Journal of Chromatography, 230 (1982) 309—317
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1278

DETERMINATION OF NICOTINAMIDE AND METABOLIC PRODUCTS IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

RALPH W. McKEE, YOUNG AE KANG-LEE, MARIO PANAQUA and MARIAN E. SWENDSEID*

Division of Environmental and Nutritional Sciences, UCLA School of Public Health, Los Angeles, CA 90024 (U.S.A.)

(First received November 6th, 1981; revised manuscript received February 23rd, 1982)

SUMMARY

A high-performance liquid chromatographic procedure has been developed for the quantitation of nicotinamide, nicotinic acid, nicotinuric acid, 1-methylnicotinamide and 1-methyl-2-pyridone-5-carboxamide in rat and human urines. The procedure utilizes a Varian Model 5020 liquid chromatograph with a UV detector, and an Altex 15 cm × 4.6 mm Ultrasphere-ODS column, employing a linear ion-pair mobile phase gradient. Solvent A contains 10 m*M* concentrations of pentanesulfonic acid (PSA), tetramethylammonium chloride (TMA) and KH_2PO_4, and solvent B contains PSA, TMA and acetonitrile. Different pH values for solvent A vary the retention times and thus the separation of the five compounds. Temperature of the system is critical. The conditions found most satisfactory were pH 3.30 and 24.5°C.

INTRODUCTION

Rapid, precise analyses of blood and urine for vitamins and their metabolites are of paramount importance in the evaluation of nutritional status. Progress in this field has been greatly accelerated by the use of high-performance liquid chromatography (HPLC). In 1973, Williams and co-workers [1] employed HPLC for separating and quantifying nicotinic acid and riboflavin in aqueous solutions. These investigators [2] modified and extended their reversed-phase HPLC procedure to separate mixtures of vitamins B_2, B_{12}, C, and the four fat-soluble vitamins. Since that time these and other vitamins and derivatives have been separated and measured employing various modifications of HPLC [3—10]. In 1980, De Vries et al. [11] determined nicotinamide in human plasma and urine by ion-pair reversed-phase HPLC.

We present a method for determining nicotinamide and several of its metabolites in rat and human urines with HPLC using a linear ion-pair mobile

0378-4347/82/0000—0000/$02.75

phase gradient. The compounds reported are nicotinic acid (niacin), nicotinamide, 1-methylnicotinamide, nicotinuric acid and 1-methyl-2-pyridone-5-carboxamide. A preliminary report of a method for separating some of these compounds has already appeared [12].

MATERIALS AND METHODS

Apparatus

The apparatus employed was a Varian Model 5020 high-performance liquid chromatograph, incorporating a Varian Vari-Chrom UV detector, a Rheodyne 7126 injector with a 20-μl loop, and an Altex 15 cm × 4.6 mm Ultrasphere-ODS column, particle size 5 μm. The HPLC system was interfaced to a Varian CDS 401 system for gradient programming and data processing.

Chemicals and solvents

The 1-pentanesulfonic acid sodium salt (PSA) and tetramethylammonium chloride (TMA) were obtained from Eastman-Kodak (Rochester, NY, U.S.A.). The water, methanol and acetonitrile were Baker Analyzed HPLC grade reagents (J.T. Baker, Phillipsburg, NJ, U.S.A.). All other chemicals used in preparing the two solvent systems were of analytical reagent grade. The nicotinamide (NAM) was a Calbiochem (Los Angeles, CA, U.S.A.) product; the nicotinic acid (NA), 1-methylnicotinamide chloride (NMN) and nicotinuric acid (NUA) were from Sigma (St. Louis, MO, U.S.A.); and the 1-methyl-2-pyridone-5-carboxamide (2-PYR) was kindly prepared by Dr. Michael Jung of the UCLA Department of Chemistry. All five compounds were kept desiccated.

Several A solvents with different amounts of mobile phase ion-pairing agents, PSA and TMA, and different pH values were prepared and evaluated. The one selected for the work reported here contained 10 m*M* concentrations of each of the two reagents, and 10 m*M* KH_2PO_4 in HPLC grade water. Solvent B consisted of 100 ml HPLC grade water containing 10 m*M* PSA and 10 m*M* TMA, plus 900 ml acetonitrile. The solvents were degassed by sonication for 2—3 min.

Standard solutions

Solutions of different known strengths were prepared in HPLC grade water for each of the five compounds (NAM, NA, NMN, NUA and 2-PYR). They were employed as standards either singly or in combinations and for adding to urine samples to confirm the position and quantification of the peaks. Use of an internal standard has been considered but a suitable one has not been determined. Therefore standard solutions were determined at least twice each day, runs were made and the values determined to be constant as described below under Sensitivity and accuracy.

Urine specimens

Urines (48 h) were collected from three-month-old Sprague-Dawley rats into 1 *N* hydrochloric acid and diluted to 0.1 *N* and a known volume. The animals were maintained on a diet containing 12% casein and 90 mg niacin per kg diet. The human urines were either 24-h collections made into toluene, or spot

samples. There was no diet supplementation with nicotinamide or nicotinic acid. Urine samples not analyzed immediately upon collection were kept frozen. Urine aliquots were filtered through 0.2-μm Metricel Gelman membrane filters and analyzed without further treatment, except that dilution was necessary for some urine samples. Trial treatment with perchloric acid to remove any protein did not alter concentrations of the compounds. No other fractionation was attempted. Repeated freezing, storing at −20°C, and thawing over a period of at least five weeks did not alter the concentrations in either the urines or standard solutions.

Operation of apparatus

The analyses were performed with the UV detector set for 254 nm, a band width of 8 nm, and an absorbance range of 0.05. The CDS 401 was programmed to record the concentration of each compound in μg/ml, with an attenuation of 512 and a plotting rate of 1 cm/min. Aliquots (20 μl) of standards or urines were injected for analysis with a flow-rate of 2 ml/min and a linear ion-pair mobile phase gradient programmed from 0% to 6% solvent B in 6 min. The five compounds were eluted within 4 min. The column was regenerated for 2 min with a solvent A/B ratio of 94:6, and then equilibrated for 5 min with an A/B ratio of 100:0.

RESULTS

Calibration of HPLC apparatus

Each of the five standard solutions was chromatographed separately at several pH values. The average of three determinations was used for the final calibration factor for each of the five standards. Mixtures of the five standards were then chromatographed (Fig. 1A and Table I).

Retention times of the standards were rechecked each time a new batch of solvent was prepared and at least twice during a day of analyzing.

TABLE I

RETENTION TIMES (min) AT 24.5°C FOR THE FIVE COMPOUNDS AT DIFFERENT pH VALUES

Solvents A and B as described in text, were used. Each value is an average of 2—4 assays, with no attempt made to show statistically significant differences. Between-assay retention time variation was ± 0.05 min. Therefore it is important to select conditions for which the retention times from one compound to another differ by at least 0.20 min.

Compound	pH value for solvent A				
	2.20	3.30	4.40	5.50	6.80
NA	1.70	1.35	1.20	1.00	0.85
NMN	1.80	1.90	1.95	2.05	2.30
NAM	2.00	2.85	3.40	3.50	3.50
NUA	3.60	3.10	2.40	1.30	1.20
2-PYR	3.30	3.40	3.35	3.35	3.30

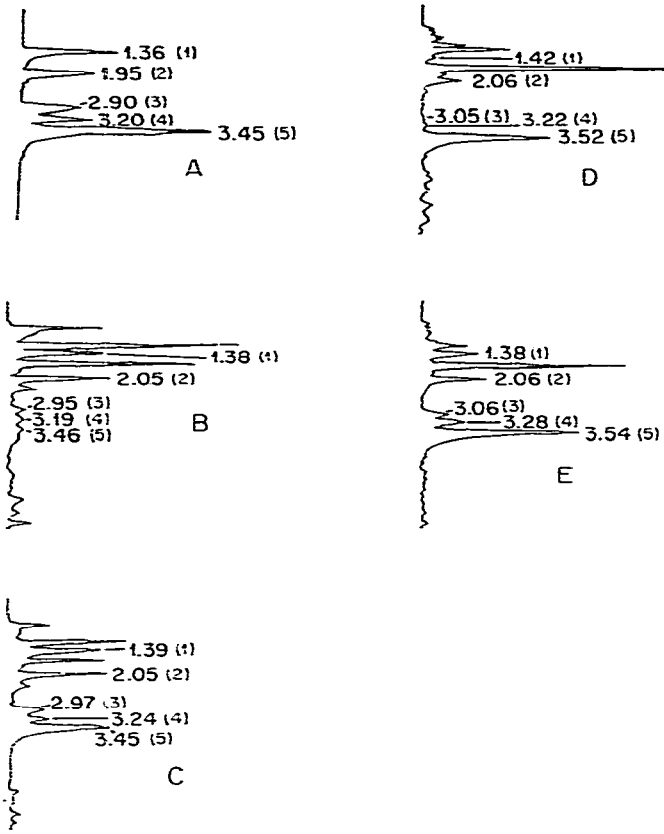

Fig. 1. Tracing of elution pattern of the five compounds: (1) NA, (2) NMN, (3) NAM, (4) NUA, and (5) 2-PYR. Analyses were made with solvent A, pH 3.30, and temperature of 24.5°C. Retention times in minutes are noted in the five tracings: (A) standard curves, (B) rat urine (see also Table III), (C) rat urine plus standards, (D) human urine (see also Table IV), (E) human urine plus standards.

Sensitivity and accuracy

Standards (1 μg) in 20-μl samples (50 μg/ml) could be determined with an accuracy of about 3% and 0.1 μg with an accuracy of about 6%. The following are examples of two 2-PYR standards analyzed over a period of two weeks. Six determinations of one standard gave a mean value of 48.0 μg/ml (46.4–48.8), S.E.M. 0.35. Twelve determinations of a second standard gave a mean value of 4.24 μg/ml (4.03–4.45), S.E.M. 0.034. Amounts as small as 0.01 μg were readily detectable but the determined values varied as much as 50%.

Composition and pH of solvent A

Eight different concentration combinations of PSA and TMA, with a constant amount of KH_2PO_4, were tested at different pH values. The retention time and calibration factors changed with each solvent. The concentrations found to be most satisfactory were 10 m*M* for each of the three components. Concentrations of PSA, TMA and acetonitrile in solvent B were held constant (see Chemicals and solvents section).

Changes in the pH of solvent A greatly altered the retention times and the calibration factors (Table I) so that in going from solvents of different pH the system had to be reprogrammed.

Effects of temperature

Maintenance of temperature of the solvents and column was found to be important for consistent retention times for the five compounds. With variations in temperature, retention times for components having a stronger affinity for the column packing were altered to a greater degree than those components eluted earlier in the run (Table II). At the lower temperature there was a somewhat greater spread of the retention times. For this study solvent A, pH 3.30 and a temperature of 24.5°C were employed.

TABLE II

RETENTION TIMES (min) AT DIFFERENT TEMPERATURES FOR THE FIVE COMPOUNDS

Solvent A, pH 3.30 was used. Each value is an average of 2—4 assays, with no attempt made to show statistical differences. Between-assay retention time variation is ± 0.05 min.

Compound	Temperature of column		
	22.5°C	24.5°C	26.0°C
NA	1.40	1.35	1.35
NMN	2.00	1.90	1.90
NAM	3.00	2.85	2.80
NUA	3.25	3.10	3.00
2-PYR	3.55	3.40	3.30

Chromatography of urine samples

Duplicate analyses were made on the urines from six rats for the five components, and values for one are shown in Table III. Fig. 1B is a tracing of the chromatogram for this urine. The compounds were identified by retention times and by addition of an equal volume of a mixture of the five standards (Fig. 1C). Percent recoveries of the amounts added are shown in Table III.

One rat urine which contained 4.65 μg 2-PYR/ml (three determinations, 4.61—4.71), when diluted with an equal volume of a 2-PYR standard containing 4.24 μg/ml, was determined to have 4.15 μg/ml (five determinations, 3.98—4.36). Theoretically the value should be 4.65/2 + 4.24/2 = 4.45 μg/ml, a difference of 7%. Another rat urine with only traces of 2-PYR gave values of 0.17—0.49 μg/ml (four determinations) and recoveries of added 2-PYR that were in error by more than 50%.

Also, duplicate analyses were made on the urines from six human subjects for the five components and the compounds identified, as with the rat urines, by retention times and by the addition of an equal volume of a mixture of the five standards (Fig. 1D and E). Data are given in Table IV. These same urines were also analyzed for NMN by the chemical method of Pelletier and Brassard [13]. There was good agreement with values averaging 7% higher for the chemical procedure.

TABLE III

AMOUNTS OF NA, NMN, NAM, NUA AND 2-PYR IN ONE RAT URINE, AND RECOVERIES OF THE FIVE COMPOUNDS ADDED TO THE ONE, AND TO SIX RAT URINES

Aliquots of 20 μl were analyzed by HPLC [(1) and (4)]; for recoveries the urines were diluted with an equal volume of an aqueous mixture of the five compounds.

Component	Rat urine analyses and recoveries					Recovered (%) from six rat urines	
	Amount present (μg/ml) (1)	Amount added (μg/ml) (2)	Sum (1) + (2) (μg/ml) (3)	Total amount measured (μg/ml) (4)	Recovered (%) (4)/(3)	Range	Average
NA	23.8	25.0	48.8	48.4	99	92—99	95
NMN	29.1	25.0	54.1	58.2	108	93—110	102
NAM	16.2	25.0	41.2	37.9	92	85—104	93
NUA	7.4	25.0	32.4	30.0	93	89—111	100
2-PYR	1.8	25.0*	26.8	30.1	112	98—133	117

*Recoveries of 2.12 μg/ml from an additional rat urine gave an error of 7%, see section Chromatography of urine samples.

TABLE IV

AMOUNTS OF NA, NMN, NAM, NUA AND 2-PYR IN ONE HUMAN URINE, AND RECOVERIES OF THE FIVE COMPOUNDS ADDED TO THE ONE URINE, AND TO SIX HUMAN URINES

Aliquots of 20 μl were analyzed by HPLC [(1) and (4)]; for recoveries the urines were diluted with an equal volume of an aqueous mixture of the five compounds.

Component	Human urine analyses and recoveries					Recovered (%) from six human urines	
	Amount present (μg/ml) (1)	Amount added (μg/ml) (2)	Sum (1) + (2) (μg/ml) (3)	Total amount measured (μg/ml) (4)	Recovered (%) (4)/(3)	Range	Average
NA	0	25.0	25.0	25.7	103	92—116	105
NMN	7.1	25.0	32.1	33.4	104	89—105	100
NAM	0	25.0	25.0	23.6	94	75—112	95
NUA	5.0	25.0	30.0	33.6	112	99—112	107
2-PYR	8.7	25.0	33.7	35.8	106	95—108	102

DISCUSSION

The presence of large numbers of components in rat and human urines made separation and quantification of nicotinamide and metabolic products very difficult. Often there was overlapping of peaks of unknown components with one or more of the five compounds studied and often this was the reason for excessively high values for recoveries of the added compounds. This was especially the situation for 2-PYR in rat urines. To obtain better separations, varying concentrations of PSA and TMA in the solvent were found to be beneficial. Varying the pH has been found to be the most beneficial (Table I). With higher pH values the retention times of 2-PYR remained fairly constant while those of the acids shifted to shorter times and the amides to longer times. It also was important to maintain the temperature of the system within 1°C (Table II).

De Vries et al. [11] have determined NMN in human plasma and urine, precleaning the samples with a small column of Sep-Pak C_{18} followed by HPLC separation and the use of an ion-pair in combination with a reversed-phase system. The sensitivity of their method is 0.1 mg/l for plasma and 1 mg/l for urine. At a plasma level of 0.5 mg/l the coefficient of variation (n = 10) was 3.4% and at a urine level of 5 mg/l the coefficient of variation was 6.8%. The variations we obtained for NMN are comparable (Tables III and IV).

It is of interest to note that the rats excreted large quantities of NA, NMN and NAM and relatively small amounts of NUA and 2-PYR. Chang and Johnson [14] reported results that were somewhat different. They found excretion of high levels of NMN, NUA and 4-PYR, and low levels of 2-PYR and NAM. In their procedure they injected 5.9 mg/kg body weight of ^{14}C-labeled NA, collected 24-h urine samples and separated the components using paper chromatography. Petrack et al. [15], employing paper chromatography, analyzed rat urines for NA, NMN, NAM, NUA, 2-PYR, N-methyl-4-pyridone-3-carboxamide and nicotinamide-N-oxide, following injection of the rats with 7-^{14}C-labeled nicotinamide and nicotinic acid. They found only very small amounts of the last three compounds in rat urine. We have not been able to obtain samples of N′-methyl-4-pyridone-3-carboxamide, or of nicotinamide-N-oxide, a metabolic product present in relatively large amounts in the urine of mice [16].

By contrast to rats, our human studies showed large excretions of NMN, NUA and 2-PYR and little or no NA and NAM. The amount of 2-PYR was usually greater than NMN. Mainardi and Tenconi [17] in their studies on humans also found basal values of 2-PYR to be higher than NMN. They utilized ultraviolet measurements before and after passing urine through multilayers of Dowex 1 and 50.

ACKNOWLEDGEMENTS

This project was supported by BRSG S07 RR5442, awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health, and by USDA grant No. 59-2065-1-1661-0, awarded by the USDA Competitive Grants Research Program, Science and Education Administration.

REFERENCES

1 R.C. Williams, R.D. Baker and J.A. Schmit, J. Chromatogr. Sci., 11 (1973) 618.
2 R.C. Williams, J.A. Schmit and R.A. Henry, J. Chromatogr. Sci., 10 (1971) 494.
3 D. Wittmer and W.G. Haney, Jr., J. Pharm. Sci., 63 (1974) 588.
4 C.K. Clifford and A.J. Clifford, J. Ass. Offic. Agr. Chem., 60 (1977) 1248.
5 D.A. Light, C. Walsh and M.A. Marletta, Anal. Biochem., 109 (1980) 87.
6 G.A. Taylor, M. Peacock, B. Pelc, W. Brown and A. Holmes, Clin. Chim. Acta, 108 (1980) 239.
7 J.F. Gregory III, Anal. Biochem., 102 (1980) 374.
8 J.T. Vanderslice, K.K. Stewart and M.M. Yarmas, J. Chromatogr., 176 (1979) 280.
9 J.T. Vanderslice and C.E. Maire, J. Chromatogr., 196 (1980) 176.
10 J.T. Vanderslice, C.E. Maire and G.R. Beecher, Amer. J. Clin. Nutr., 34 (1981) 947.
11 J.X. de Vries, W. Günthert and R. Ding, J. Chromatogr., 221 (1980) 161.
12 Y.K. Lee, M. Panaqua, R.W. McKee and M.E. Swendseid, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 40 (1981) 864.
13 O. Pelletier and R. Brassard, Amer. J. Clin. Nutr., 30 (1977) 2108.
14 W.M. Chang and B.C. Johnson, J. Biol. Chem., 234 (1959) 1817.
15 B. Petrack, P. Greengard and H. Kalinsky, J. Biol. Chem., 241 (1966) 2367.
16 S. Chaykin, M. Dagani, L. Johnson and M. Samli, J. Biol. Chem., 240 (1965) 932.
17 L. Mainardi and L.T. Tenconi, Acta Vitaminol., 18 (1964) 249.