Erythrocyte niacin co-enzymes as a measure of vitamin B3 status

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ABSTRACT

This study reports a new Cobas Mira method for measuring erythrocyte NAD and NADP, which can be used in the diagnosis of vitamin B3 deficiency and as a tool for monitoring vitamin B3 treatment. It also has potential for the study of oxidative stress states.

In vitamin B3 deficiency, levels of NAD and NADP in the erythrocyte are disproportionately maintained; NAD levels fall, while NADP remains stable. In niacin deficiency we found the molar NAD/NADP ratio to be below 2.10, while in nutritionally adequate states the ratio was greater than 2.50 (population mean 3.50, n = 50).

This method has proved to be more sensitive than the measurement of erythrocyte NAD on its own and is suitable for the routine assessment of vitamin B3 status in clinical samples.

METHODS

A method for measuring erythrocyte NAD and NADP levels, based on the enzymatic cycling method of Nisselbaum and Green [3] has been developed for the Cobas Mira.

NAD^+ is a cofactor for alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase, ADH). In the metabolism of ethanol to ethanal (acetaldehyde), NAD^+ is converted to $NADH_2$:

$$CH_3CH_2OH + NAD^+ \rightarrow CH_3CHO + NADH + H^+$$

With the use of additional downstream electron acceptors to regenerate NAD from $NADH_2$, the rate of this reaction is proportional to NADH concentration. A re-cycling mixture of phenazine methosulfate (PMS, Sigma P9625) and thiazolyl blue (MTT, Sigma M2128), which forms a formazan dye upon accepting electrons from PMS, was used in conjunction with ADH and ethanol to measure NADH [2,3].

$NADP^+$ is a cofactor for glucose-6-phosphate dehydrogenase (G6PD). In the metabolism of glucose-6-phosphate (G6P) to 6-phosphoglucono-6-lactone, $NADP^+$ is converted to $NADPH_2$:

$$G6P + NADP^+ \rightarrow 6\text{-phosphoglucono-6-lactone} + NADPH + H^+$$

A re-cycling mixture of PMS and MTT was used in conjunction with G6PD and G6P to determine NADP levels [2,3].

The rate of formation of the formazan dye was measured at 550 nm and the concentrations of NAD and NADP in µmol/L of red cells derived by comparison with aqueous standards. The ratio of NAD to NADP was calculated as: B3 niacin (pyridine nucleotide ratio) = (erythrocyte NAD) / (erythrocyte NADP).

Final reactant concentrations in the measurement cuvettes were:
NAD: 169 µmol/L MTT, 751 µmol/L PMS , 10200 U/L ADH, 0.019 % v/v ethanol
NADP: 169 µmol/L MTT, 783 µmol/L PMS , 3 mmol/L G6P, 11952 U/L G6PD

NAD stock standard (800 µM) and NADP stock standard (400 µM) were prepared fresh for each analysis in HPLC water.

CONCLUSIONS

In vitamin B3 deficiency, levels of NAD and NADP in the erythrocyte are disproportionately maintained, with NAD levels falling while NADP remains stable. A method has been developed for the Cobas Mira to measure the ratio between the erythrocyte concentrations of these pyridine nucleotides as a marker of niacin status.

In niacin sufficiency we found the molar NAD/NADP ratio to be greater than 2.50, with no clearly defined upper limit of normality.

A ratio of 2.10 - 2.50 is consistent with a mild niacin deficiency.

A ratio of less than 2.10 is consistent with a marked niacin deficiency.

The ratios of NAD^+ to $NADH_2$ and of $NADP^+$ to $NADPH_2$ are also of interest as a measure of the cellular response to oxidative stress. In particular, a decline in cellular $NADH_2$ may be associated with the ageing process. Examples of these changes have been observed in the study of sickle-cell anaemia and of glucose-6-phosphate dehydrogenase deficiency. Red cells from subjects in a sickling crisis show a decrease in NAD redox potential that may be a reflection of their increased sensitivity to oxidative stress [4]. When production of $NADPH_2$ is impaired in glucose-6-phosphate dehydrogenase deficiency, then the ingestion of certain drugs or toxic agents results in depletion of reduced cellular glutathione and consequently haemolysis and anaemia [5].

The classical treatment for vitamin B3 deficiency [1] is to give 100 mg of nicotinamide five times per day for a period of months, with prolonged B3 washout into the urine while tissue levels are repleted. Other nutritional deficiencies (for example of magnesium and zinc) may be corrected at the same time by oral supplementation. Under these circumstances, direct measurement of niacin in the blood may not give an accurate reflection of the level of tissue de-saturation. The method reported above provides the basis for a functional assessment of niacin metabolism in the erythrocyte during the treatment of vitamin B3 deficiency.

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INTRODUCTION

Dietary niacin requirements vary with energy expenditure, since NAD and NADP act as carriers of H^+ ions from substrates through the mitochondrial electron transport chain. They are also required for the functioning of respiratory enzymes, in DNA repair, cellular differentiation and apoptosis, as well as the metabolism of carbohydrates and fats. Since NAD is primarily involved in intracellular oxidation reactions, while NADP is required for intracellular reduction reactions, oxidative stress favours increased synthesis of NADPH and reduced synthesis of NADH.

Inadequate supply of niacin causes pellagra, a potentially fatal deficiency disease, which is characterised by a red "butterfly rash" and defects in energy metabolism. Symptoms of niacin deficiency include mucosal atrophy in the gastrointestinal tract (leading to diarrhoea) and progressive CNS disturbances. Associated depressive symptoms may be due to concurrent deficiency of tryptophan for serotonin synthesis. In the first decade of the 20th century, when pellagra was described in the southern USA, medical opinion favoured the view that it was caused by an infectious organism [1]. Because of the high dosage of vitamin B3 required to treat pellagra (e.g. 100 mg of nicotinamide, 5 x per day) and the ease with which such high doses are washed out in the urine, direct measurement of niacin in the plasma may not always reflect the degree of tissue de-saturation of the vitamin.

Red cell NAD levels have been shown to be a good indicator of niacin status [2]. In contrast, red cell NADP levels are maintained at a constant level, even during periods of niacin depletion [3]. Since NAD concentrations alone are affected by niacin intake, the ratio of NAD to NADP reflects niacin status. Thus the red cell NAD/NADP ratio can be used as a functional test for tissue de-saturation of vitamin B3.

SUBJECTS AND SAMPLE PREPARATION

The red cell NAD/NADP ratio in the blood of 5 volunteers was assessed on 3 consecutive days (measured in triplicate). The results are shown in figure 1, which depicts the daily variation in B3 status, as measured by this method.

Red cells from blood samples were washed three times in cold phosphate buffered saline, pH 7.40, then diluted 1 in 100 in carbonate buffer, pH 10.70 at 4° C. Nicotinamide (10 mM) was added to the buffer to prevent conversion between the different coenzyme forms; the buffer contained 10 % v/v Triton X-100 to ensure complete haemolysis. After adequate mixing samples were stored at 4° C until analysis.

Samples from 50 sequential subjects (27 males, 23 females, age range 20 – 60 years) presenting for functional B vitamin assessment were used to establish a preliminary reference interval for red cell niacin coenzyme ratio.

RESULTS

Comparison of erythrocyte niacin coenzyme ratios with NAD concentrations in 50 subjects showed that the detection of vitamin B3 deficiency was more sensitive when the coenzyme ratio was derived. The mean niacin coenzyme ratio was 3.54 ± 0.73 and the mean erythrocyte NAD level was 182 ± 29 µmol/L (n = 50).

Subsequent experience with the investigation of subjects with clinical niacin deficiency suggested that, in niacin deficiency, the erythrocyte niacin coenzyme ratio falls below 2.50 due to the maintenance of cellular NADP levels at the expense of NAD. A ratio of less than 2.10 was consistent with a marked niacin deficiency. A ratio of 2.10 - 2.50 was consistent with a mild niacin deficiency.

In niacin sufficiency we found the molar NAD/NADP ratio to be greater than 2.50, with no clearly defined upper limit of normality.