Automated microbiological assay of thiamin in serum and red cells

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Abstract

Aims—To develop a sensitive, direct, automated method for the measurement of serum and red cell thiamin.

Methods—A microbiological assay using a chloramphenicol resistant strain of *Lactobacillus fermenti* as the test organism was developed. Addition of chloramphenicol and cycloheximide to the assay medium suppressed bacterial and yeast contamination and enabled tests to be automated without recourse to aseptic procedures. Evaluation of the assay included precision analysis and estimation of thiamin recovery. Results obtained on red cell extracts were compared with an established colorimetric (thiochrome) method.

Results—Acceptable intrabatch and interbatch precision was obtained and good recovery of thiamin added to serum was obtained. Non-parametric reference ranges based on the results from 505 healthy people were: serum thiamin 11.3–35.0 nmol/l and red cell thiamin 190–400 nmol/l. Results were not age or gender related. The method gave results for red cell thiamin which were significantly higher than those obtained with an established thiochrome method.

Conclusions—This automated microbiological assay is sensitive to 2.0 nmol/l of thiamin and allows tests to be set up at the rate of 100 per hour and after 20–22 hours allows incubation results to be read at 60 per hour. The method has proved reliable, suitable for the assay of large numbers of samples, and relatively inexpensive to perform.

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Several methods have been proposed for the determination of thiamin concentration in man. An indirect approach has often been used, involving the measurement of red blood cell transketolase activity.^{1,2} Although convenient to perform, other non-thiamin dependent enzymes may interfere and several reports have appeared which cast doubt on the validity of this test. Leevy *et al*³ reported that there was no correlation between blood thiamin (as measured by microbiological assay with *Ochromonas danica*) and red cell transketolase activity in hospital patients. Baker also noted a similar discrepancy, especially in patients with liver disease.⁴

Direct methods based on the oxidation of thiamin to its fluorescent derivative thiochrome have also been used.^{5–8} The major drawback with most thiochrome methods has been poor sensitivity, making them unsuitable for assay of concentrations found in plasma. More recently increased sensitivity has been achieved by coupling high pressure liquid chromatography (HPLC) separation with thiochrome derivatisation.^{9–11}

Several microbiological methods for thiamin assay have been described.^{12–15} However, to date none has gained widespread acceptance largely due to methodological complexities.

Methods

The assay organism used was a chloramphenicol resistant strain of *Lactobacillus fermenti* developed from a standard strain obtained from the American Type Culture Collection (ATCC 9338). Chloramphenicol resistance was achieved using the method described by Davis *et al*¹⁶ and the organism was maintained by drying on to small ceramic beads.¹⁷

Double strength assay medium was prepared according to the protocol shown in table 1. Solution was effected using a magnetic stirrer without heating.

Table 1 Preparation of assay medium

Glucose	200 g
Sodium acetate	100 g
Maltose	50 g
Casamino acids (vitamin free)*	25 g
Casein hydrolysate enzymatic (vitamin free)**	250 ml
Di-potassium hydrogen phosphate	5 g
Potassium di-hydrogen phosphate	5 g
Ascorbic acid	2 g
Magnesium sulphate (7H ₂ O)	2 g
L-cysteine	5 g
L-cystine	1 g
L-tryptophan	125 mg
Manganous sulphate (H ₂ O)	125 mg
Adenine sulphate†	100 mg
Guanine hydrochloride†	100 mg
Uracil†	100 mg
Sodium chloride	50 mg
Ferrous sulphate (7H ₂ O)	50 mg
Riboflavin	1 mg
Nicotinic acid	1 mg
Pyridoxine hydrochloride	1 mg
Calcium D pantothenate	1 mg
Biotin	4 µg
Folic acid	2.5 µg
Tween 80	5 ml
Distilled water (make up volume to)	5 litres
Adjust pH to 6.5 with 8 mol/l potassium hydroxide, dispense into 250 ml amounts and store at –20°C	

*Difco Laboratories, Detroit, Michigan, USA

**ICN Biomedicals, Costa Mesa, California, USA, (5% sterile solution)

†Dissolve by suspending in a small volume of water and then adding 8 mol/l potassium hydroxide dropwise until solution is complete.

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For maintenance of the test organism in liquid culture, chloramphenicol (30 mg/l) and cycloheximide (30 mg/l) were added to single strength assay medium which had been supplemented with thiamin nitrate (10 nmol/l).

On the day before the test, a tube containing 10 ml of maintenance medium was inoculated with one ceramic bead coated with the dried organism. After overnight incubation the suspension was centrifuged at $1200 \times g$ for five minutes, the supernatant fluid discarded, and the organism resuspended in single strength assay medium. The absorbance (700 nm) of the suspension was adjusted to 0.68–0.70.

Blood was collected into plain disposable phials. Serum was separated and stored at -20°C until required for assay.

For red cell thiamin, 5 ml of heparin or EDTA anticoagulated blood was collected and centrifuged at $1200 \times g$ for 10 minutes. The plasma and buffy coat layers were removed and discarded. Packed red blood cells (0.5 ml) were diluted in 4.5 ml of acetate buffer (0.05 mol/l, pH 5.0) and mixed well. This preparation was stored at -20°C until required for assay. On the day of the assay, red cell dilutions were thawed, mixed well, heated at 90°C for five minutes, allowed to cool, and centrifuged at $1200 \times g$ for 10 minutes. The supernatant fluid was separated and assayed as for serum.

Thiamin mono-nitrate (2.0 mmol) was dissolved in 1 litre of 1.6 mmol/l hydrochloric acid. This stock standard solution has been found to be stable for at least two years at 4°C . On the day of the assay the stock standard was diluted 1 in 10 000 in distilled water. This working solution was used to prepare standard solutions containing: 5, 10, 15, 20, 40, 60, 80 and 120 nmol/l.

A Gilson model 222 robotic diluting system, linked to a Gilford Stasar III spectrophotometer and controlled by an Epson PC, was used for setting up and reading the results of the assay.

Serum samples, red cell extracts, and standards were presented to the analyser in 2.5 ml capacity disposable analyser cups and dilutions were made into 9 ml capacity polypropylene tubes loaded into 44 place racks.

To each litre of single strength assay medium was added 10 mg of chloramphenicol base, 10 mg of cycloheximide, and 0.8 ml of an overnight culture of the test organism which had been adjusted to a standardised absorbance (see preparation of inoculum). The organism was kept in suspension by the use of a magnetic stirrer.

The system was programmed to sample 0.2 ml of serum, red cell extract, or standard solution and dilute it with 4.8 ml of the inoculated assay medium. Standards were set up in triplicate. A control serum was included after every 10 test samples.

After dilution assay racks were removed to a 37°C incubator for 20–22 hours. The racks were then returned to the Gilson system which was programmed to mix the contents of each tube and record absorbance readings at 640 nm. To increase precision, five readings

for each tube were taken over two seconds. Samples were flagged where any reading greater than 0.02 absorbance units from the mean of the five absorbance readings was obtained. The interfaced PC was programmed to generate results by interpolation from the standards.

Intrabatch precision was determined by assaying one serum sample 24 times in one batch; the interbatch precision was determined using three serum samples (one in the deficient range, one borderline normal and one clearly normal) which were aliquoted and stored at -20°C and assayed 12 times in different batches over nine months.

To determine recovery thiamin pyrophosphate (the biologically active and most abundant intracellular form of the vitamin) was added to four serum samples which had been previously assayed.

Ten red cell samples were assayed by both the microbiological and an established thiochrome method.⁶ Due to the insensitivity of this thiochrome method it was not possible to compare results obtained with serum samples.

To determine reference ranges, serum and red cell thiamin was assayed on 505 apparently healthy individuals ranging in age from 18 to 90 years.

Results

The intrabatch coefficient of variation (CV) was 3.4% (range 16.2–18.1 nmol/l, mean (SD) 17.1 (0.56)). The interbatch precision is shown in table 2. Acceptable reproducibility was obtained at all thiamin concentrations.

Results for the recovery of thiamin pyrophosphate are shown in table 3. Recovery ranged from 101 to 110%.

Table 2 Interbatch precision of microbiological assay

Mean thiamin (nmol/l)	No	Range	(SD)	CV(%)
5.69 (serum)	12	4.5–6.4	(0.57)	10.0
13.57 (serum)	12	12.1–15.1	(0.94)	6.9
26.0 (serum)	12	24.9–28.3	(1.58)	6.1
154.5 (red cell)	19	131.9–173.3	(11.46)	7.4

Table 3 Recovery of thiamin pyrophosphate added to serum

Sample No	Initial assay (nmol/l)	Thiamin added (nmol/l)	Repeat assay (nmol/l)	% Recovery
1	3.8	40.0	47.7	110
2	3.8	20.0	24.0	101
3	55.8	100.0	160.5	105
4	43.3	75.0	124.4	108

Table 4 Comparison of thiochrome and microbiological assay results

Sample	Red cell thiamin (nmol/l)	
	Thiochrome method	Microbiological method
1	279	369
2	211	268
3	170	226
4	260	332
5	226	298
6	128	173
7	252	347
8	188	252
9	305	415
10	203	286

Results for the comparison with a thiochrome method are shown in table 4. Although correlation between the two methods was good ($r = 0.99$), the microbiological assay gave results which were 21–28% higher than the thiochrome method. The slope of the correlation line was 1.33 (SE 0.064) and the intercept was 1.16 (SE 14.61).

No correlation was observed between serum or red cell thiamin and age and there was no significant difference between the serum or red cell thiamin of men and women (Student's t test). Reference ranges (non-parametric estimates) of 11.3–35.0 nmol/l for serum and 190–400 nmol/l for red cells were adopted.

Discussion

The major drawback of most previously described methods for the direct measurement of thiamin in blood, both conventional thiochrome and HPLC methods, has been poor sensitivity.^{6,18,19} At best, these methods can measure thiamin in whole blood but not in serum where concentrations are only 10% of those found in red cells. The development of improved HPLC procedures incorporating thiochrome derivatisation has addressed this problem.^{9–11} However, HPLC requires considerable investment in instrumentation and is outside the scope of many smaller laboratories. Although the microbiological method described here has been automated using a robotic liquid handling system for high throughput, it can be easily performed using manual pipetting. Adaptation of the assay to microtitre plates as has been recently described for folate assay²⁰ would offer greatly improved efficiency and work is continuing on this modification.

It has already been shown that the thiamin activity of red cells is stable at 90° C for up to 20 minutes under conditions of the assay method but declines by up to 30% by 30 minutes.²¹ The higher red cell thiamin concentrations obtained by the present method when compared with those obtained by the thiochrome method are probably due to the destruction of thiamin during the heat hydrolysis stage of the latter method. In the new microbiological method samples are heated at 90° C for only five minutes. Comparisons with serum thiamin concentrations were not possible due to the insensitivity of the thiochrome method used. The new method was sensitive to 2.0 nmol/l of thiamin. The concentration of total thiamin found in the serum of healthy subjects (11.3–35.0 nmol/l) compares well with concentrations reported using sensitive HPLC methods. Weber and Kewitz⁹ reported a range of 6.6–42.0 nmol/l and Bettendorff *et al*¹¹ reported a range of 5.9–20.7 nmol/l. It should be noted, however, that these data were derived from the study of relatively small numbers (91 subjects and 42 subjects, respectively).

One of the drawbacks of all microbiological assays is the potential for organism inhibition by therapeutic drug concentrations in blood (particularly antibiotics) and this must be taken into account in the interpretation of low results. This inhibition usually only occurs with serum assay. With red cell assay, the greater dilution used minimises the problem. Repeat assay using a different dilution of test material is usually sufficient to alert to the presence of inhibitors.

Using the method described, it is possible to set up assays at the rate of 100 per hour and, after 20–22 hours of incubation, read at 60 per hour. The method is particularly well suited for use in population and institutional surveys.

The automated microbiological method has been used in this laboratory for a number of years and to date more than 60 000 assays have been performed. The test has proved a reliable and relatively inexpensive addition to the diagnostic repertoire of the laboratory.

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