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DETERMINATION OF NIACIN METABOLITES 1-METHYL-5-CARBOXYLAMIDE-2-PYRIDONE AND N-1-METHYLNICOTINAMIDE IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A test suitable for detecting latent niacin deficiency was developed. It measures the 24-h urinary output of the two major metabolites of niacin, 1-methyl-5-carboxylamide-2-pyridone and N-1-methylnicotinamide. The two metabolites were isolated from urine using separate ion-exchange extractions. They and their two internal standards were quantitated simultaneously by high-performance liquid chromatography using a reversed-phase ion pairing separation. Detection was by absorbance at 254 nm.

INTRODUCTION

Niacin or nicotinic acid is one of the B vitamins. Its active form, nicotinamide, serves as a precursor in the biosynthesis of the two coenzymes, NAD and NADP, which are involved in a myriad of enzymatic reactions and metabolic pathways. The deficiency of niacin manifests itself as the disease known as pellagra. Pellagra, while once common in corn-eating areas of the world two centuries ago, is now rarely seen except in occasional cases of alcoholism.

The evaluation of niacin status involves the determination of two major urinary metabolites, 1-methyl-5-carboxylamide-2-pyridone (2-PY) and N-1-methylnicotinamide (N1MN). The weight ratio of 2-PY to N1MN is regarded as an important index of niacin status. De Lange and Joubert [1] defined a 2-PY to N1MN ratio of 1.3 to 4.0 as normal and of less than 1.0 as indicative of latent niacin deficiency.

Until the present method, it has been necessary to use two different tests to quantitate both niacin metabolites and thus calculate the metabolite ratio.

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N1MN in urine has been measured by fluorometric methods [2—5] as well as by high-performance liquid chromatography (HPLC) [6, 7]. 2-PY in urine has been measured by a fluorometric method [8], colorimetric methods [9, 10], spectrophotometry [11], and HPLC [12]. The present HPLC method measures both niacin metabolites simultaneously after separate ion-exchange extractions of the two metabolites and their two internal standards.

EXPERIMENTAL

Reagents

N-1-Methylnicotinamide chloride, trigonelline · HCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide · HCl, and nicotinic acid were obtained from Sigma (St. Louis, MO, U.S.A.). Sodium acetate · $3H_2O$, potassium ferricyanide, boric acid, sodium hydroxide, ammonium chloride, sodium carbonate, and methyl iodide were analytical reagent grade and were obtained from Mallinckrodt (St. Louis, MO, U.S.A.). Sodium heptane sulfonate was purchased from Regis (Morton Grove, IL, U.S.A.). Glacial acetic acid was electronic grade from DuPont (Wilmington, DE, U.S.A.). Omnisolve® methanol and reagent grade concentrated HCl were obtained from MCB Manufacturing Chemists Inc. (Cincinnati, OH, U.S.A.). Bio-Rex 70 resin (100—200 mesh, Na^+), AG-1-X4 (100—200 mesh, Cl^-), and prepacked 4 cm × 0.7 cm I.D. polypropylene Econo-Columns® filled with either AG-50W-X8 (200—400 mesh, Na^+) or AG-1-X8 (200—400 mesh, AcO^-) were all purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). Norit "A" neutral pharmaceutical grade decolorizing carbon was obtained from Amend Drug and Chemical Co. (Irvington, NJ, U.S.A.). Thionyl chloride was obtained from Aldrich (Milwaukee, WI, U.S.A.). Methyl ethyl ketone, *n*-butanol, and ethanolamine were reagent grade and were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). N,N-Dimethylformamide (DMF) was obtained from Pfaltz and Bauer (Stanford, CT, U.S.A.). The ethanolamine and DMF were redistilled before use.

The compound 1-methyl-5-carboxyl-2-pyridone was synthesized by the method of Huff [13]. The synthesis of niacin metabolite 1-methyl-5-carboxylamide-2-pyridone, internal standards 1-methyl-5-carboxyl-[N-(2-ethoxy)]-amide-2-pyridone and 1-methyl-3-carboxyl-[N-(2-ethoxy)]amidepyridine chloride, and internal standard precursor 3-carboxyl-[N-(2-ethoxy)]amidepyridine are described below. Melting points were performed on a micro hot stage melting point apparatus from Arthur H. Thomas Company (Philadelphia, PA, U.S.A.) and are uncorrected. Elemental analyses were done by Dow Chemical Western Division Analytical Lab. (Walnut Creek, CA, U.S.A.).

1-Methyl-5-carboxylamide-2-pyridone. 1-Methyl-5-carboxy-2-pyridone (5.00 g, 0.0327 moles) in 15 ml of water was adjusted to pH 5.9 with 5 *N* NaOH. Ammonium chloride (1.76 g, 0.0327 moles) was then added to the sodium salt solution followed by a solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide · HCl (6.89 g, 0.0360 moles) in 11 ml of water. The resulting solution was stirred at 4° C for three days during which time the pH was held below 6.5 by the occasional addition of 1 *N* HCl. The reaction mixture was purified by passage through a 16.5 cm × 11 cm I.D. AG-1-X4 (100—200 mesh, Cl^-) ion-exchange column eluted with water. Fractions containing the product were

distilled under reduced pressure to dryness giving an off-white solid which was recrystallized from *n*-butanol utilizing a decolorizing charcoal hot filtration. After vacuum drying, 2.20 g of a white crystalline solid, m.p. 209—210° C, was obtained. Yield was 44%.

Elemental analysis $C_7H_8N_2O_2$. Theoretical: C 55.25%, H 5.30%, N 18.41%. Found: C 55.25%, H 5.35%, N 18.48%.

1-Methyl-5-carboxyl-[N-(2-ethoxy)]amide-2-pyridone. 1-Methyl-5-carboxy-2-pyridone (14.0 g, 0.0914 moles) was refluxed under nitrogen with 70 ml of thionyl chloride for 1 h, at which time the excess $SOCl_2$ was distilled off under reduced pressure. The resulting solid acid chloride was dissolved in 70 ml of DMF and added dropwise to a suspension consisting of sodium carbonate (9.69 g, 0.0914 moles), ethanolamine (5.61 g, 0.919 moles) and 280 ml of DMF stirring under nitrogen at 4°C. After the 45-min addition, the reaction mixture was stirred for another 0.5 h at 4° C, at which time the reaction mixture was filtered. The filtrate was distilled under reduced pressure to dryness giving a yellowish solid which was recrystallized from *n*-butanol utilizing a decolorizing charcoal hot filtration. One more *n*-butanol recrystallization without the charcoal gave a white crystalline solid which after vacuum drying weighed 12.3 g, a yield of 69%. The m.p. was 173—174°C.

Elemental analysis $C_9H_{12}N_2O_3$. Theoretical: C 55.09%, H 6.17%, N 14.28%. Found: C 54.83%, H 6.06%, N 14.19%.

3-Carboxyl-[N-(2-ethoxy)]amidepyridine. Nicotinic acid (10.0 g, 0.0813 moles) and ethanolamine (4.98 g, 0.0813 moles) were dissolved in 20 ml of water. The pH of the solution was adjusted to 6.0 with concentrated HCl. A solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide - HCl (17.2 g, 0.0894 moles) in 16.5 ml of water was added to the reaction mixture stirring at 4°C. The pH of the reaction mixture was held at 6.0 by the occasional addition of 6 *N* HCl. After 24 h at 4°C the reaction mixture, a light yellow solution, was purified by passage through a 16.5 cm × 11 cm I.D. AG-1-X4 (100—200 mesh, Cl^-) ion-exchange column eluted with water. Fractions containing the product were distilled under reduced pressure to dryness resulting in a yellow solid. This was recrystallized from methyl ethyl ketone utilizing a decolorizing charcoal hot filtration. After vacuum drying, 9.78 g of a white crystalline solid, m.p. 88—90° C, were obtained. The yield was 72%.

Elemental analysis $C_8H_{10}N_2O_2$. Theoretical: C 57.84%, H 6.07%, N 16.86%. Found: C 57.67%, H 6.11%, N 17.00%.

1-Methyl-3-carboxyl-[N-(2-ethoxy)]amidepyridine chloride. 3-Carboxyl-[N-(2-ethoxy)]amidepyridine (11.0 g, 0.0662 moles), methyl iodide (94 g, 0.66 moles), and 110 ml of methanol were refluxed for 7 h, at which time the excess methyl iodide and the methanol were distilled off under reduced pressure. The resulting oil was diluted with 11 ml of water and then purified by passage on a 23 cm × 2.5 cm I.D. AG-1-X4 (100—200 mesh, Cl^-) ion-exchange column eluted with water. The fractions containing the product were distilled under reduced pressure to dryness giving a light yellow solid. This was recrystallized from *n*-butanol utilizing a decolorizing charcoal hot filtration. After vacuum drying, 10.7 g of a white crystalline compound, m.p. 161—162° C, were obtained. The yield was 75%.

Elemental analysis $C_9H_{13}N_2O_2Cl$. Theoretical: C 49.89%, H 6.05%, N 12.93%, Cl 16.36%. Found: C 49.73%, H 6.03%, N 13.04%, Cl 16.18%.

Working standard and controls. A working standard solution consisted of 15 μg/ml 2-PY and 10 μg/ml N1MN in a matrix composed of 0.25 *M* sodium acetate and 0.16 *M* boric acid. Controls were normal urine containing 0.16 *M* boric acid. The low control was a urine having a 24-h volume of greater than 2 l. The high control was a urine having a 24-h volume of less than 1 l.

Storage of reagents. Standards and controls were stored at —20° C. Mobile phase buffer and the two solutions containing the two working internal standards were stored at 4° C. Bio-Rad prepacked AG-1-X8 Econo-Columns were stored at 4°C. Bio-Rad prepacked AG-50W-X8 Econo-Columns, 2 *N* HCl, methanol, and Bio-Rex 70 resin were stored at room temperature.

Extraction of metabolites from standard solution and urine

One milliliter of urine or standard solution was mixed with 100 μl of 150 μg/ml 1-methyl-5-carboxyl-[N-(2-ethoxy)]amide-2-pyridone (2-PY internal standard) in water and 100 μl of 2 *N* HCl. This solution was added to a 4 cm × 0.7 cm AG-50W-X8 ion-exchange column which dripped directly into a 4 cm × 0.7 cm AG-1-X8 ion-exchange column. The urine solution was eluted with 7 ml of water added to the AG-50W-X8 column. The last 5 ml of the effluent from the AG-1-X8 column were collected, saved, and labeled "2-PY extract". One milliliter of urine or standard solution was diluted with 19 ml of water and added to a 4 cm × 0.7 cm Bio-Rex 70 ion-exchange column. The column was then washed with two 5-ml aliquots of water. This was followed by the addition of 5 ml of 0.2 *M* sodium acetate solution containing 2.4 μg/ml 1-methyl-3-carboxyl-[N-(2-ethoxy)]amidepyridine chloride (N1MN internal standard). The last 4 ml of effluent from the Bio-Rex 70 column were collected, saved, and labeled "N1MN extract." Fifty microliters of each 2-PY extract were mixed with 600 μl of the corresponding N1MN extract to make a solution for injection into the chromatograph.

High-performance liquid chromatography

The chromatography was performed on an Altex Model 330 isocratic liquid chromatograph (Altex Scientific, Berkeley, CA, U.S.A.) equipped with a 15 cm × 4.6 mm I.D. Ultrasphere Ion Pairing analytical column of 5 μm particle size (Altex Scientific) and a 3 cm × 4.6 mm I.D. Brownlee MPLC LiChrosorb C-18 guard column of 10 μm particle size (Rheodyne, Berkeley, CA, U.S.A.).

The temperature of the analytical column was kept at 35° C by an aluminum column temperature control block (Waters Assoc., Milford, MA, U.S.A.) connected to a Haake Model FJ temperature-controlled circulating water bath (Haake Instruments, Saddle Brook, NJ, U.S.A.). The volume of injection was 50 μl. The mobile phase used was 91% buffer (containing 0.15 *M* acetate, pH 5.0, and 0.01 *M* sodium heptanesulfonate) and 9% methanol. The flow-rate was 1.0 ml/min. Detection of the analytes and internal standards was by UV absorbance at 254 nm, 0.01 absorbance units full scale. Analytes and internal standards were quantitated by manually measuring peak heights as recorded by a Hewlett-Packard strip chart recorder, Model 7123A (Hewlett-Packard, Avondale, PA, U.S.A.).

RESULTS

HPLC separation

Typical chromatograms obtained from a standard, a low control, and a high control are shown in Fig. 1. The metabolite 2-PY eluted at 3.5 min, its internal standard eluted at 4.4 min, the metabolite N1MN eluted at 5.6 min, and its internal standard eluted at 8.0 min. The low control 2-PY level was 6.64 μg/ml of urine and its N1MN level was 1.90 μg/ml of urine. From the chromatogram it can be seen that detection limits of 0.3 μg/ml of urine for both metabolites were possible.

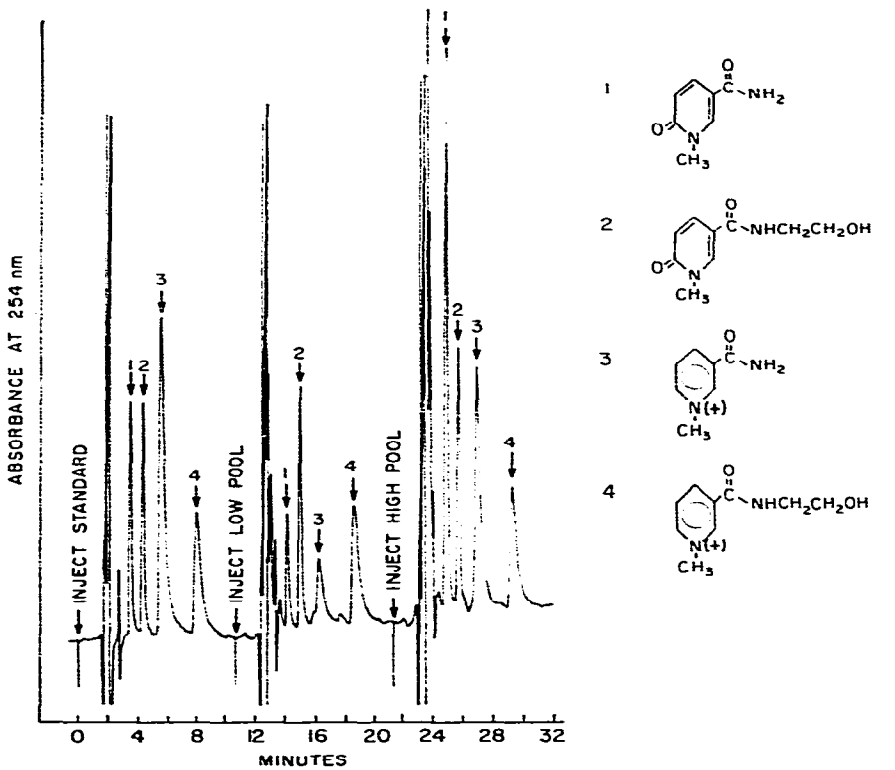

Fig. 1. Chromatogram of standard, low control, and high control with relevant structures. Compound 1 is niacin metabolite 1-methyl-5-carboxylamide-2-pyridone. Compound 2 is internal standard 1-methyl-5-carboxyl-[N-(2-ethoxy)]amide-2-pyridone. Compound 3 is niacin metabolite N-1-methylnicotinamide. Compound 4 is internal standard 1-methyl-3-carboxyl-[N-(2-ethoxy)]amidepyridine.

Method linearity

Linearity of the method was demonstrated using urine from a donor after ingestion of a vitamin preparation containing nicotinamide. The undiluted specimen contained 99 μg/ml of the 2-PY metabolite and 23 μg/ml of the N1MN metabolite. These concentrations were approximately four times higher than the mean of the reference ranges for these metabolites. The specimen (containing 0.16 *M* boric acid) was diluted with the same solution used as

the matrix for the 15 μg/ml 2-PY and 10 μg/ml N1MN standard. A linear relationship was observed for both metabolites with up to a five-fold dilution of this high specimen when the peak height ratio of metabolite to its internal standard was plotted against the dilution of the urine sample.

Method precision

Precision of the assay was studied by running three different controls in duplicate with one standard in each of ten different batches. Table I shows the mean, intra-run coefficient of variation (C.V.), and the inter-run coefficient of variation for the metabolites in each control. The inter-run C.V. of the 2-PY metabolite assay was approximately 3% and the inter-run C.V. of the N1MN metabolite assay was approximately 5%. The C.V. was relatively constant with concentration for both metabolites and the ratio of intra-run C.V. to inter-run C.V. was approximately 0.5.

TABLE I

METHOD IMPRECISION

Data are based on ten runs with two determinations per run.

	Low control	Medium control	High control
2-PY metabolite			
Mean, $\bar{X}$ (μg/ml)	6.64	14.91	32.33
Intra-run C.V. (%)	1.6	1.1	1.3
Inter-run C.V. (%)	2.7	2.5	2.8
N1MN metabolite			
Mean, $\bar{X}$ (μg/ml)	1.90	4.17	8.04
Intra-run C.V. (%)	2.9	2.2	2.5
Inter-run C.V. (%)	5.1	4.7	4.7

Metabolite extraction study

The elution of the niacin metabolites and their internal standards from the ion-exchange columns was studied by collecting 0.5-ml fractions of effluent from the ion-exchange columns and analyzing 50 μl of each 0.5-ml fraction with the HPLC system. Peak heights of each metabolite or internal standard detected in the 0.5-ml fractions were measured in millimeters and plotted against volume of effluent coming from either the Bio-Rex 70 columns or the AG-1-X8 column. Fig. 2 and 3 show the elution profile for the standard and a spiked control containing 14.9 μg/ml 2-PY and 10.2 μg/ml N1MN. Based on Fig. 2, the 2—7-ml fraction of the distilled water eluant contained most of the 2-PY and its internal standard and this fraction was used for the assay. Likewise, most of the N1MN was eluted in the 1—5-ml fraction of the 0.2 *M* sodium acetate eluant. It should be noted that the elution curve of the N1MN internal standard was not a "peak". This was a consequence of adding the N1MN internal standard to the eluting buffer rather than to the specimen prior to its introduction onto the Bio-Rex 70 column. The N1MN internal standard could not be added to the specimen prior to the Bio-Rex 70 extraction because of erratic recovery.

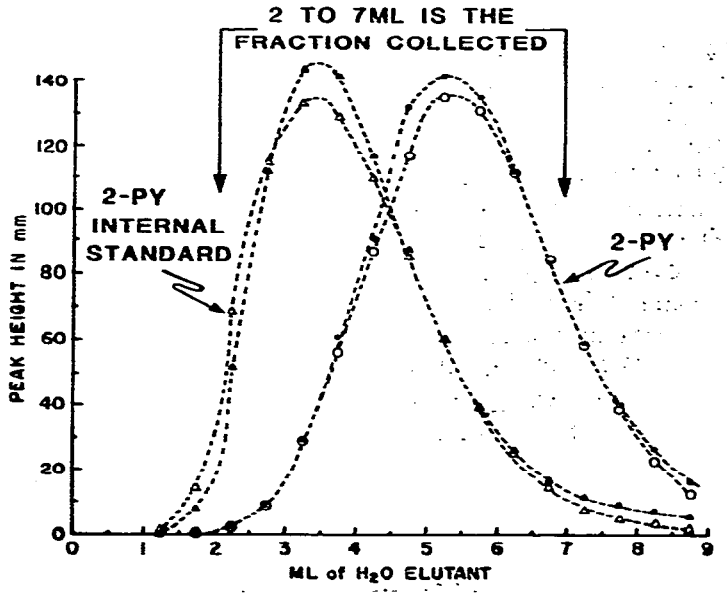

Fig. 2. Elution profile of 2-PY metabolite and 2-PY internal standard from sequential AG-50W-X8 and AG-1-X8 columns. (▲), 2-PY internal standard, 15 μg, spiked into 1 ml of medium urine control; (●), 2-PY metabolite, 14.9 μg/ml, in medium urine control; (△), 2-PY internal standard, 15 μg, spiked into 1 ml of standard; (○), 2-PY metabolite, 15.0 μg/ml, in standard.

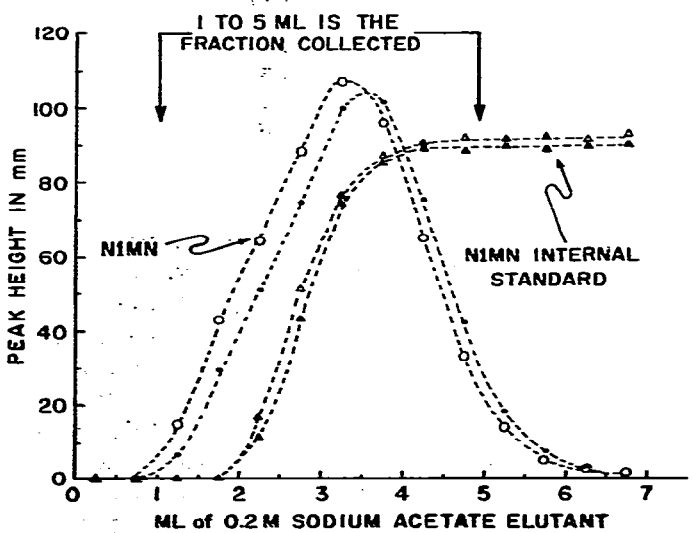

Fig. 3. Elution profile of N1MN metabolite and N1MN internal standard from Bio-Rex 70 columns. (▲), N1MN internal standard, 4.8 μg/ml, in 0.2 *M* sodium acetate buffer used to elute spiked medium control; (●), N1MN metabolite, 10.2 μg/ml, in spiked medium urine control; (△), N1MN internal standard, 4.8 μg/ml, in 0.2 *M* sodium acetate buffer used to elute standard; (○), N1MN metabolite, 10.0 μg/ml, in standard.

Recovery studies

An experiment was designed to determine whether urine ionic strength affected recovery since normal random urines range in osmolality from 50 to 1200 mosm/kg H_2O. N1MN (5 and 10 μg/ml) and 2-PY (10 and 20 μg/ml) were added to five different sodium acetate solutions (containing 0.16 *M* boric acid) varying from 200 to 1000 mosm/kg H_2O.

The extraction recoveries and the recovery ratios of metabolites to internal standards were calculated. The recovery of 2-PY ranged from 71% to 78% and the 2-PY internal standard recovery ranged from 87% to 89%. The recovery of the N1MN internal standard ranged from 54% to 61%. The recovery of N1MN ranged from 82% to 90% at matrix osmolality 200—800 mosm/kg H_2O but fell to about 77% at 1000 mosm/kg H_2O. The result was that the recovery ratio of N1MN to N1MN internal standard was around 1.5 at matrix osmolality 200—800 mosm/kg H_2O, but was only 1.3 at a matrix osmolality of 1000 in osm/kg H_2O (see Table II).

TABLE II

RECOVERY RATIOS OF METABOLITES TO INTERNAL STANDARDS VS. MATRIX OSMOLALITY

Each matrix consisted of the proper amount of sodium acetate to produce the desired osmolality in addition to 0.16 *M* boric acid.

Matrix osmolality (mosm/kg H_2O)	Recovery ratios			
	2-PY spike 10 μg/ml	2-PY spike 20 μg/ml	N1NM spike 5 μg/ml	N1MN spike 10 μg/ml
200	0.82	0.82	1.52	1.54
400	0.85	0.85	1.46	1.55
600	0.84	0.85	1.52	1.61
800	0.86	0.88	1.40	1.46
1000	0.84	0.83	1.32	1.29

The recovery of 2-PY and N1MN from three different urines having osmolalities of 201, 561, and 945 mosm/kg H_2O was determined. The three urines were spiked with both 2-PY and N1MN to add an additional 10 μg of each metabolite to 1 ml of urine. After adding boric acid to a level of 0.16 *M*, the three baseline urines and the three spiked urines were run through the assay in duplicate with a single standard solution. Based on the standard solution, recovery of the 2-PY metabolite ranged from 93% to 96% while recovery of the N1MN metabolite ranged from 93% to 95% (see Table III).

An experiment was performed to establish whether the recoveries of the N1MN and its internal standard would vary with the bed heights of the Bio-Rex 70 columns. Unlike the prepacked AG-50W-X8 and AG-1-X8 ion-exchange columns, the Bio-Rex 70 columns were individually packed just prior to starting the assay and the bed heights were found to vary slightly. The resin bed heights in the experiment ranged from 36 mm to 44 mm. Percentage recovery and the ratio of N1MN peak height to that of its internal standard

TABLE III

RECOVERY OF 2-PY AND N1MN FROM THREE SPIKED URINES WITH DIFFERENT OSMOLALITIES

Specimens and their spikes were run in duplicate. Recovery was calculated against a standard solution run singly.

	Specimen 1	Specimen 2	Specimen 3
Urine osmolality (mosm/kg H_2O)	201	561	945
Baseline urine, 2-PY level (μg/ml)	4.6	17.4	31.5
10 μg/ml spiked urine, 2-PY level (μg/ml)	13.9	26.8	41.1
Recovery of 10 μg/ml 2-PY spike (%)	93	94	96
Baseline urine, N1MN level (μg/ml)	1.1	5.8	10.9
10 μg/ml spiked urine, N1MN level (μg/ml)	10.4	15.1	20.4
Recovery of 10 μg/ml N1MN spike (%)	93	93	95

TABLE IV

RECOVERY OF N1MN METABOLITE AND INTERNAL STANDARD VS. BIO-REX 70 RESIN BED HEIGHT

Data based on a solution containing 10 μg/ml N1MN, 0.25 *M* sodium acetate, and 0.16 *M* boric acid.

Resin bed height (mm)	Recovery (%)		N1MN:N1MN internal standard peak height ratio
	N1MN metabolite	N1MN internal standard	
36	95.5	62.7	3.0
38	95.1	60.3	3.1
40	93.5	57.1	3.0
42	83.7	50.0	3.3
44	75.6	42.9	3.4

peak height are shown in Table IV. As the resin bed height in the column increased, the recoveries of both the metabolite and its internal standard decreased. The N1MN metabolite to N1MN internal standard ratio was relatively constant. An ideal internal standard would have given a constant recovery ratio, but bed heights of 36—40 mm provided acceptable recovery and ratios.

Sample stability

Seven-day stability of urine specimens was achieved for both niacin metabolites at 30° C, 4° C, and —20° C by the addition of 1 g of boric acid per 100 ml (0.16 *M* boric acid) of urine. Each stability study had a total of sixteen data points accumulated over four independent runs. Table V lists the calculated rectangular regression lines and the correlation coefficient for the six separate stability studies.

TABLE V

STABILITY STUDY RESULTS FOR NIACIN METABOLITES (μg/ml) IN URINE PRESERVED WITH 0.16 *M* BORIC ACID AT 30°C, 4°C, AND —20°C

Metabolite	Storage condition	Calculated rectangular regression line*	Correlation coefficient
2-PY	30°C	$Y = 1.003X + 0.11$	0.9922
2-PY	4°C	$Y = 0.997X - 0.09$	0.9942
2-PY	—20°C	$Y = 0.973X + 0.06$	0.9925
N1MN	30°C	$Y = 0.991X + 0.06$	0.9952
N1MN	4°C	$Y = 0.984X + 0.10$	0.9945
N1MN	—20°C	$Y = 0.969X + 0.01$	0.9934

*X is day 0 and Y is day 7.

Reference range

A reference range study was conducted on 27 female and 23 male adults. All 50 donors in this study stated that to their knowledge they had no liver damage, were not in the second or third trimester of pregnancy, and did not take oral contraceptives or B vitamins. Boric acid (1 g) was used to preserve 100 ml of each 24-h urine collection. Normal range histograms for mg 2-PY per 24 h, mg N1MN per 24 h, and the 2-PY to N1MN weight ratio over 24 h are shown in Figs. 4—6. All three normal ranges form skewed (non-gaussian) distributions with normal range estimates [14] of 6.0—51.3 mg 2-PY per 24 h, 1.6—14.8 mg N1MN per 24 h, and a 2-PY to N1MN weight ratio of 1.76 to 5.90 (95% confidence limits).

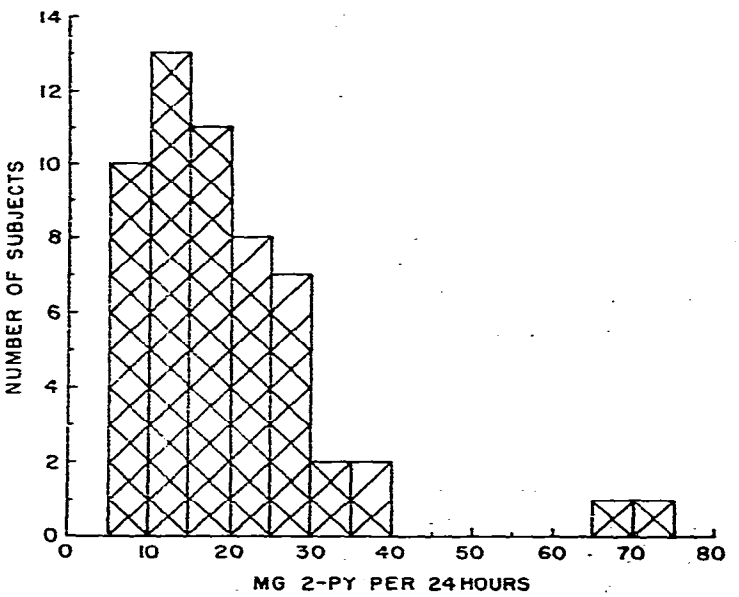

Fig. 4. Reference range histogram of 24-h 2-PY excretion. For 50 normals, reference range estimate (95% confidence limits) was 6.0—51.3 mg of 2-PY per 24 h.

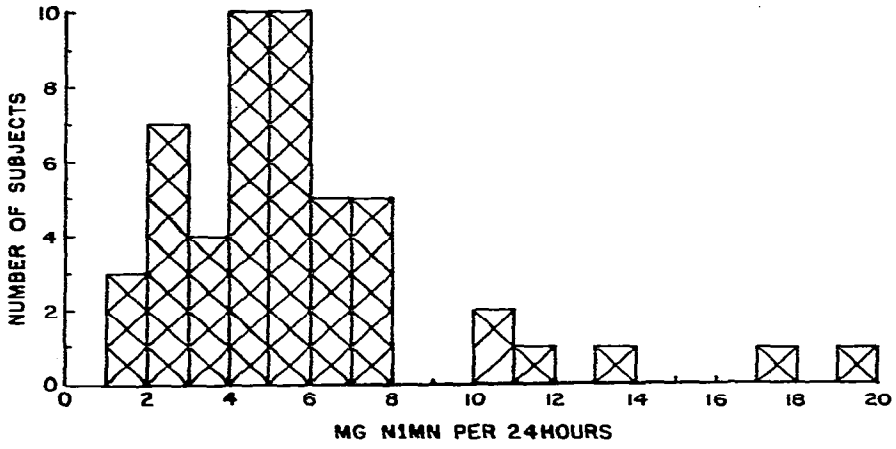

Fig. 5. Reference range histogram of 24-h N1MN excretion. For 50 normals, reference range estimate (95% confidence limits) was 1.6—14.8 mg of N1MN per 24 h.

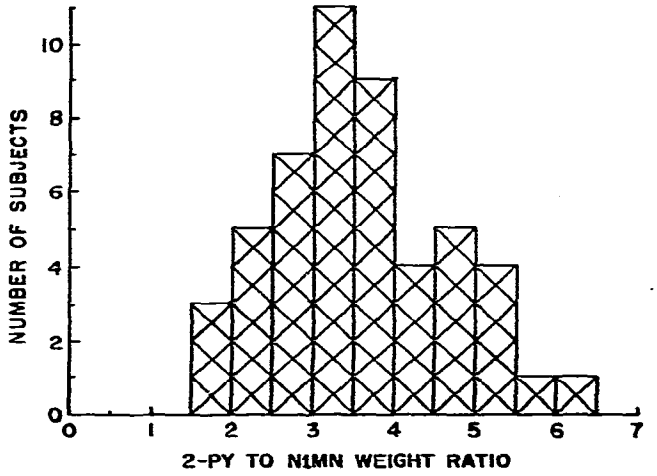

Fig. 6. Reference range histogram of 24-h 2-PY to N1MN weight ratio. For 50 normals, reference range estimate (95% confidence limits) was 1.76—5.90.

Interference study

A total of 37 drugs and drug metabolites were examined for interference (Table VI). No interference with any of the niacin metabolites or internal standards was seen.

DISCUSSION

The simultaneous measurement of both major human metabolites of niacin represents a novel step forward in the determination of niacin status. The present method utilized a modification of the 2-PY extraction of Price [11] and an approach similar to that used by Shaikh et al. [6] in the extraction of N1MN. The result was a very clean niacin metabolite extract providing an ex-

TABLE VI

COMPOUNDS TESTED THAT DO NOT INTERFERE WITH THE ASSAY

Compound	Concentration (μg/ml)	Compound	Concentration (μg/ml)
Acetylisoniazid	200.0	Isoniazid	100.0
Amitriptyline	5.0	Lidocaine	2.5
Brompheniramine	2.5	Meperidine	2.5
Caffeine	20.0	Meprobamate	20.0
Chlorpheniramine	5.0	Methadone	5.0
Chlorpromazine	10.0	Methadone metabolite	5.0
Codeine	10.0	Methapyrilene	5.0
Desalkylflurazepam	10.0	Methaqualone	5.0
Desipramine	5.0	Methyprylon	5.0
N-Desmethyldiazepam	10.0	Norpropoxyphene	10.0
N-desmethyldoxepin	5.0	Nortriptyline	5.0
Dextromethorphan	10.0	Oxycodone	10.0
Diazepam	10.0	Pentazocine	10.0
Diphenhydramine	2.5	Phenacetin	20.0
Doxepin	5.0	Phencylidine	2.5
Doxylamine	5.0	Promazine	5.0
Flurazepam	10.0	Propoxyphene	5.0
Glutethimide	20.0	Trihexyphenidyl	5.0
Imipramine	5.0		

cellent baseline for HPLC. The selectivity gained in the double ion-exchange extraction procedure was demonstrated by the lack of interference from the 37 compounds tested.

The excellent precision of the 2-PY analysis was due to the consistency of the prepacked Bio-Rad AG-50W-X8 and AG-1-X8 ion-exchange columns. These columns exhibited unusual uniformity with respect to resin volume. However, efforts to utilize prepacked Bio-Rex 70 (100—200 mesh, Na^+) ion-exchange columns were unsuccessful because they contained substantial quantities of "fines" causing unacceptable variation in flow-rate.

Recovery experiments showed that concentrated urine with osmolalities greater than 1000 mosm/kg H_2O caused recovery of the N1MN metabolite to be reduced. Concentrated urines with 24-h total volumes near 500 ml (as compared with more commonly found 1000—1500-ml outputs) should be diluted 1 plus 1 with distilled water containing 0.16 *M* boric acid prior to the 1 plus 19 dilution of the urine with distilled water. This results in a urine solution which gives a good recovery of the N1MN metabolite when run on the 4 cm × 0.7 cm I.D. Bio-Rex 70 columns.

With so many different analytical techniques used in the past to measure the two major niacin metabolites it was not surprising that reference ranges published in the literature vary considerably. Table VII shows some reported reference ranges for comparison with Figs. 4—6 along with three pathological subpopulations selected from the literature. Relatively good agreement was evident in the normal daily N1MN excretion. However, different researchers have reported varying 24-h urine values for the 2-PY metabolites of niacin as well as the 2-PY to N1MN weight ratio.

Prinsloo et al. [21] studied eleven children with pellegra and showed clearly that they had low urinary levels of niacin metabolites. A metabolite ratio of 0.6 ± 0.4 was extremely low compared to the reported normal values shown in Table VII. Alcoholics and people with cirrhosed livers may also exhibit low 2-PY to N1MN metabolite weight ratios. This may be due to the fact that their damaged livers were slow to oxidize the N1MN to the 2-PY metabolite and consequently the N1MN was excreted before the oxidation could occur. The difference between a well-fed alcoholic and a true pellagrin would be that the absolute 24-h excretion values of niacin metabolites are low for a pellagrin and high for the well-fed alcoholic. A 24-h urine collection is required for the analysis of niacin status because conversion of the results from an untimed, random specimen to 24-h excretion values on the basis of creatinine concentration can lead, in the case of a pellagrin, to an incorrect interpretation. This is due to the abnormally low creatinine values which may be found in cases of pellagra [21].

TABLE VII

REFERENCE RANGES WITH STANDARD DEVIATIONS

Author Date	2-PY (mg per 24 h)	N1MN (mg per 24 h)	2-PY:N1MN by weight	Sample population
Present 1982	20.1 ± 12.9	5.8 ± 3.6	3.60 ± 1.06	50 normals
Vivan et al. [15] 1958	9.1 ± 2.5	5.9 ± 2.1	1.57 ± 0.16	4 normals
Motegi [16] 1960	11.8	4.6	—	24 normals
Joubert and DeLange [17] 1962	13.9 ± 5.7	7.7 ± 2.3	—	10 normals
DeLange and Joubert [1] 1964	—	—	2.06 ± 0.74	12 normals
Marnardi and Tenconi [18] 1964	12.2	6.7	—	50 normals
Leklem et al. [19] 1975	17.8 ± 3.5	6.0 ± 1.8	3.00 ± 0.72	9 normals
Joubert and DeLange [17] 1962	4.4 ± 3.9	7.0 ± 3.2	—	10 alcoholics
Gabuzda and Davidson [20] 1962	—	11.3 ± 3.0	—	10 cirrhosis patients
Prinsloo et al. [21] 1968	1.0 ± 0.7	1.7 ± 1.0	0.6 ± 0.4	11 pellagra children

In summary, a new HPLC method has been developed to measure niacin metabolites in urine. The method showed excellent precision (C.V. 2.5—5%), recovery and linearity. Samples could be stored for seven days at 30°C, 4°C or —20°C. No interfering peaks were found from 37 drugs tested and the reference range was consistent with others that have been reported.

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