pH Metric Method for the Determination of Nicotinic Acid in Plasma

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Received for publication 10 January 1977

The acidimetric method for the determination of nicotinic acid (NA) using *Lactobacillus plantarum* ATCC 8014 (*Lactobacillus arabinosus* 17-5) has been simplified and thus made less time consuming, and the sensitivity has been increased fivefold by replacement of the titration by a pH determination. As the regression of the decrease in pH on the amount of NA was found linear within a range of 1 to 4 ng of NA per ml, the calculations were performed according to the slope-ratio principle. The NA concentration of plasma was determined with a coefficient of variation of 5 to 7%, which rose to about 10% at low NA concentrations. Assays of fasting plasma samples from 13 hyperlipidemic male patients showed a group mean NA concentration of 80 ± 55 ng/ml (mean $\pm$ 2 standard deviation), before treatment, and 705 ± 544 ng/ml (mean $\pm$ 2 standard deviation) during therapy with sustained release NA preparations, of which a single dose, ingested during steady-state conditions, doubled or tripled the plasma concentration within 1 to 3 h.

The concentration of the majority of the B-vitamins can be determined by titration of the acids produced by various microorganisms. However, the titrimetric standard curve is non-linear within the range in question, 5 to 50 ng of nicotinic acid (NA) per ml (6, 9, 10). Moreover, the titration procedure is generally time consuming and the color change of the indicator may be difficult to recognize, so electrometric/automatic titration has been recommended (4, 7).

The pH in an assay tube was readily determined before the electrometric titration starts (5). As preliminary investigations in our laboratory indicated agreement between pH metric and titrimetric values, the present study was undertaken. On the one hand, the aim was to ascertain that the results of a time-saving pH metric method and of the titrimetric method were in accordance; on the other hand, the object was to design a more sensitive method suitable for diluted plasma in order to avoid the interference of the natural buffering effect of undiluted plasma. Therefore, the method has been worked out primarily for use within the NA range of 1 to 4 ng/ml.

MATERIALS AND METHODS

Preparation of assay tubes. Five-milliliter portions of rehydrated double-strength niacin assay medium (Difco) were poured into glass tubes. To duplicate standard tubes 10, 20, 30, and 40 ng of NA were added, respectively, and distilled water was

added to a final volume of 10 ml. Distilled water up to 9.5, 9.0, 8.5, and 8.0 ml was added to sets of duplicate test tubes, and all tubes were autoclaved at 121°C for 10 min.

The test samples (heparin plasma, freeze-stored at -21°C immediately after aseptic drawing and centrifugation) were defrosted in cold water and centrifuged at 3,000 rpm for 20 min, and the supernatant was poured into another sterile tube. Five hundred or 1,000 μ l was withdrawn from each of the samples, which were diluted with sterile distilled water to approximately 20 ng of NA per ml (at least a 10 $\times$ dilution). One-half, 1.0, 1.5, and 2.0 ml of each of these dilutions were added to sets of duplicate test tubes.

Inoculation and incubation. The assay tubes were inoculated with a 50- μ l suspension of *Lactobacillus plantarum* (a 24-h culture, washed once with 0.9% NaCl solution and diluted to 10⁶ to 10⁷ bacteria/ml) and incubated at $37 \pm 0.5^\circ\text{C}$ for 66 h.

pH determination. A digital pH meter (Radiometer Standard pH meter, PHM 62, fitted with a combined electrode GK 2302 C) was adjusted to pH 7.00 and 4.01 at $25 \pm 0.1^\circ\text{C}$. Each assay tube was placed in a water bath at $25 \pm 0.1^\circ\text{C}$ for 15 min before the addition of a Teflon-coated magnet and stirring at 300 rpm for 0.5 min, just before the pH was recorded at 100 rpm.

Calculation. The slope-ratio principle (1) is used for the calculations. As the pH decrease was nonlinear for NA amounts over 4 ng/ml, duplicate test tubes with a mean pH below the mean pH of the 4-ng/ml standard tubes were excluded from the calculations together with sporadically occurring duplicates which differed by more than a 0.15-pH unit.

The following calculations were made. (i) For

each sample and level the mean pH values were calculated which were used in the further calculations. (ii) The differences between the duplicates were included in the calculation of the pooled variance. (iii) The regression line for each sample was calculated and an F-test was made for linearity of all these lines. (iv) A multiple regression analysis was performed, forcing all the regression lines through the same intercept. The goodness of fit of the fanlike statistical model was examined by an F-test. (v) The NA concentration $\pm$ standard deviation (SD) was calculated for each sample.

RESULTS AND DISCUSSION

The regression of the pH decrease on the NA content is approximately linear at low NA values (1 to 4 ng/ml), while it is exponential at higher NA values (2 to 50 ng/ml) (Fig. 1).

The pH is defined as $-\log C_{H^+}$, and a regression analysis of pH on log of milliliters of 0.1 N NaOH within the whole range of 10 to 1,000 ng of NA shows a correlation coefficient of 0.998 ($n = 14$, $P < 0.001$), thus confirming the close definitional agreement between the pH metric and titrimetric values.

The following pH values, found in 10-fold standard tubes each with the addition of 20, 100, or 500 ng of NA, demonstrate the reproducibility of the pH values (mean $\pm$ SD): 5.82 ± 0.05 , 5.07 ± 0.02 , and 4.42 ± 0.06 , respectively. The pooled SD equaled 0.05, so 1% of the duplicates were excluded from the calculations because of a pH difference exceeding 0.15 unit (provided normal distribution).

NA forms part of the endogenous pyridine complex, and the *L. plantarum* assay is not

specific for unchanged NA. The present method applied on aqueous solutions of nicotinamide, nicotinuric acid, and nicotinamide adenine dinucleotide did indeed result in a recovery of more than 90%, whereas less than 10% of 1-methylnicotinamide chloride (Sigma) and *N*¹-methyl-2-pyridone-5-carboxylamide (2-pyridone) were recovered. However, during NA therapy, nicotinuric acid is presumably the only one of these substances likely to be present in diluted plasma in amounts adequate for interference (8). The use of diluted plasma is essential, as the natural buffering effect of undiluted plasma would interfere with the results, especially in regard to low levels of NA (Table 1). Furthermore, the test range of 1 to 4 ng of NA per ml implies a fivefold increase of the sensitivity compared with the test range of the titrimetric method (5 to 50 ng of NA per ml).

The estimation of SD in different intervals of NA concentrations of the plasma samples included 366 assays (Table 2). Some of these samples were not optimally diluted, thus contributing relatively high SD values. In general, NA determination according to this method can be performed with a coefficient of variation (CV) of 5 to 7% when the plasma contains more than 500 ng/ml. In the range 100 to 500 ng/ml, CV gradually rises to about 10% at low levels, which is the upper limit normally accepted for microbiological assays (4). With plasma concentrations below 100 ng of NA per ml, an SD with the numerical value of about 10 ng/ml is to be expected.

TABLE 1. Buffer capacity of plasma^a

0.1 N lactic acid (ml)	Plasma (ml)				
	0 ^b	0.1	0.5	1.0	2.0
0	6.75	6.73	6.95	6.99	7.08
0.25	6.57	6.55	6.77	6.82	6.90
0.5	6.35	6.37	6.59	6.65	6.75
1.0	5.99	6.05	6.25	6.33	6.43
2.0	5.64	5.64	5.77	5.85	5.95
5.0	5.09	5.09	5.15	5.24	5.26
10.0	4.63	4.61	4.67	4.74	4.74
15.0	4.29	4.29	4.35	4.42	4.40
25.0	3.83	3.83	3.89	3.96	3.92
Total	2.92	2.90	3.06	3.03	3.16
pH decrease					
<i>t</i> -test, <i>P</i> ^c		0.8	<0.001	<0.001	<0.001

^a Sterilized niacin assay medium was added to 0 to 2 ml of pooled human plasma and titrated with lactic acid.

^b The pH values shown were read from automatically drawn pH curves.

^c Paired sample *t*-test for testing systematic differences between the zero column and the indicated ones.

FIG. 1. The pH decrease (■, ●, ▲) and base consumption (○) as a function of the NA content of 10-ml standard tubes. Mean of duplicates.

In a clinical crossover trial, fasting plasma samples from 13 hyperlipidemic male patients (on low-fat and low-cholesterol diet—no vitamin supplements) were collected on three occasions at intervals of at least 2 weeks before the NA therapy was started. Determinations by the method described showed a group mean NA

TABLE 2. Pooled standard deviations of 366 NA assays of plasma

NA concn (ng/ml)	No. of analyses	Pooled SD (ng)	CV (%)
0-100	113	9.5	35.2
1-200	50	13.0	10.1
2-300	6	26.3	10.9
3-400	22	31.2	8.9
4-500	13	35.0	7.6
5-600	24	36.7	6.6
6-700	20	43.1	6.5
7-800	34	46.1	6.1
8-900	22	49.4	5.6
9-1,000	16	61.2	6.5
10-1,100	12	58.2	5.6
11-1,200	8	67.9	6.0
12-1,300	6	62.4	5.1
13-1,400	4	81.5	6.2
14-1,500	6	79.8	5.5
>1,500	10		7.0

FIG. 2. The influence of a single dose (510 mg of NA) of two sustained-release NA preparations on plasma NA levels in four hyperlipidemic male patients after treatment with NA for 3 weeks (510 mg of NA three times a day). Mean + standard error of the mean of preparation (A), mean - standard error of the mean preparation (B).

concentration of 80 ng/ml (95% limits: 25 to 135 ng/ml), which is in accordance with basal NA serum levels, as determined by use of the flagellate *Tetrahymena pyriformis* (2, 3). During treatment with sustained-release NA preparations (1,530 mg/day) the mean fasting NA level of this group of patients was found to be 705 ng/ml (95% limits: 172 to 1,238 ng/ml), showing a wide variation between subjects. In the hours shortly after NA ingestion by four patients in steady-state treatment, a more or less sharp rise in NA concentration was observed, reflecting the different release rates of the two preparations (Fig. 2).

The relatively simple and time-saving pH metric method described in this paper seems particularly convenient for large series of analyses and standardizations. Moreover, the principle of pH measurement instead of titration may be applicable also for corresponding determinations of other B-vitamins with acid-producing microorganisms.

ACKNOWLEDGMENTS

I thank Irene Laurentiussen for technical assistance, Karsten Schmidt for statistical assistance, and Per O. Gravesen for developing the computer program.

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