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Whole Blood NAD and NADP Concentrations Are Not Depressed in Subjects with Clinical Pellagra[1–3]

Paul I. Creeke,[4] Filippo Dibari,[4] Edith Cheung,[5] Tina van den Briel,[6] Eustace Kyroussis,[7] and Andrew J. Seal[4]*

[4]Centre for International Health and Development, Institute of Child Health, London, WC1N 1EH, UK; [5]World Food Programme, Luanda, Angola; [6]World Food Programme, 00148 Rome, Italy; and [7]Medecins sans Frontieres-Belgium, Kuito, Angola

Abstract

Population surveys for niacin deficiency are normally based on clinical signs or on biochemical measurements of urinary niacin metabolites. Status may also be determined by measurement of whole blood NAD and NADP concentrations. To compare these methods, whole blood samples and spot urine samples were collected from healthy subjects ($n = 2$) consuming a western diet, from patients ($n = 34$) diagnosed with pellagra and attending a pellagra clinic in Kuito (central Angola, where niacin deficiency is endemic), and from female community control subjects ($n = 107$) who had no clinical signs of pellagra. Whole blood NAD and NADP concentrations were measured by microtiter plate-based enzymatic assays and the niacin urinary metabolites 1-methyl-2-pyridone-5-carboxamide (2-PYR) and 1-methylnicotinamide (1-MN) by HPLC. In healthy volunteers, inter- and intra-day variations for NAD and NADP concentrations were much lower than for the urinary metabolites, suggesting a more stable measure of status. However, whole blood concentrations of NAD and NADP or the NAD:NADP ratio were not significantly depressed in clinical pellagra. In contrast, the concentrations of 2-PYR and 1-MN, expressed relative to either creatinine or osmolality, were lower in pellagra patients and markedly higher following treatment. The use of the combined cut-offs (2-PYR <3.0 μmol/mmol creatinine and 1-MN <1.3 μmol/mmol creatinine) gave a sensitivity of 91% and specificity of 72%. In conclusion, whole blood NAD and NADP concentrations gave an erroneously low estimate of niacin deficiency. In contrast, spot urine sample 2-PYR and 1-MN concentrations, relative to creatinine, were a sensitive and specific measure of deficiency. J. Nutr. 137: 2013–2017, 2007.

Introduction

Pellagra is a disease caused by a severe dietary deficiency in niacin (generic name for nicotinic acid and nicotinamide) and/or tryptophan (which is metabolized to niacin) or by an inability to absorb and process these nutrients. Diagnosis is normally based on clinical signs, which include a characteristic dermatitis and changes in the gastrointestinal tract and nervous system (1). However, the correct diagnosis may be overlooked in patients where clinical signs are atypical or absent (2,3). Although outbreaks are now rare, sporadic cases of pellagra are reported in chronic alcoholics and in individuals taking medication affecting tryptophan metabolism, such as isoniazid therapy for tuberculosis.

In 1990 and 2000, pellagra epidemics were documented in Malawi and central areas of Angola (4–6). In these areas, the pellagra outbreaks were associated with a high dietary dependence on unfortified maize. Given that many people in southern and central Africa are similarly dependent on unfortified maize, it is likely that seasonal subclinical niacin deficiency is widespread. The accurate biochemical diagnosis of niacin status is also of increasing interest due to its possible association with HIV-related conditions (7) and cancer (8).

Previous studies have assessed niacin status by the quantification of the major urinary niacin metabolites 1-methylnicotinamide (1-MN)[8] and 1-methyl-2-pyridone-5-carboxamide (2-PYR), and sometimes the more minor metabolite 1-methyl-4-pyridone-5-carboxamide (9,10), in timed urine collections. Status is usually expressed as the concentration ratio 2-PYR:1-MN or as the concentrations of the individual metabolites relative to creatinine (10). However, it has been shown in human subjects consuming controlled niacin diets that changes in 1-MN and 2-PYR concentrations, but not 2-PYR:1-MN, correlated with dietary niacin intake, casting doubt on the utility of the ratio indicator (11,12).

In previous field studies, timed urine collections have been used; however, these are often not practical due to compliance issues and the difficulty of relocating individuals and households. Random spot urine sampling avoids these issues and has

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[3] Details of the laboratory protocol for the analysis of NAD/H and NADP/H are available with the online posting of this paper at jn.nutrition.org.
* To whom correspondence should be addressed. E-mail: a.seal@ich.ucl.ac.uk.

[8] Abbreviations used: Hb, hemoglobin; 1-MN, 1-methylnicotinamide; 2-PYR, 1-methyl-2-pyridone-5-carboxamide.

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been suggested as an alternative (10,13,14). We previously showed that in spot urine samples, the niacin metabolite ratio 2-PYR: 1-MN was highly variable due to the different excretion rates of the 2 metabolites and was very dependent on recent dietary intake (15). Status was best described as the concentration of the metabolites relative to creatinine.

An alternative method for assessing niacin status is the measurement of the active coenzymes of niacin, NAD and NADP. Erythrocyte NAD and NADP concentrations are, in principle, a more direct measure of functional niacin status and have been shown to respond to changes in niacin intake. In a dietary depletion study, large decreases in erythrocyte NAD concentrations were observed and these were reversed during dietary repletion (16). Due to the relative stability of NADP concentrations, it has been proposed that the NAD:NADP ratio may be a useful indicator of niacin status (16–18).

Nicotinamide nucleotide metabolism has been investigated in pellagra patients, consuming a staple diet of jowar (*Sorghum vulgare*), in India. In contrast to the data from the dietary depletion studies, this work showed no significant change in the concentration of NAD or NADP during clinical pellagra. However, there was evidence of impaired synthesis due to a reduction in the activity of nicotinic acid mononucleotide adenyl transferase (19–21). This somewhat surprising finding raised important questions about the etiology of pellagra and appropriate methods for biochemical assessment, which have not subsequently been answered. To address these questions, we developed an HPLC method for the analysis of the urinary niacin metabolites 1-MN and 2-PYR (15) and a modified microtiter plate method, initially described by Jacobson and Jacobson (18), for the enzymatic determination of whole blood NAD and NADP.

We recently reported data on the use of urinary metabolite excretion to assess the prevalence of pellagra and niacin deficiency in a pellagra endemic area of Angola (22). Here, we present data on whole blood NAD and NADP in healthy control subjects, subjects with clinical signs of pellagra, and patients undergoing treatment. We discuss the relationship among NAD, NADP, and other indicators and the implications for the biochemical assessment of niacin status.

Subjects and Methods

Subjects. Three groups of subjects were included in this study. Two healthy male volunteers, consuming a western-style diet, were recruited in London for the indicative study of inter- and intra-day variability. Thirty-four patients suffering from clinical pellagra and attending the treatment clinic in Kuito, Central Angola, on 13 December 2004, were recruited to the study. Cases were admitted to the clinic on the basis of an assessment of clinical signs performed by local medical officers. In a representative population survey in the security cleared areas of Kuito Municipality, Bie Province, in December 2004, 107 women aged between 15 and 49 y were recruited to act as community control subjects.

Ethical approval. Ethical approval for the surveys was obtained from the Angolan Ministry of Health and a letter of support issued by the ethical review board of the Institute of Child Health, London. Individual informed consent was obtained from all participants before samples were taken. No material benefits, other than feedback on their nutritional and health situation, were offered to encourage participation.

Anthropometry and clinical signs and symptoms. Anthropometric measurements were taken using standard equipment and methods (23). Weight was measured to the nearest 100 g and height to the nearest millimeter. Subjects were examined for clinical signs of niacin deficiency by medically trained clinic staff. Subjects were defined as suffering from pellagra if they showed bilateral dermatitis on 1 or more sun-exposed areas, or Casal's necklace, or a "butterfly" sign on the face. The subjects were asked whether they had had any gastrointestinal (diarrhea) or neurological signs of deficiency (headaches, depression, or insomnia) in the previous 7 d.

Following diagnosis, patients were treated with a nicotinamide supplement of 100 mg (Ucemine PP, UCB Healthcare) and a B-complex tablet 3 times daily for 17 d. In addition, all patients received a weekly food supplement of 400 g of a fortified blended food [corn-soy blend (6.2 mg niacin/100 g), oil, and sugar] for 3 wk. Families of pellagra patients were also eligible for a food ration from the United Nations World Food Program, which was distributed monthly for 3 mo. Patients were required to return to the clinic once per week during their course of treatment, which typically lasted for 6 wk, and at which they were given more vitamin supplements and food rations.

Sample collection. Urine samples were collected in 100-mL disposable cups (VWR International), transferred into 10-mL Monovette urine collection tubes (VWR), and stored at 0–8°C. At the end of the day, the urine samples were transferred into 2-mL Nalgene cryovials (VWR) and frozen at –20°C.

Venous blood was collected using lithium heparin-coated 2-mL Vacutainer tubes (BD) and stored in vaccine boxes at 4–8°C until the end of each day. The blood was then thoroughly mixed and aliquoted (50 μL) into 2 2-mL Nalgene cryovials. The aliquots were frozen at –20°C for up to 2 wk before being transported on dry ice from Kuito to London via Luanda. In London, urine and whole blood samples were stored at −80°C until analysis.

Measurement of hemoglobin (Hb) was performed using a portable Hemocue Photometer (HemoCue), which utilizes the azidemethemoglobin principle.

To measure inter- and intra-day variations in niacin metabolites, paired fasting early morning peripheral blood and urine samples were collected from 2 subjects on 4 consecutive days. On d 4, another 3 nonfasting samples were collected at 3-h intervals. In total, 7 paired samples were collected from each subject. Peripheral blood was collected from finger pricks, made using safety lancets, into lithium heparin-coated Microtainer tubes (BD). The samples were aliquoted (50 μL) into 2-mL Nalgene cryovials and stored at –80°C. Urine samples were collected as above and stored at –80°C.

Urine analysis. Urine samples were analyzed by investigators who did not know the clinical status of the individual. The urinary niacin metabolites 1-MN and 2-PYR were measured by ion-pairing reverse-phase HPLC (15). Limits of detection for 1-MN and 2-PYR were 0.4 and 0.1 μmol/L, respectively, and limits of quantification were 2.6 and 0.5 μmol/L, respectively. A urine quality control sample ($n = 47$, analyzed for 9 d) following purification and analysis had a 3.0% CV for 2-PYR (mean concentration, 37.0 μmol/L; range, 34.8–39.4 μmol/L) and a 6.6% CV for 1-MN (13.3 μmol/L; range, 11.6–16.0 μmol/L). Urine samples were analyzed for creatinine (using Vitros CREA slides, Ortho-Clinical Diagnostics) and osmolality (using an Advanced Micro Osmometer Model 3300, Advanced Instruments) by Camelia Botna Laboratories.

Whole blood NAD and NADP concentrations. Whole blood samples were analyzed by investigators who did not know the clinical status of the individual. Analysis of whole blood for NAD and NADP was performed using modified versions of the microtiter plate-based enzymatic methods described by Jacobson and Jacobson (18) (the modified methods are described in detail in the online supporting material).

Limits of detection and quantification in whole blood (assessed using the pure standards) for NAD were 750 and 2438 nmol/L and 750 and 1875 nmol/L for NADP, respectively. Upper limits, measured using the pure standards, were equivalent to NAD and NADP concentrations of 34,613 nmol/L and 33,413 nmol/L, respectively, in whole blood (additional details of the assay performance are given in the online supporting material).

Statistical analysis. Means and SD are presented in the text. All statistical tests were performed in SPSS version 13.0 (SPSS) and EpiInfo

6.04d (CDC). Correlations were tested using the Pearson correlation test and differences in means tested by ANOVA and Tamhane's T2 post-hoc test, which does not assume equal variance. Differences were considered significant at $P < 0.05$. Assessment of inter- and intra-day variation was by percent CV. To adjust for hematocrit variations between blood samples, the Hb concentrations were measured and their relationship to NAD and NADP concentrations described by regression formulae. The concentrations of NAD and NADP were adjusted using the following formulae:

$$\text{AdjNAD} = [\text{NAD}] + (1518 \times (14.175 - [\text{Hb}])).$$

$$\text{AdjNADP} = [\text{NADP}] + (1272 \times (14.175 - [\text{Hb}])).$$

The niacin index (molar ratio) was calculated as [NAD]/[NADP] × 100.

Results

Inter- and intra-day variation in NAD, NADP, 2-PYR, and 1-MN concentrations in healthy subjects. Variations in the concentration of NAD, NADP, 2-PYR, and 1-MN were monitored over a 4-d period in paired whole blood and urine samples in 2 healthy volunteers (**Fig. 1**). Inter- and intra-day variations for NAD and NADP were much less then those for the urinary metabolites 2-PYR and 1-MN (**Table 1**).

Descriptive data on patients vs. nonpatient controls. The characteristics of the pellagra patients have been previously described (22). Of the 34 patients recruited, 11 patients were attending for the first time and were classified as new untreated cases. The other 23 had been receiving treatment for a period of between 1 and 6 wk. The control women ($n = 107$) showed no clinical signs of pellagra and were aged between 15 and 49 y (27.4 ± 9.4 y). Their BMI was 21.13 ± 3.25 ($n = 104$) and their Hb concentration 134 ± 21 g/L ($n = 106$).

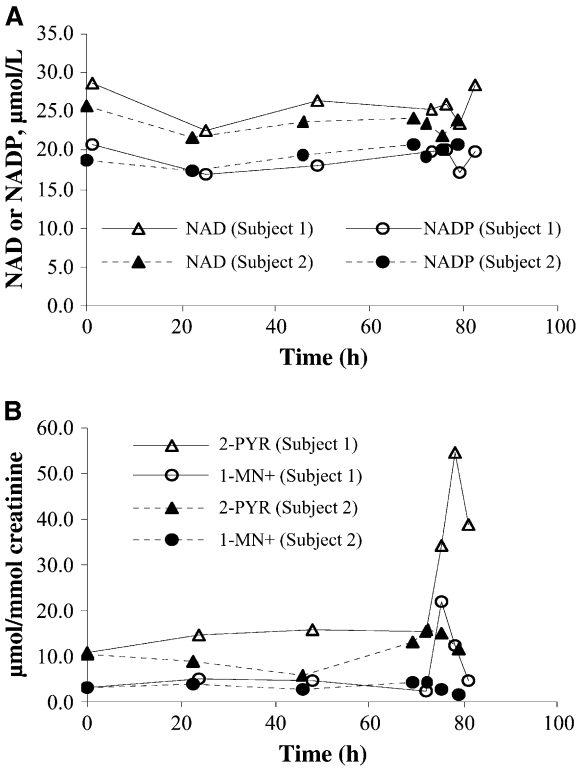

FIGURE 1 Variation in the concentration of whole blood NAD and NADP concentrations (*A*) and niacin urinary metabolites, 1-MN and 2-PYR (*B*), in 2 healthy volunteers. Samples were collected over 4 d and the results illustrate the relatively low variation in the concentrations of NAD and NADP.

TABLE 1 Inter- and intra-day variations in the concentrations of whole blood NAD and NADP and urinary 2-PYR and 1-MN in 2 healthy male subjects

Analyte	Subject 1	Subject 2	Subject 1	Subject 2
	Inter-day[1] % *CV*		*Intra*-day[2] % *CV*	
NAD	9.9	7.2	7.7	4.2
NADP	9.3	7.7	7.1	3.4
2-PYR	16	32	45	13
1-MN	34	19	85	40

[1] Measurements from the same time point on d 1–4 were used to calculate the inter-day percent CV.
[2] Intra-day percent CV was calculated using the 4 measurements taken on d 4.

Unadjusted NAD and NADP concentrations in whole blood were paradoxically higher in pellagra patients than healthy controls (**Table 2**). However, whole blood concentration depends on the hematocrit of the sample and this artifact was removed when the concentrations were expressed as the NAD:NADP ratio, or when adjusted for the Hb concentration of the sample. The NAD:NADP ratio did not differ between new admissions and community controls or patients after treatment.

In contrast, the urinary concentrations of the niacin metabolites relative to creatinine were significantly reduced in new admissions compared with community controls. Concentrations of 2-PYR were 69% lower in new admissions ($P < 0.001$) and 1-MN concentrations were 61% lower ($P = 0.002$). In patients who had commenced treatment, urinary concentrations of 2-PYR and 1-MN were 23 times ($P = 0.013$) and 38 times ($P = 0.023$) greater, respectively, than those in the control subjects.

Taking a niacin index of <100 as a cut-off (16), whole blood NAD and NADP concentrations classified only 36% of new admissions as niacin deficient. Using previously established urinary metabolite excretion cut-offs (10) of 2-PYR <4.0 mg/g creatinine (<3.0 μmol/mmol creatinine) and 1-MN <1.6 mg/g creatinine (<1.3 μmol/mmol creatinine), both measures individually identified 82% of new admissions as niacin deficient. However, if the 2 urinary metabolite excretion cut-offs were used sequentially, then 91% (10 of 11 patients) of new admissions were classified as deficient.

To assess the sensitivity and specificity of the biochemical measures against clinical diagnosis, data on the 11 newly admitted pellagra patients were combined with that from the 95 control subjects with complete datasets. Use of the urinary metabolite cut-offs yielded a much greater sensitivity than the use of the niacin index (**Table 3**). Using the combined urine metabolite cut-offs [2-PYR <4.0 mg/g creatinine (<3.0 μmol/mmol creatinine) and 1-MN <1.6 mg/g creatinine (<1.3 μmol/mmol creatinine)] achieved the best sensitivity and reasonable specificity in identifying clinical pellagra in the study population. In comparison, a low niacin index was an insensitive measure for the detection of pellagra in this population.

As creatinine concentrations may be dependent on the nutritional status of the subject, we investigated whether the use of osmolality might be a more appropriate means to adjust for urine concentration and the hydration status of the individual. Osmolality and creatinine concentrations were correlated ($r = 0.638$; $P < 0.001$). However, there was no correlation between creatinine concentration and BMI ($r = -0.001$; $P = 0.990$).

TABLE 2 Whole blood NAD and NADP and urinary metabolite concentrations in community controls and newly admitted and treated pellagra patients[1]

	Community controls	New admissions	Treated patients	*P*
n	107	11	23	
Whole blood				
NAD, *μmol/L*	24.3[b] (23.0, 25.6)	29.7[a] (22.4, 37.0)	29.1[a] (25.0, 33.2)	0.005
NADP, *μmol/L*	19.6[b] (18.3, 20.8)	26.1[a] (21.3, 30.9)	24.4[a] (21.5, 27.4)	<0.001
Adjusted NAD, *μmol/L*	25.4 (24.3, 26.5)	26.5 (17.9, 35.2)	26.4 (23.1, 29.7)	0.747
Adjusted NADP, *μmol/L*	20.5 (19.5, 21.4)	23.5 (18.3, 28.7)	22.1 (20.0, 24.3)	0.087
Niacin index	126 (121, 130)	114 (95, 134)	120 (109, 131)	0.222
Urine				
1-MN,[2] *mg/g creatinine*	3.1[b] (2.6, 3.5)	1.2[c] (0.3, 2.1)	45.3[a] (14.3, 76.2)	<0.001
2-PYR,[3] *mg/g creatinine*	7.6[b] (6.3, 8.9)	2.4[c] (1.0, 3.7)	55.2[a] (21.8, 88.7)	<0.001

[1] Values are means (95% CI). Means in a row with superscripts without a common letter differ, $P < 0.05$.
[2] To convert 1-MN from mg/g creatinine to μmol/mmol creatinine, multiply by 0.825.
[3] To convert 2-PYR from mg/g creatinine to μmol/mmol creatinine, multiply by 0.743.

There was also no correlation between total metabolite excretion and the NAD:NADP ratio when using creatinine- or osmolality-adjusted metabolite concentrations. In this sample, the use of osmolality had no apparent advantages over creatinine for adjusting niacin metabolite concentrations.

Discussion

The most prominent finding of this work was that the ratio of whole blood NAD and NADP concentrations were not significantly depressed in clinical pellagra, despite the inter- and intra-day stability of the ratio and the individual metabolite concentrations in 2 healthy volunteers. This counter-intuitive finding contrasts with results from dietary depletion studies (16) but agrees with earlier work on pellagra patients (19–21). In contrast to the unresponsive nature of NAD and NADP, low excretion of urinary niacin metabolites was shown to be a sensitive and specific indicator of clinical pellagra in this study population.

Microtiter plate-based enzyme cycling assays were selected for the analysis of NAD and NADP because of the small sample volumes required and their suitability for analyzing large numbers of samples. Base and acid were used to extract total NAD, NADH, NADP, and NADPH. The extracts were stored at −80°C prior to analysis and were reportedly stable at −20°C for >2 y (18). During analysis of the extract, NAD^+ and $NADP^+$ were used for the oxidation of ethanol and isocitrate by the enzymes alcohol dehydrogenase and isocitric dehydrogenase, respectively.

TABLE 3 Sensitivity and specificity of biochemical measures in the detection of clinical pellagra[1]

Measure	Sensitivity[2]	Specificity[3]	Positive predictive value[4]
		%	
Low urine metabolites	90.9 (57.1, 99.5)	71.6 (61.3, 80.1)	27.0 (14.4, 44.4)
2-PYR <4 mg/g creatinine	81.8 (47.8, 96.8)	75.8 (65.7, 83.7)	28.1 (14.4, 47.0)
1-MN <1.6 mg/g creatinine	81.8 (47.8, 96.8)	78.9 (69.1, 86.4)	31.0 (16.0, 51.0)
Niacin index <100	36.4 (12.4, 68.4)	88.4 (79.8, 93.8)	26.7 (8.9, 55.2)

[1] Values are percentages (95% CI).
[2] Sensitivity is the probability of a subject having a positive biochemical test result for pellagra if exhibiting clinical signs.
[3] Specificity is the probability of having a negative biochemical test if not exhibiting clinical signs.
[4] The positive predictive value is the probability of a subject having clinical signs for pellagra if they had a positive biochemical test.

The reactions produced NADH and NADPH, which were reoxidized by reduction of the dye thiazolyl blue tetrazolium bromide via the electron transport reagent phenazine ethosulfate. Reduction of thiazolyl blue tetrazolium bromide converted it from an initial yellow color to a blue formazan. The amount of blue formazan produced was dependent on the quantity of NAD and NADP present and was quantified by absorption measurement at 570 nm.

The assays had low limits of detection and quantification and these were less than the lower limits (~3500 nmol/L for NAD and NADP) previously seen in study subjects consuming a niacin-deficient diet (16). The upper limits for the assays were sufficient to measure the majority of NAD and NADP concentrations, likely to be found in population samples, without further dilution. Stability studies (see online supporting material) showed venous whole blood samples to be most stable when frozen; however, if thawed, cell lysis resulted in a slow decrease in NADP and rapid loss of NAD. The samples were relatively stable for a period of 7.5 h (the longest time that samples would normally spend "in the field" during a survey) in an unfrozen state between 5.5 and 37°C. Consequently, samples were maintained at 4–8°C in cool packs during collection and not frozen. Finger prick blood sampling was considered as an alternative to venous sampling and has been used under laboratory conditions (24). However, from our experience in field surveys, hemolysis was more likely to occur and this would have lead to NAD and NADP depletion.

To investigate whether status changed with collection time, paired peripheral whole blood and urine samples were collected from 2 subjects over 4 consecutive days. Concentrations of whole blood NAD and NADP (Fig. 1) were similar to those found by other workers (24–26) and showed less change than the urinary niacin metabolite concentrations (Table 1). The greater variation in the urinary metabolite concentrations resulted from recent dietary intake (15). We concluded that whole blood NAD and NADP concentrations would give the most stable measurement of status, because they varied least with collection time.

In pellagra patients, the excretion of urinary metabolites, relative to creatinine, declined sharply, whereas the NAD:NADP ratio did not change. Only 4 of 11 cases identified using clinical signs had a niacin index <100. In treated patients, there was a dramatic increase in the concentration of urinary metabolites but little change in the whole blood NAD:NADP ratio. This lack of change in the NAD:NADP ratio contrasts with that in dietary depletion studies (16) and remains an enigma in need of

explanation. It is likely that a number of factors are operating. One possibility is that erythrocytes are not subject to as much oxidative stress as sun-exposed skin. NAD concentrations as a result do not fall as quickly and can be maintained by the available niacin, even while dermatitis is developing. Another possibility is that there are genetic factors in the survey population that allow NAD concentrations in some cell types (such as erythrocytes) to be maintained at the expense of others (such as the skin) during periods of niacin deficiency.

The control group used in this study consisted of women from the local area, ~29% of whom had low niacin metabolite excretion (22). Further work is recommended to obtain a reference dataset from populations where niacin deficiency is rare.

We conclude, from the data currently available, that the NAD:NADP ratio cannot be used to diagnose clinical pellagra and would lead to erroneously low estimates of niacin deficiency if used in population surveys. Quantification of the urinary metabolites remains the method of choice for determining the epidemiology of niacin deficiency.

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