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Assessment of Vitamin B₁, B₂ and B₆ Status by Coenzyme Activation of Red Cell Enzymes Using a Centrifugal Analyser

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Summary: We describe a fully automated method for the assessment of vitamin B₁, B₂ and B₆ status using a centrifugal analyser. The activation of the red cell enzymes transketolase, glutathione reductase and aspartate aminotransferase¹⁾ by their respective coenzymes were measured in freshly prepared haemolysate. The enzyme catalytic activities in the sample were measured with (maximal activity) and without (basal activity) the coenzyme, and the percentage activation was calculated. The between run precision for red cell transketolase, glutathione reductase and aspartate aminotransferase were 8.5%, 10.3% and 9.5% respectively. When whole blood was stored at room temperature for 6 hours, red cell aspartate aminotransferase activity significantly decreased ($n = 10$, $p < 0.05$). There were no significant changes in the activities of the other two enzymes.

For a group of 30 healthy young subjects, the mean (standard deviation) values for the percentage activation of transketolase, glutathione reductase and aspartate aminotransferase were 11.9% (7.3), 35.1% (19.1) and 85.3% (18.0), respectively. The vitamin status of a group of 86 pregnant women was assessed by this method; 2.3%, 8.1% and 8.1%, respectively, of the pregnant women showed a higher percentage activation for transketolase, glutathione reductase and aspartate aminotransferase than that found in the young subjects.

Both groups correlated well with respect to the basal activity and the percentage activation of each enzyme. Basal activity was inversely proportional to the percentage activation. It is therefore suggested that the basal activity can be used as a second criterion in the assessment of vitamin status.

Introduction

The nutritional survey of a population requires the analysis of a large number of specimens. Vitamin B assessment by in vitro coenzyme activation of red cell enzymes is known to be widely accepted (1, 2) and manual spectrophotometric methods are well established (3–5). However they are time consuming and not suitable for handling a large number of specimens. Here we describe an automated version of these methods.

¹⁾ Enzymes:

1. Aspartate aminotransferase, *L*-Aspartate: 2-oxoglutarate aminotransferase (EC 2.6.1.1)
2. Glutathione reductase, NAD(P)H: glutathione oxidoreductase (EC 1.6.4.2)
3. Transketolase, Sedoheptulose-7-phosphate: *D*-glyceraldehyde-3-phosphate glyceraldehyde transferase (EC 2.2.1.1)

Materials and Methods

Instrument and reagents

The kit for aspartate aminotransferase was obtained from Baker Instrument Ltd. (Allentown, PA, USA). Glycerol phosphate dehydrogenase/triose phosphate isomerase, glutathione and thiamine pyrophosphate were from Boehringer Mannheim GmbH (Mannheim, Germany). Tris was from BDH Chemical Co. (Poole, England). All other chemicals were from Sigma Chemical Co. (St. Louis, MO, USA). All analyses were performed on a Cobas Bio centrifugal analyser (Roche Diagnostic, Basle, Switzerland).

Specimen

Blood was collected into heparinized tube and centrifuged within 2 hours. After separation of plasma, 1 ml of cells was transferred into a 5 ml tube and 4 ml of cold isotonic magnesium chloride solution (osmolality = 290 mosmol/kg) was added. The tube was inverted gently several times then centrifuged at 1000 *g* for 10 minutes. The clear supernate was discarded, and the washing was repeated twice. After the final

washing, two 0.1 ml aliquots of cells were stored at -20°C . Analysis was usually done within 1 week. Before analysis, haemolysate was prepared by adding 1 ml of distilled water to each tube followed by vigorous mixing. haemolysates were kept on ice during the assay of enzyme activities.

Procedures

Previously reported procedures were modified for the automated method (3, 4). All reagents were freshly prepared except the buffers.

Transketolase¹⁾

The reagent for the Cobas Bio was 100 mmol/l Tris-HCl buffer, pH 7.6, containing 16.4 mmol/l of ribose-5-phosphate, 154 $\mu\text{mol/l}$ of NADH, with or without 360 $\mu\text{mol/l}$ of thiamine pyrophosphate. Glycerol phosphate dihydrogenase/triosephosphate isomerase suspension was diluted 4 times with tris buffer and used as the start reagent. The Cobas Bio program is shown in table 1.

Glutathione reductase¹⁾

Phosphate buffer, 80 mmol/l, pH 7.4, containing 2.22 mmol/l glutathione (oxidizing form), 1.78 mmol/l EDTA, with or without 11.1 $\mu\text{mol/l}$ flavin adenine dinucleotide, was used as the reagent for the Cobas Bio. Start reagent was 3 mmol/l of NADPH in 10 g/l sodium bicarbonate. The Cobas Bio program is shown in table 1.

Aspartate aminotransferase¹⁾

The aspartate aminotransferase kit from Baker Instrument Ltd. was used by reconstituting each vial with 2 ml of the diluent supplied. This solution was used as the start reagent for the Cobas Bio. The reagent was 80 mmol/l Tris-HCl buffer, pH 7.8, with or without 25 $\mu\text{mol/l}$ pyridoxal-5-phosphate. The Cobas Bio program is shown in table 1.

Calculation

Enzyme catalytic activity per gram of haemoglobin (Hb) is calculated according to the following equation.

Transketolase:

Activity (U/g Hb)

$$= \frac{\Delta A}{\text{min}} \times 1000 \times \frac{180}{10} \times \frac{1}{6.22} \times \frac{1}{\text{Hb (g/l)}}$$

$$= \frac{\Delta A}{\text{min}} \times 2894 \times \frac{1}{\text{Hb (g/l)}}$$

Aspartate aminotransferase/glutathione reductase:

Activity (U/g Hb)

$$= \frac{\Delta A}{\text{min}} \times 1000 \times \frac{260}{5} \times \frac{1}{6.22} \times \frac{1}{\text{Hb (g/l)}}$$

$$= \frac{\Delta A}{\text{min}} \times 8360 \times \frac{1}{\text{Hb (g/l)}}$$

The factors, 2894 and 8360, were incorporated into Cobas Bio programs for transketolase and aspartate aminotransferase/glutathione reductase, respectively.

Percentage activation is calculated according to the following formula.

Percentage activation

$$= \frac{\text{activity with vitamin} - \text{activity without vitamin}}{\text{activity without vitamin}} \times 100.$$

Haemoglobin was determined by adapting the cyanmethaemoglobin method (6) to the Cobas Bio. The program is shown in table 1. The reagent was 500 mg/l of potassium ferricyanide and 125 mg/l of potassium cyanide in 2.5 g/l of sodium bicarbonate solution. Haemoglobin standard (Sigma 525-18) was prepared by reconstitution with an appropriate amount of reagent.

Tab. 1. Cobas Bio program for the three red cell enzymes and haemoglobin.

Parameter listing	Transketolase	Glutathione reductase	Aspartate aminotransferase	Haemoglobin
Alpha code	—	—	—	+
1 Units	U/l	U/l	U/l	g/l
2 Calculation factor	2894	8360	8360	0
3 Standard 1 conc	0	0	0	9.0
4 Standard 2 conc	0	0	0	27.0
5 Standard 3 conc	0	0	0	0
6 Limit	0	0	0	0
7 Temperature ($^{\circ}\text{C}$)	37.0	37.0	37.0	25.0
8 Type of analysis	3	3	3	1
9 Wavelength (nm)	340	340	340	540
10 Sample volume (nm)	10	05	05	10
11 Diluent volume (μl)	12	10	15	50
12 Reagent volume (μl)	150	225	200	90
13 Incubation time (s)	990	900	900	10
14 Start reagent volume (μl)	08	20	40	0
15 Time of first reading (s)	5.0	5.0	10.0	0.5
16 Time interval (s)	60	10	10	180
17 Numbers of readings	30	30	30	10
18 Blanking mode	0	0	0	1
19 Printout mode	1	1	1	1

Result and Discussion

Precision

Precision of the method was evaluated by determination of the analytical standard deviation (SD) and the analytical coefficient of variation (CV) (7) from duplicate analysis results of specimens in separate batches (tab. 2). The CVs for basal activities were between 4.1 and 6.3%, which are similar to those previously reported (3, 4). For percentage activation, the CV was calculated for normal and low values. The CVs of percentage activation for all 3 enzymes were between 8.5 and 10.3% at a mean value near the lower limit of normal (tab. 4).

Stability

Stability was evaluated in both washed cells and whole blood. The stability of washed cells was studied by comparing the percentage activation of enzymes after storage at -20°C for a week; the results are shown in table 3 A. There was no significant change (by paired t-test) in percentage activation after one week of storage.

Secondly, we studied the stability of enzymes in whole blood stored at room temperature. Ten fresh specimens were divided into 2 portions; one was washed immediately and one was left at room temperature for 6 hours. The results are shown in table 3 B. Only the aspartate aminotransferase percentage activation showed a small but statistically significant decrease after 6 hours at room temperature, while the other two enzymes were stable. Therefore, in our protocol, cells were washed within 2 hours of blood collection to ensure minimal deterioration of enzyme activity.

Control study

Blood samples were obtained from a group of 30 healthy subjects (medical students and laboratory staff, age 20–40) and assessed by the above method (tab. 4). The mean and range of results obtained are comparable to those reported in the literature (2, 3, 8). If the mean percentage activation plus 2 SD is taken as the lower limit of normal, a percentage activation of greater than 26.5, 73.3 and 121.4% for transketolase, glutathione reductase and aspartate aminotransferase, respectively, can be considered to indicate deficiency of the respective vitamins.

Tab. 2. Precision study.

	No. of pair	Overall mean	Analytical SD	Analytical CV (%)
Percentage activation (%)				
Transketolase	6	27.2*	2.3	8.5
	36	11.6	2.5	23.2
Glutathione reductase	20	66.2*	6.8	10.3
	36	20.0	3.5	17.5
Aspartate aminotransferase	21	124.2*	11.9	9.5
	32	68.8	7.1	10.3
Basal activity (U/g Hb)				
Transketolase	42	0.499	0.023	4.7
Glutathione reductase	56	7.29	0.30	4.1
Aspartate aminotransferase	53	4.84	0.30	6.3

* Values near the lower limit of normal (see tab. 4)

Tab. 3 A. Stability of washed cell aliquots stored at -20°C for one week.

	Percentage activation, mean (SD)		
	Transketolase	Glutathione reductase	Aspartate aminotransferase
No. of pairs	42	56	53
Day 0	14.1 (8.4)	40.3 (29.2)	89.0 (40.80)
Day 7	12.9 (7.7)	40.6 (29.7)	92.5 (37.8)

Tab. 3B. Stability of the 3 red cell enzymes in whole blood.

Time stored at room temperature	Percentage activation, mean (SD)		
	Transketolase	Glutathione reductase	Aspartate aminotransferase
0 h	12.3 (5.7)	40.7 (17.5)	84.2 (22.3)*
6 h	13.9 (9.0)	39.6 (17.8)	80.1 (22.1)*

* $p < 0.05$, by paired t-test

Tab. 4. Basal activity, maximal activity and percentage activation of the 3 red cell enzymes in 30 healthy young adults.

		Transketolase	Glutathione reductase	Aspartate aminotransferase
Basal activity (U/g Hb):	mean	0.557	7.78	6.43
	SD	0.134	1.57	1.39
	mean - 2 SD	0.289	4.65	3.65
Maximal activity (U/g Hb):	mean	0.618	10.22	10.00
	SD	0.126	1.73	2.07
Percentage activation:	mean	11.9	35.1	85.4
	SD	7.3	19.1	18.0
	mean + 2 SD	26.5	73.3	121.4

Tab. 5 A. Basal activity, maximal activity and percentage activation of the 3 red cell enzymes in 86 pregnant women.

		Transketolase	Glutathione reductase	Aspartate aminotransferase
Basal activity (U/g Hb):	mean	0.558	7.09	6.29
	SD	0.169	1.64	2.30
Maximal activity (U/g Hb):	mean	0.622	9.51	10.66
	SD	0.187	1.68	2.82
Percentage activation:	mean	11.9	38.1	77.3
	SD	7.2	24.6	27.3
	mean + 2 SD	26.3	87.3	131.9
Number of deficient subjects		2	7	7
Percent of deficient subjects		2.3	8.1	8.1

Tab. 5 B. Percentage activation of the three enzymes in pregnant women.

	Percentage activation
Transketolase	33.0, 31.5
Glutathione reductase	101.2, 73.6, 87.3, 93.6 110.8, 105.9, 82.9
Aspartate aminotransferase	122.2, 130.2, 124.2, 129.1 130.2, 129.2, 133.5

Pregnant women study

Blood samples from a group of 86 pregnant women were assessed (tab. 5 A). Using the criterion mentioned above, 2 (2.3%), 7 (8.1%) and 7 (8.1%) out of the 86 subjects were found to have abnormally high percentage activation for vitamin B₁, B₂ and B₆, respectively. Individual values of these percentage activations are given in table 5 B.

Basal activity

It has been suggested that vitamin deficiency is usually associated with a low basal activity due to the low levels of apoenzyme (9). Therefore we correlated the basal activity with percentage activation by *Spearman* rank for the 3 different vitamins. The correlation

coefficient showed (tab. 6) a significant correlation in both groups for glutathione reductase and aspartate aminotransferase, and in the control group for transketolase.

If the mean basal activity minus 2 SD of the control group is taken as the lower limit of normal, then a basal activity of less than 0.289, 4.65 and 3.65 U/g Hb (tab. 4) for transketolase, glutathione reductase and aspartate aminotransferase, respectively, can be considered to indicate deficiency of the respective vitamins. Using this criterion, 0 (0%), 1 (1.2%) and 9 (10.5%) of the 86 pregnant women were identified as abnormal for vitamin B₁, B₂ and B₆, respectively.

Tab. 6. Correlation of percentage activation and basal activity by *Spearman* rank.

	Control group (n = 30)	Pregnant group (n = 86)
Transketolase	0.411*	0.046**
Glutathione reductase	0.720*	0.641***
Aspartate aminotransferase	0.512*	0.814***

* p < 0.01
 ** p > 0.01
 *** p < 0.001

If both the percentage activation and basal activity limits are considered, then only 0 (0%), 1 (1.2%) and 4 (4.7%) of the pregnant women would be classified as abnormal for vitamin B₁, B₂ and B₆, respectively.

Assessment of vitamin status using solely percentage activation, which reflects the functional aspect of vitamin (1), is controversial (9, 10). Because of our demonstration of good correlation between basal activity and percentage activation, it is suggested that

the mean minus 2 SD of the basal activity should be used as a second criterion, in addition to percentage activation, in the assessment of vitamin status. Use of both criteria would give a more specific and sensitive assessment. Further investigations are in progress.

The analysis of all 3 red cell enzymes in 39 samples can be performed in one day. The method is therefore suitable for use in nutritional surveys.

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