuric chloride ($HgCl_2$) although not as stable as $K_3Fe(CN)_6$ provides greater fluorescence intensity.

In 1979 Gubler and Hemming illustrated the potential of HPLC by outlining two methods for the separation of simple mixtures of thiamine and its phosphate esters [87]. The first method was an anion-exchange technique using a Vydac column with phosphate buffer gradient elution followed by both UV and fluorescent detection. The effluent from the UV detector was fed into a postcolumn $K_3Fe(CN)_6$ oxidation system and then into a fluorescent detector connected in series. Their second technique used a reversed-phase isocratic ion-pair mode with a 10 μm C_{18} Bondapak column and a 50 m*M* tetrabutylammonium hydroxide in 1% acetic acid mobile phase. They did not apply either method to any more complex samples.

At the same time as Gubler and Hemming, Ishii et al. published a more straight forward precolumn isocratic technique for the separation of simple mixtures of thiochrome and its phosphate esters [88]. A LiChrosorb NH_2 column eluted with acetonitrile–phosphate buffer, pH 8.4 (60:40) with fluorimetric detection was described. The elution order was thiochrome, thiochrome monophosphate, diphosphate and triphosphate. Cyanogen bromide was used to form thiochrome and its esters. They described the excitation and fluorescence spectra of thiochrome and its ester derivatives at pH 8.4. All the compounds showed identical excitation spectra (maximum excitation wavelength 375 nm) but slightly different emission spectra (maximum emission wavelength 432–435 nm). More importantly they found the relative ratio of fluorescence intensity was different for each compound: 100:65:80:85 for thiochrome, thiochrome monophosphate, diphosphate and triphosphate, respectively. In contrast Levin and Wei reported the same values for thiochrome, thiochrome monophosphate and diphosphate [75]. The fluorescence intensity was pH dependent and reached a plateau above 8.5 for thiochrome triphosphate and above 8.0 for the others. It seems therefore appropriate that any quantitative determination of thiochrome and its esters should involve calibration with standard mixtures of all four compounds.

The choice of precolumn or postcolumn thiochrome formation is contentious. Precolumn offers convenience, sharper peaks and better resolution. Although postcolumn requires additional equipment, less caustic mobile phases can be used (pH greater than 8.0 is required for maximum thiochrome fluorescence) hence extending column stability and lifetime. This is particularly important with C_{18} silica based columns. Iwata et al., using precolumn derivitization and by mixing the acidic effluent mobile

phase with an alkaline methanol solution prior to fluorescence detection, were able to improve the stability of the ODS column used in their method [89], although this still required extra equipment.

2.2. Modern methods

2.2.1. Food, multivitamin preparations and other medical products

In 1978 Kirchmeier and Upton first used reversed-phase ion-pair HPLC for the simultaneous determination of niacin, niacinamide, pyridoxine, thiamine and riboflavin in multivitamin blends [90]. An ODS 10 μm column, methanol–acetic acid mobile phase and a sodium hexanesulphonate counter ion was used. Due to the high vitamin levels in such blends and no nucleotide interference, UV spectroscopic detection was sufficiently sensitive and hence thiochrome derivitization was not needed. Indeed, the sample was simply dissolved in mobile phase prior to injection. Walker et al. described the same assay but with the addition of hydroxybenzoic acid as an internal standard [91]. They also emphasized the problem of thiamine instability in vitamin–mineral formulations. In the presence of ferrous and cupric salts thiamine is unstable; addition of a metal chelation agent minimized this problem. Lam et al., using a similar method, but with two UV detectors connected in series, added vitamin C to the panel of vitamins measured [92]. Amin and Reusch also used reversed-phase ion-pair HPLC but with an electronically controlled glass extraction apparatus for their determination of the water soluble vitamins in pharmaceutical preparations [93,94]. Hilker and Clifford used UV detection for their determination of thiamine in fortified breakfast cereals and urine (a curious mixture) [95]. Cereal samples were hydrolysed with HCl and passed through disposable 10 μm C_{18} clean up columns. Urine samples avoided the hydrolysis step. A 5 μm NH_2 Microlab AX5 column, a weak anion-exchange column with a ammonium phosphate buffer, pH 2.85 mobile phase, was used.

Hurst et al. of the Hershey Foods Corporation published a method for the determination of total thiamine in milk chocolate in 1983 [96]. Reversed-phase ion-pair chromatography with a C_{18} column and hexanesulphonic acid counter ion was used with postcolumn oxidation to thiochrome with $K_3Fe(CN)_6$. Samples were defatted with light petroleum, hydrolysed with H_2SO_4, incubated with takadiastase and papain and finally filtered through a small polyamide clean up column prior to injection.

Ang and Mosely used a precolumn, Spherisorb 20 μm silica column, normal-phase technique for the determination of total thiamine in meat [97]. Sample pretreatment was similar to that of Van de Weerdhof [82], however, importantly, HCl was used instead of H_2SO_4 for the acid hydrolysis step. Isobutanol was used to extract the thiochrome and this was injected straight onto the column. For routine work isobutanol extraction is probably best avoided since it is a suspected carcinogen [98]. Ang and Mosely confirmed that pork is a rich source of thiamine with almost seven-times the levels of beef and chicken. Although the authors did not provide a reason for the change from H_2SO_4 to HCl, H_2SO_4 contains sulphites that can cause increased breakdown of thiamine [32,73]. Using this principle, Soliman improved the manual thiochrome method by using benzenesulphonyl chloride to provide a more representative blank sample for the determination of any background fluorescence [99]. Gauch et al., in their HPLC method for determination of B vitamins in milk, suggested better repeatability was possible if the thiochrome reaction was deliberately stopped by the addition of sodium sulphite after a fixed time [100].

Ohta et al. reported a straightforward reversed-phase method to analyse total thiamine content in rice flour [101]. A Nucleosil 5 μm C_{18} column with a pH 2.2 phosphate buffer and postcolumn derivitization with $K_3Fe(CN)_6$ were used. Takadiastase was used for the enzymatic hydrolysis of the rice flour prior to injection. They found typical values for the thiamine level of brown rice of 0.46 mg/100 g, whereas polished rice had concentrations of 0.15 mg/100 g.

Nicholson et al. vividly demonstrated the standardization problems associated with differing HPLC methodology for the determination of thiamine in food [102]. Seven laboratories were sent three samples, two were fortified breakfast cereal and the other a mixture of thiamin, vitamin C and glucose. The results for the simple mixture were acceptable with an interlaboratory relative standard deviation (RSD)

of 6.9%, however the results for the two cereal samples were not (47.2% and 34.1%). Each of the laboratories used different sample preparation procedures which appeared adequate for simple mixtures but not for the extraction of thiamine from complex matrices. The authors concluded that further investigation leading to the standardization of extraction procedures was urgently needed. Since the paper was published in 1984 little appears to have been done in this regard, it would be interesting to repeat a similar study now. No external quality control scheme exists for thiamine determination in food or biological fluids.

Mauro and Wetzel determined riboflavin and thiamine simultaneously in enriched cereal products using an ion-pair method with two fluorescence detectors in series [103]. A μBondapak C_{18} column with a methanol–water–acetic acid mobile phase and a hexanesulphonic acid counter ion was used. Native riboflavin fluorescence was measured in acidic conditions immediately post column, where it is not inhibited. Thiochrome was then produced by $K_3Fe(CN)_6$ oxidation and its fluorescence measured under alkaline conditions using the second detector in series. Wimalasiri and Wills applied a similar system to a broader range of foods [104]. They found that with pure compounds, UV detection gave detection limits of 0.005 μg for both riboflavin and thiamine, while using fluorescence detection the limits were lower, being 0.003 μg for riboflavin and 0.002 μg for thiamine. The difference in the detection limits between UV and fluorescence in more complex matrices would be expected to be higher.

In 1984 Augustin reviewed previous methods and introduced a new ion pair method for the simultaneous determination of thiamine and riboflavin in food [105]. The new method, which was poorly described, used an ODS 5 μm column and a pH 7.5 mobile phase composed of 0.005 *M* tetrabutyl ammoniumphosphate in methanol–water. It was unclear whether pre- or postcolumn derivitization was used.

In 1988 Dong et al. published a comprehensive study into the factors affecting the ion pair chromatography of pure water soluble vitamin mixtures [106]. Using a 3 μm C_8 column the optimum mobile phase was found to be methanol–water (15:85) containing 5 m*M* hexanesulphonate, 1% acetic acid and 0.1% triethylamine at a pH of 3.2. The effect of C_5–C_{10} alkyl sulphonate ion-pair reagents was studied. Thiamine is a cation which ion-pairs with alkyl sulphonates under acidic conditions, the longer chain alkyl sulphonates showed excessive retention times for thiamine and pentanesulphonate showed relatively poor resolution thus hexanesulphonate was chosen. Increasing the pH of the mobile phase resulted in a reduction in retention time for thiamine. The addition of triethylamine not only improved the peak shape of thiamine but also column to column performance thought to be caused by residual silanol groups or trace metals in the column material. C_8 columns were preferable since they yielded comparable resolution in shorter assay times. As expected, 3 μm particles generated higher performance than 5 μm. Ultraviolet detector wavelength programming enhanced analytical sensitivity.

Nicolas and Pfender also used ion pair chromatography for the determination of thiamine in infant formula and milk [107]. Sample pretreatment was simply acid treatment to precipitate protein followed by filtration. A μBondapak C_{18} column with an aqueous mobile phase consisting of 0.05 *M* hexanesulphonate, 1.5% acetic acid and 20% methanol with UV detection was used. They also recommended that due to the nature of ion-pair chromatography certain components of the sample could cause peak distortion. Therefore, quantification should be done with peak areas rather than peak heights. Also, since retention of thiamine is strongly affected by the concentration of the ion pair reagent, alteration of the concentration is a useful tool for separating the thiamine peak from any potential interfering peaks.

Vidal-Valverde and Reche used a mixture of hexane and heptanesulphonate as their ion-pair reagent [108]. Because of their sample clean up, the method was not only applicable to milk but to meat and legumes as well. In the case of legumes HPLC analysis without a previous thorough purification step is not recommended. Polyphenols and other interfering compounds were removed by Amberlite CG-50 resin columns. Prior to this, acid hydrolysis with HCl and treatment with takadiastase were performed on each sample. They found the use of filtration through membrane filters (0.22–0.45 μm) or the use of Sep-Pak disposable cartridges was inadequate.

Chase et al. published a method for the simulta-

neous determination of thiamine, riboflavin and pyridoxine in infant formula [109] and later modified it to allow determination in medical foods [110]. A NovaPak C_{18} column, a mobile phase of water–acetonitrile and an hexanesulphonic acid ion-pair reagent was used. A programmable fluorometric detector was used to detect riboflavin and pyridoxine on the first injection. A switching system followed by a second injection allowed postcolumn derivitization to thiochrome with $K_3Fe(CN)_6$ for the determination of thiamine. Samples were simply extracted with perchloric acid and flitered prior to injection.

Sims and Shoemaker used a more comprehensive clean up procedure in their reversed-phase method for the determination of thiamine and riboflavin in a wide variety of food [111]. Excellent separation was achieved using a μBondapak C_{18} column with an aqueous ammonium acetate–methanol (72:28) mobile phase. No ion-pairing agents were needed; this not only improved reproducibility but sensitivity as well, due to an increase in the quantum yield of thiochrome. Sample clean up included hydrochloric acid hydrolysis and a C_{18} Sep-Pak cartridge step after derivitization with $K_3Fe(CN)_6$. Fluorescence detection using wavelength switching was used. The sample clean up procedure omitted an enzymatic hydrolysis step, but this could easily be included.

Barna and Dworschak used ion-pair HPLC with UV detection for the determination of thiamine and riboflavin in meat [112]. Spare rib, chop, ham and liver were homogenized. Hydrochloric acid hydrolysis was followed by enzymatic digestion with papain, takadiastase and claradiastase. The extracts were then cleaned using Nucleosil C_{18} cartridges. Separation was achieved on a 3 μm Nucleosil C_{18} column with a pH 3.2 phosphate buffer with 16% acetonitrile. Heptanesulphonate was used as the ion-pair reagent. Papain was added to the enzyme mix since it was found that proteolytic treatment led to clearer chromatograms. Even with this additional enzyme the chromatograms were not totally clean; indeed, quantification was based upon calibration using three levels of standard addition. The detection limits were 0.1 and 0.03 μg/ml for thiamine and riboflavin, respectively.

In 1994 Hagg published the interesting results from a study investigating the effect of various commercially available enzymes on the HPLC determination of thiamine and riboflavin in food [113]. Different enzymes, as well as the same enzyme produced by different manufacturers, strongly affected the determination of both vitamins. A precolumn reversed-phase HPLC system consisting of a Bondapak C_{18} column with a methanol–phosphate buffer, pH 7.0 (35:65) was used for the study. Separate runs using different wavelengths for fluorimetric detection were used for each vitamin. Careful sample preparation involved hydrochloric acid hydrolysis followed by enzymatic hydrolysis and sample clean up with C_{18} Sep-Pak cartridges. For thiamine determinations, results with Fluka claradiastase were 38% higher compared to those obtained with Fluka takadiastase. Papain gave a result that was 66% of that obtained with claradiastase. Fluka and Serva takadiastase also gave different results. Since the results using claradiastase were nearest to whose found with an interlaboratory comparison study, it was recommended. Different mixtures of enzymes were not studied, which perhaps would provide the best results. Earlier in 1984 Sauberlich did comment that problems may arise due to between batch variation with takadiastase; indeed, some batches are inactive [114].

Baumgartner et al. investigated the stability of thiamine in total parenteral nutrition solutions, using a Zorbax phenyl column with a phosphate buffer containing 0.01% triehylamine–acetonitrile–50 m*M* sodium octanesulphonate (70:15:15) and UV detection [115]. Samples were simply injected straight on to the column. In their total parenteral nutrition solutions thiamine remained stable for at least 7 days under refrigeration, but it underwent significant degradation (more than 10%) in less than 24 h at room temperature.

Selected methods, including those mentioned above, for the determination of thiamine in foodstuffs are summarized in Table 1. The method outlined by Sims and Shoemaker [111] is recommended, it is a relatively simple reversed-phase technique with a good clean up procedure and is applicable to a wide variety of food. The addition of an enzymatic hydrolysis step should be included.

2.2.2. Biological fluids and tissues

The first HPLC method for the determination of

Table 1
Summary of selected methods for the determination of thiamine in food

Ref.	Sample type	Extraction/clean up	Column	Mobile phase	Detection/derivatization (limit of detection)	Analyte
[90]	Multivitamin preparations	None	10 μm C_{18} ODS	CH_3OH–water (25:75), 1% CH_3COOH, 5 m*M* hexanesulphonate	UV 270 nm (not quoted)	Thiamine
[91]	Multivitamin preparations	None (*p*-hydroxybenzoic acid internal standard)	C_{18} ODS	CH_3OH–water (25:75), 1% CH_3COOH, 3–8 m*M* hexanesulphonate	UV 254 nm (0.2 ng/injection)	Thiamine
[93]	Multivitamin preparations	Electronically controlled CH_3OH, water	5 μm LiChrosorb RP-18	CH_3OH–water (50:50)	UV 254 nm (5.0 ng/injection)	Thiamine
[94]	Multivitamin preparations	Electronically controlled CH_3OH, water	5 μm LiChrosorb RP-18	CH_3OH–water–H_3PO_4–octanesulphonate	UV 254 nm (5.0 ng/injection)	Thiamine
[96]	Chocolate	Light petroleum H_2SO_4 Takadiastase, papain	ES industries M-C_{18}	0.01 *M* H_3PO_4, 0.005 *M* octanesulphonate–CH_3CH_2OH (67:33)	Fluorescence $K_3Fe(CN)_6$ postcolumn (0.8 ng/100 g chocolate)	Total thiamine
[97]	Meat	HCl Papain, takadiastase TCA Isobutanol	20 μm Spherisorb silica	$CHCl_3$–CH_3OH (90:10)	Fluorescence 367/418 nm $K_3Fe(CN)_6$ precolumn (0.05 ng/injection)	Total thiamine
[101]	Rice flour	HCl Takadiastase	5 μm C_{18} Nucleosil	0.01 *M* Na_2HPO_4, pH 2.2–0.15 *M* $NaClO_4$	Fluorescence 375/435 nm $K_3Fe(CN)_6$ postcolumn (not quoted)	Total thiamine
[103]	Enriched cereals	H_2SO_4 Mylase P	C_{18} μBondapak	CH_3OH–water (36:64), 1% CH_3COOH, 0.05 *M* hexanesulphonate	Fluorescence $K_3Fe(CN)_6$ postcolumn (0.28 ng/injection)	Thiamine
[104]	Pork Beef Lamb Cereals	HCl Clarase C_{18} Sep-Pak	10 μm C_{18} μBondapak	CH_3OH–water (40:60), 5 m*M* Pic B6	Fluorescence 360/425 nm $K_3Fe(CN)_6$ postcolumn (0.002 ng/injection)	Total thiamine

[107]	Infant milk formula	HCl Filter	10 μm C_{18} μBondapak (5 μm Zorbax Rx Octyl)	Hexanesulphonate–EDTA–CH_3COOH–CH_3OH–water	UV 248 nm (60 ng/injection)	Thiamine
[108]	Peas, beans, lentils Pork Milk	HCl Takadiastase Filter Amerlite resin C_{18} Sep-Pak columns	10 μm C_{18} μBondapak	Hexanesulphonate–heptanesulphonate– CH_3COOH–CH_3OH–water % difference according to food type	UV 254 nm (0.5 ng/injection)	Total thiamine
[110]	Medical foods	$HClO_4$	C_{18} NovaPak	Hexanesulphonate–CH_3CN–water–H_3PO_4	Fluorescence $K_3Fe(CN)_6$ postcolumn (0.01 ng/injection)	Thiamine
[111]	Whole grains Broccoli	HCl Sep-Pak columns	C_{18} μBondapak	0.5 m*M* $CH_3CH_2ONH_4$–CH_3OH (72:28)	Fluorescence 370/435 nm $K_3Fe(CN)_6$ precolumn (0.5 ng/injection)	Thiamine
[112]	Meat Liver	HCl Papain Takadiastase Claradiastase C_{18} Nucleosil	3 μm C_{18} Nucleosil	0.01 *M* KH_2PO_4, pH 3.0–CH_3CN (84:16) 5 m*M* heptanesulphonate	UV 254 nm (5.0 ng/injection)	Total thiamine
[115]	Total parenteral nutrition solution	None	Zorbax phenyl	0.01 *M* Na_2HPO_4–0.01% $(CH_3CH_2)_3N$– CH_3CN–50 m*M* octanesulphate (70:15:15)	UV 280 nm (50 ng/injection)	Thiamine
[146]	Rodent feed	HCl	Synchro pak SCD-100	CH_3OH–0.05 *M* pentanesulphonate, pH 4.0 (40:60)	Fluorescence 370/470 nm $K_3Fe(CN)_6$ postcolumn (5 pg/injection)	Thiamine

thiamine was published in 1978 when Roser et al. described a normal-phase method for urinary thiamine determination [116]. Precolumn thiochrome formation using $K_3Fe(CN)_6$ and its subsequent fluorescent detection was used. Separation was performed on a LiChrosorb 5 μm column with a methanol–diethyl ether mobile phase. Prior to injection an elaborate sample clean up procedure was employed using Decalso cation-exchange resin columns. The final step of the clean up was isobutanol thiochrome extraction. The sensitivity of the method was 0.03 μg/ml.

In 1979 Ishii et al. applied their isocratic technique, described in Section 2.1, to the analysis of rat brain tissue [117]. The last eluting broad thiochrome triphosphate peak did, however, give rise to problems and the same authors published a more robust reversed-phase method [118]. A Toyo Soda TSK-gel ODS column with a 2.5% dimethylformamide in phosphate buffer, pH 8.5 mobile phase was used. This resulted in a reversing of the elution order to thiochrome triphosphate, diphosphate and finally monophosphate. Thiochrome itself was not eluted from the column under these conditions and a change of mobile phase composition to dimethylformamide–phosphate buffer (50:50) was required. The stability of the column was not reported under these alkaline conditions.

In early 1982 Warnock [119] and Schijver et al. [120] published the first descriptions of the determination of thiamine in blood. Schrijver et al. modified the original LC technique of Van de Weerdhof [82]. Sample preparation included removal of the plasma and buffy coat, washing the erythrocytes with saline, lysing with Steros-SE and protein precipitation with trichloroacetic acid. At this stage an enzymatic dephosphorylation step using takadiastase was included, so that the total thiamine concentration was measured as one peak. Postcolumn oxidation and fluorescent detection was used. Warnock measured thiamine diphosphate levels by precolumn oxidation with BrCN. Sample preparation was different from above; trichloroacetic acid was directly added to the washed erythrocytes and later removed from the aqueous supernatants by repeated washing with diethyl ether. No enzymatic dephosphorylation was used. Separation was achieved with a Bondapak NH_2 column with a methanol–phosphate buffer, pH 7.5 (50:50) mobile phase and fluorescent detection. The method was used to measure erythrocyte thiamine diphosphate in rats given a thiamine deficient diet. After 10 days levels fell from normal baseline levels by 80%. Unfortunately concurrent transketolase measurements were not carried out. Standard mixtures of thiamine and its esters can be separated using this method but endogenous fluorescent compounds mask all but the thiamine diphosphate peak when blood samples are used. Floridi et al. published a similiar technique in 1984 [121].

Kimura et al. published a similar method for the determination of total thiamine in blood to that of Schrijver in 1982 [122]. In their case a Shodex OH Pak column and pure phosphate buffer mobile phase was chosen. In 1983 the same group demonstrated a postcolumn method using a Bondapak C_{18} column for the determination of thiamine and its phosphate esters in blood [123]. Thiamin triphosphate was eluted first followed by diphosphate, monophosphate and finally free thiamine. They found that in whole blood more than 70% of thiamine was in the form of diphosphate, with the monophosphate and free thiamine forms hardly detectable. In erythrocytes only diphosphate and triphosphate esters were found and in plasma low levels of monophosphate and free thiamine but no triphosphate and diphosphate were found. Kimura and Itokawa republished the above method in 1985, measuring the blood thiamine content of small animals [124]. Thiamine triphosphate and thiamine diphosphate were found in rat erythrocytes and larger amounts of thiamine monophosphate and free thiamine were detected in rat plasma than in human. They concluded that the distribution of free thiamine and its esters differs according to species.

In 1984 Bontempts et al. used a gradient elution with methanol–phosphate buffer, pH 8.4 and a 5 μm ODS column reversed-phase technique to investigate thiamine and its esters in nerves and electric organs [125]. They carried out a thorough investigation of thiochrome oxidation by $K_3Fe(CN)_6$, preferring this over the hazardous cyanogen bromide. Under their chromatographic conditions, the concentration of $K_3Fe(CN)_6$ and NaOH, had a strong influence on peak areas and retention times. Final concentrations of $K_3Fe(CN)_6$ had varied from 0.1 to 30 m*M* and

NaOH from 0.04 to 1.9 *M* in previous methods. They found the optimum concentrations were 0.15 m*M* and 1.44 *M* for $K_3Fe(CN)_6$ and NaOH, respectively. They also demonstrated the excellent stability of thiochrome and its esters when stored at room temperature in the dark. Contrary to some earlier reports [88] but in agreement with others [75] they found no difference in the fluorescence quantum yield between thiochrome and its esters. They did attempt to lower the pH of the phosphate buffer in order to increase column stability, but this lead to a loss of sensitivity. The typical distribution of thiamine in the nerve tissue they studied was 70% thiamine diphosphate, 3% triphosphate, 13% monophosphate and 14% free thiamine. Brunnekreeft et al. later published a modification of this method [126]. They again used gradient elution, but with a mobile phase of pH 7.0. At this pH they found fluorescence intensity was 20% lower than the plateau level seen above pH 8.0. They investigated postcolumn addition of sodium hydroxide, but although the signal intensity was increased it was ultimately omitted due to sample dilution and peak broadening.

Baines modified Warnock's earlier method [119] by developing a simpler sample clean up and derivitization procedure [127]. It included thiamine extraction directly into methanol (a mobile phase component) and its subsequent oxidation by $K_3Fe(CN)_6$. They found a similar reference range to Warnock, 130–282 compared to 165–286 n*M*. In subsequent papers [21] they applied their method to measure the thiamine levels of hospitalized alcoholics before and after oral or parenteral replacement. They concluded that except for alcoholics requiring thiamine urgently, for whom the parenteral route is demonstrably quicker at raising tissue levels, oral supplementation achieves the same result as parenteral. They also provided evidence that erythrocyte thiamine diphosphate was at least as sensitive as the thiamine diphosphate effect and more sensitive than transketolase activity in reflecting thiamine uptake and utilization in erythrocytes [9].

Weber and Kewitz, using normal-phase technique, measured total thiamine levels in plasma [128]. Plasma was deproteinized using perchloric acid. Overnight enzymatic hydrolysis using acid phosphatase converted any thiamine esters to their free form, and the thiamine was then oxidized to thiochrome which was then extracted into isobutanol. The isobutanol extracts were then injected directly onto a 5 μm NH_2 LiChrosorb column. The increased sensitivity over previous methods, required for thiamine measurement in plasma, was due to the use of mercuric chloride as the oxidizing reagent and the use of a totally organic methanol–diethyl ether mobile phase leading to less quenching of the thiochrome fluorescence by water. The plasma levels of 91 control subjects ranged from 6.6 to 43 n*M* (median 11.6).

Bettendorff et al. published an extremely sensitive method for thiamine and its phosphate esters in serum [129]. Precolumn derivitization with $K_3Fe(CN)_6$ was followed by isocratic reversed-phase HPLC. A phosphate buffer, pH 8.5–methanol (90:10) was used as the mobile phase for the separation of thiamine esters; for thiamine, due to its higher retention time, tetrahydrofuran was used in place of the methanol. A big advantage of this method was the use of a PRP-1 column. This styrene–divinylbenzene copolymer column is very stable in the pH range 1–13, so that the high pH necessary for optimum thiochrome fluorescence does not cause the rapid column deterioration seen with silica based columns. For 42 normal control subjects the total thiamine concentration in serum was 14.1 n*M* (SD 4.5 n*M*) range 5.9–20.7 n*M*. This level agreed with the levels quoted earlier by Weber and Keewitz [128]. They found that, typically, free thiamine accounts for 84% and thiamine monophosphate for 16% of this total. In agreement with Kimura and Itokawa [124], thiamine triphosphate and diphosphate were undetectable in serum. Kimura et al. [122] gave a total thiamine value for whole blood of 46.2 μg/l corresponding to 130 n*M*. This agrees well with those found by Weilders and Mink [84] (117 n*M*) and Burch et al. using a microbiological method [130] (128–154 n*M*). A major disadvantage of the above Bettendorff et al. method was that two different mobile phases were required. This problem was overcome when the same group modified the method using ion-pair reversed-phase HPLC and applied it to the determination of thiamine and its phosphate esters in cultured neurons and astrocytes [131]. The same column was used, but the mobile phase was sodium phosphate buffer, pH 9.0–25 m*M* tetrabutylammonium hydrogen sulphate with

4% tetrahydrofuran. The change in mobile phase resulted in a reverse of the elution order, with thiamine triphosphate eluting last.

In 1987 Botticher and Botticher described a method for the simultaneous detection of vitamins B_1, B_2 and B_6 in serum and whole blood [132]. They developed a sample pretreatment procedure suitable for all three vitamins. Claradiastase was used for the enyzmatic hydrolysis of serum or haemolysed whole blood. Trichloroacetic acid was added and following centrifugation the sample was passed down an octadecyl solid-phase extraction column. HPLC separation was via a 5 μm C_{18} Novapak column with an amino precolumn; methanol–hexanesulphonic acid (20:80) was used for the mobile phase. No derivitization was required since UV detection was used for both riboflavin and thiamine. In line fluorescence detection was needed for vitamin B_6. The detection limit of the method for thiamine was quoted as 2.0 ng/ml, but the chromatograms produced looked noisy.

Lee et al. published a gradient elution method for the determination of thiamine and its esters using a 5 μm ODS column and postcolumn derivatization with $K_3Fe(CN)_6$ [133]. The use of the gradient system avoided the use of an ion pairing reagent and organic modifier. The two mobile phases were 15 m*M* citric acid, pH 4.2 and 0.1 *M* formic acid, pH 3.2 with 4% diethylamine. Blood and serum samples were deproteinized with perchloric acid, mixed with buffer, centrifuged and injected. The elution order was thiamine, thiamine monophosphate, diphosphate and finally triphosphate. The average total blood thiamine of eight control samples was 95.8 n*M* (range 68.7–122.1). More than 70% was in the diphosphate form, the rest as mono or triphosphate; free thiamine was hardly detected. In serum the mean total level was 12.9 n*M* (range 5.7–23.3); thiamine triphosphate and diphosphate were not detected in serum.

Tallaksen et al. used a precolumn technique to separate thiamine and its esters in blood and serum [134,135]. Sample preparation included deproteinization with trichloroacetic acid and oxidation to thiochrome using cyanogen bromide. A Supelcosil NH_2 column using a 85 m*M* phosphate buffer, pH 7.5–acetonitrile mobile phase was used. A step gradient was programmed: for the elution of thiamine the ratio was 90:10, for thiamine esters the ratio was 60:40. Interestingly, a standard mixture of thiamine and its esters could be separated using the 60:40 buffer, with thiamine eluted first and thiamine triphosphate last. However, in biological samples thiamine was not separated from the solvent front. This method has the advantage over the method of Lee et al. [133] of using the same components in the two mobile phases required. Samples of whole blood and serum from 30 normal adults were analyzed. The following reference ranges were found for 15 males/15 females [n*M*, mean (SD)]. In serum: thiamine, 10.9 (2.9)/16.9 (3.3); thiamine monophosphate, 8.3 (1.5)/3.7 (1.5). In whole blood: thiamin, 29.6 (10.0)/33.4 (10.4); thiamine monophosphate, 9.7 (2.3)/10.9 (5.1); thiamine diphosphate, 121 (29.6)/165 (40.4). They later applied their method to alcoholic patients [136] and to alcoholics with liver cirrhosis [137]. They concluded that in these patients complete blood thiamine status discloses not only deficiency but also impairment of thiamine metabolism, possibly due to altered protein binding, and that thiamine supplementation is effective even in currently abusing patients. Cerebrospinal fluid (CSF) thiamine levels, treated in the same way as serum, were also measured by the same group in a subsequent paper [138]. The levels in CSF were significantly higher than in serum for each compound. The CSF–serum ratio was 2.1 (SD 0.8) for thiamine and 8.3 (SD 4.3) for thiamine monophosphate. Low serum levels correlated well with low CSF levels. These results support the existence of an active transport mechanism from serum to CSF.

In 1990 Bailey and Finglas used a normal-phase HPLC technique to measure total thiamine in blood and tissue [139]. A 5 μm silica LiChrosorb Li60 column with a chloroform–methanol (80:20) mobile phase was used. Hydrolysis using hydrochloric acid, taka amylase and acid phosphatase was followed by oxidation with $K_3Fe(CN)_6$; thiochrome was then extracted with isobutanol and injected. They later used this method to study thiamine indices in adolescents [11], where, contrary to others [9,15,16,24], they found no relationship between erythrocyte thiamine levels and transketolase activity or thiamine diphosphate effect.

Herve et al. published a postcolumn isocratic method for the determination of thiamine and its

esters in erythrocytes in 1994 [140]. A 10 μm μBondapak C_{18} column with a ammonium citrate buffer, pH 4.2–0.4% diethylamine (90:10) in formic acid mobile phase was used. Thiochrome was formed postcolumn using alkaline $K_3Fe(CN)_6$. Erythrocytes were haemolysed and deproteinized with trichloroacetic acid, and the samples were then washed with ether and hexane before injection. Thiamine triphosphate was eluted first followed by diphosphate, monophosphate and then free thiamine. No differences were found between the 28 females and 24 males studied. Total erythrocyte thiamine levels were 186 (SD 30.0) n*M*, which was mainly thiamine diphosphate 176 (28.0), followed by triphosphate 7.0 (6.0), free thiamine 4.0 (2.0) and monophosphate <2. In a subsequent paper the authors compared transketolase activity with erythrocyte thiamine and thiamine ester levels in chronic alcoholics [24]. Thirty-eight percent of alcoholics showed a thiamine deficiency, thiamine diphosphate levels being 90.8 (25.7) n*M*. Interestingly 43% of these deficient patients had a normal transketolase activity, illustrating the greater sensitivity of direct measurement.

Table 2 contains a summary of selected methods for the determination of thiamine in body fluids and tissues. As a routine indicator of thiamine nutriture the authors recommend the measurement of thiamine diphosphate in washed erythrocytes. The method outlined by Baines [127] and later improved by Lynch et al. [145] is adequate for this purpose. For the determination of thiamine and its esters, the isocratic method of Herve et al. should be considered [140]. If postcolumn apparatus is not available the step gradient method of Tallaksen et al. [134,135] is recommended.

2.2.3. Methods applicable to both food and biological fluids

Botticher and Botticher developed a versatile HPLC method for total thiamine in food, body fluids, urine and faeces [141]. The same chromatographic conditions were used for each sample type, including a 5 μm NH_2 Nucleosil column with a phosphate buffer–acetonitrile mobile phase, but different sample preparation was required. Food and faeces were hydrolysed with sulphuric acid and claradiastase. Thiochrome formed using $K_3Fe(CN)_6$ was extracted with isobutanol. Sodium chloride was added to give a ‘‘salting out’’ effect and the samples were then injected after filtering. Trichloroacetic acid precipitation and enzymatic hydrolysis was used for whole blood. Urine was simply oxidized directly to thiochrome, since it does not contain any thiamine esters.

Laschi-Loquerie et al. used a normal-phase technique with a 5 μm LiChrosorb NH_2 column and a mobile phase of methanol–dichloromethane (10:90) for the determination of total thiamine in food, blood and serum [142]. Their straightforward and versatile method included acid and enzymatic hydrolysis with takadiastase for all samples, followed by oxidation to thiochrome with mercuric chloride and extraction into isobutanol. The detection limit achieved was 1 ng/ml. They investigated the influence of the type of anticoagulant on blood thiamine levels. Oxalate and citrate seemed to show a redox reaction, blocking thiochrome formation and resulting in much lower values; heparin and EDTA exhibited the highest values.

Methods for the determination of thiamine applicable to both food and biological fluids are outlined in Table 3.

3. Internal standarization

The vast majority of methods for thiamine determination use external standardisation. However, a number of internal standards have been suggested. The first attempt, by Vanderslice and Huang [143] used amprolium, but it is not ideal due to its structural differences with thiamine. Walker et al. used hydroxybenzoic acid in their method for the determination of thiamine in multivitamin products [91]. Chase et al. also used hydroxybenzoic acid in their method for the simultaneous determination of thiamine, riboflavin and pyridoxine in infant formula [109]. In a subsequent modification, however, it was abandoned due to fluctuations in peak response and interfering peaks [110]. All the methods using hydroxybenzoic acid employ UV detection since it would be unsuitable for fluorescent detection. Sander et al., using both pre- and postcolumn techniques, successfully used chloroethylthiamine as an internal standard [144]. Although it worked well, it is unfortunately not commercially available.

Table 2
Summary of selected methods for the determination of thiamine in biological fluids

Ref.	Sample type	Extraction/clean up	Column	Mobile phase	Detection/derivatization	Analyte
[84]	Animal tissue	TCA Diethyl ether	Shim Pak CLC-ODS	100 m*M* NaH_2PO_4, pH 2.5–CH_3OH (92:8)	Fluorescence BrCN precolumn (NaOH/CH_3OH mixed with column effluent) (0.2 ng/injection)	Thiamine and thiamine esters
[116]	Urine	Decalso columns Isobutanol	5 μm LiChrosorb	CH_3OH–$(CH_3CH_2)_2O$ (22:78)	Fluorescence 360/400 nm $K_3Fe(CN)_6$ precolumn (0.75 ng/injection)	Thiamine
[119]	EDTA whole blood Erythrocytes	NaCl TCA Diethyl ether	5 μm Bondapak NH_2	CH_3OH–0.1 *M* KH_2PO_4 (50:50)	Fluorescence 365/460 nm BrCN precolumn (not quoted)	Thiamine diphosphate
[120]	Heparin erythrocytes	NaCl Takadiastase Sterox SE TCA	10 μm LiChrosorb SI-100	0.04 *M* Na_2HPO_4, pH 6.8, 0.03 *M* KH_2PO_4 –CH_3CH_2OH (87:13)	Fluorescence 367/430 nm $K_3Fe(CN)_6$ postcolumn (0.3 ng/injection)	Total thiamine
[121]	Heparin whole blood Erythrocytes	NaCl TCA	10 μm NH_2 μBondapak	50% 70 m*M* pH7.8 KH_2PO_4–50% CH_3CN	Fluorescence 375/435 nm BrCN precolumn (0.4 ng/injection)	Thiamine diphosphate
[122]	Whole blood	Takadiastase, amylase acid, phosphatase protease TCA	Shodex OH Pak M-414	0.2 *M* NaH_2PO_4	Fluorescence 375/450 nm $K_3Fe(CN)_6$ postcolumn (not quoted)	Total thiamine
[123]	Erythrocytes	TCA	10 μm μBondapak NH_2	0.2 *M* NaH_2PO_4–CH_3CN (99.7:0.3)	Fluorescence 375/450 nm $K_3Fe(CN)_6$ postcolumn (0.01 ng/injection)	Thiamine and thiamine esters
[124]	Human, animal whole blood	TCA	μBondapak C_{18}	0.2 *M* NaH_2PO_4–CH_3CN (99.7:0.3)	Fluorescence 375/450 nm $K_3Fe(CN)_6$ postcolumn (0.01 ng/injection)	Thiamine and thiamine esters
[125]	Nerves Electric organs	TCA Diethyl ether	5 μm Ultrasphere ODS	Gradient 25 m*M* NaH_2PO_4, pH 8.4–CH_3OH	Fluorescence 390/475 nm $K_3Fe(CN)_6$ precolumn (0.02 ng/injection)	Thiamine and thiamine esters
[126]	Heparin whole blood	$HClO_4$ Filter	3 μm Chromsphere ODS	Gradient 140 m*M* K_2HPO_4, pH 7.0–CH_3OH–*tert*.-butylammonium hydroxide	Fluorescence $K_3Fe(CN)_6$ precolumn (0.006 ng/injection)	Thiamine and thiamine esters

[127]	Heparin erythrocytes	NaCl CH_3OH	5 mm Spherisorb NH_2	0.1 *M* KH_2PO_4, pH 7.5–CH_3OH (60:40)	Fluorescence 340–380/437 nm $K_3Fe(CN)_6$ precolumn (not quoted)	Thiamine diphosphate
[128]	Heparin plasma	HCl $HClO_4$ Acid phosphatase Isobutanol	5 μm LiChrosorb NH_2	CH_3OH–$(CH_3CH_2)_2O$ (25:75)	Fluorescence 365/440 nm $HgCl_2$ precolumn (0.002 ng/injection)	Total thiamine
[129]	Serum	TCA Diethyl ether	10 μm PRP-1	15 m*M* Na_2HPO_4, pH 8.5 with 10% CH_3OH or 10% THF	Fluorescence 365/433 nm $K_3Fe(CN)_6$ precolumn (0.002 ng/injection)	Thiamine and thiamine esters
[131]	Cultured neurons Astrocytes	TCA	5 μm PRP-1	50 m*M* Na_2HPO_4, pH 9.0, 50 m*M* *tert.*-butyl ammoniumhydroxide–4% THF (96:4)	Fluorescence 365/433 nm $K_3Fe(CN)_6$ precolumn (0.02 ng/injection)	Thiamine and thiamine esters
[132]	EDTA whole blood Serum	Claradiastase TCA SPE column	5 μm NovaPak C_{18} (amino precolumn)	0.003 *M* hexanesulphonate in 1% CH_3COOH–CH_3OH (80:20)	UV 254 nm (1.0 ng/injection)	Total thiamine
[134,135,138]	Heparin whole blood Serum CSF	TCA Diethyl ether Filter	Supelcosil NH_2	85 m*M* Na_2HPO_4, pH 8.5 with 10% or 40% CH_3CN	Fluorescence 375/450 nm BrCN precolumn (0.006 ng/injection)	Thiamine and thiamine esters
[139,11]	Heparin erythrocytes	HCl Takadiastase, amylase acid, phosphatase Isobutanol	5 μm LiChrosorb Li60	$CHCl_3$–CH_3OH (80:20)	Fluorescence 375/430 nm $K_3Fe(CN)_6$ precolumn (not quoted)	Total thiamine
[140]	Heparin whole blood Erythrocytes	TCA Diethyl ether Hexane	10 μm μBondapak C_{18}	15 m*M* ammonium citrate, pH 4.2– 0.1 *M* formic acid, 0.4% $(CH_3CH_2)_2NH$ (90:10)	Fluorescence 365/435 nm $K_3Fe(CN)_6$ postcolumn (0.009 ng/injection)	Thiamine and thiamine esters
[144]	Rat intestine	TCA	5 μm ODS Hypersil	Gradient 25 m*M* KH_2PO_4, pH 8.4–CH_3OH	Fluorescence 365/460 nm $K_3Fe(CN)_6$ pre- and postcolumn (0.009 ng/injection)	Thiamine and thiamine esters (chloroethylthiamine as internal standard)

Table 3
Summary of selected methods for the determination of thiamine applicable to both food and biological fluids

Ref.	Sample type	Extraction/clean up	Column	Mobile phase	Detection/derivatization	Analyte
[95]	Breakfast cereal	HCl	5 μm NH_2-MicroPak AX-5	5 m*M* $NH_4H_2PO_4$, pH 2.85	UV 254 nm	Thiamine
	Urine	SPE C_{18}			(0.5 ng/injection)	
[141]	Food	H_2SO_4	5 μm Nucleosil NH_2	40 m*M* KH_2PO_4, pH 4.4–CH_3CN (25:75)	Fluorescence	Total thiamine
	EDTA whole blood	Claradiastase			$K_3Fe(CN)_6$ precolumn	
	Urine	TCA				
	Faeces	NaCl			(0.04 ng/injection)	
		Isobutanol				
[142]	Cereal, flour, muesli, milk, desserts	HCl	5 μm LiChrosorb NH_2	MeOH–CH_2Cl_2 (10:90)	Fluorescence 365/440 nm	Total thiamine
	EDTA or heparinized whole blood	Takadiastase			$HgCl_2$ precolumn	
		Isobutanol			(0.04 ng/injection)	

Acetylaneurine, a close structural relation of thiamine, with similar chemical, physical and chromatographic properties was used by Lynch et al. [145] in their modification of the method used by Baines [127]. It proved ideal since it was detected with similar sensitivity as TDP, does not occur naturally in the human body and was chemically stable in the sample and throughout the isolation procedure.

4. Conclusion

Many methods for the HPLC determination of thiamine have been published. A wide variety of chromatographic modes and elution techniques have been employed. Some methods have used ultraviolet detection but the more sensitive methods require fluorescence detection. Fluorescence detection requires pre- or postcolumn conversion of thiamine to thiochrome. The major problem with the analysis of thiamine by HPLC is the lack of interlaboratory standardization, particularly as far as sample preparation is concerned.

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