Journal of Chromatography, 417 (1987) 173–177
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3603

Note

Microdetermination of N^1-methyl-2-pyridone-5-carboxamide, a major metabolite of nicotinic acid and nicotinamide, in urine by high-performance liquid chromatography

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(First received November 5th, 1986; revised manuscript received January 5th, 1987)

N^1-Methyl-2-pyridone-5-carboxamide (2-Py) was first isolated from human urine in 1946 [1], and identified as a metabolite of N^1-methylnicotinamide (MNA) derived from nicotinic acid and nicotinamide in 1947 [2]. The pyridone is now known to be an end-product of the metabolic pathway of nicotinic acid and nicotinamide in mammals.

The estimation of the bioavailability of niacin is complicated by the conversion of the amino acid tryptophan into niacin, as well as the questionable bioavailability of the bound forms of nicotinic acid in foods, especially in cereals. These factors must be considered when niacin nutrition is evaluated. Therefore, it is important to establish biological methods for assessing the nutritional status of niacin. In many cases, only the urinary excretion of MNA is measured since the method for measuring 2-Py is more complicated than that for MNA [3–5]. In this paper, we desribe a high-performance liquid chromatographic (HPLC) method for the determination of 2-Py in urine. The method is sensitive, simple and reproducible.

0378-4347/87/$03.50

TABLE I

DAILY URINARY EXCRETION OF 2-Py IN RATS

The rats were fed ad libitum a 20% casein diet (20% casein, 0.2% L-methionine, 68.8% sucrose, 5% corn oil, 5% Harper's salt mixture, 1% Oriental's vitamin mixture) with or without 0.5% nicotinamide diet (100 g of the 20% casein diet and 0.5 g of nicotinamide) for 32 days. The urine was collected for the last three days.

Diet	Rat	2-Py (μmol per day)
20% Casein	No. 1	1.01
	No. 2	0.93
	No. 3	0.99
	No. 4	1.03
Mean ± S.E.		0.99 ± 0.02
20% Casein plus nicotinamide	No. 1	16.15
	No. 2	24.08
	No. 3	5.88
	No. 4	8.08
Mean ± S.E.		13.55 ± 4.15

EXPERIMENTAL

Materials

2-Py was synthesized from MNA by the method of Pullman and Colowick [6]. All other chemicals used were of the highest purity obtainable from commercial sources.

Liquid chromatography

The HPLC system employed consisted of an LC-4A liquid chromatograph (Shimadzu, Kyoto, Japan), a Model 7125 syringe loading sample injector (Rheodyne, Cotati, CA, U.S.A.), a 5-ODS-H column, 150×4.6 mm I.D., 5 μm (Chemco Scientific, Osaka, Japan), an SPD-2AS UV detector (Shimadzu) and a Chromatopac CR-3A (Shimadzu). The mobile phase was 10 m*M* potassium dihydrogen phosphate–acetonitrile (96:4, v/v; pH 4.5), the flow-rate 1.0 ml/min, the detection wavelength 260 nm, and the oven temperature 25°C.

Animal experiments and preparation of urine samples

Male rats of the Sprague–Dawley strain (five weeks old) were purchased from Clea Japan (Tokyo, Japan). Harper's salt mixture [7] and Oriental's vitamin mixture [8] were obtained from Oriental Yeast (Tokyo, Japan). The rats were kept individually in wire-bottomed cages. The light–darkness schedule was 06:00–18:00 (light) and 18:00–06:00 (dark). The room temperature and the humidity were 22 ± 2°C and ca. 60%, respectively. Food (described in Table I) and water were supplied ad libitum. Urine was collected in a flask containing 0.5 ml of toluene and 1.0 ml of 1 *M* hydrochloric acid using metabolic cages and stored at −25°C until use.

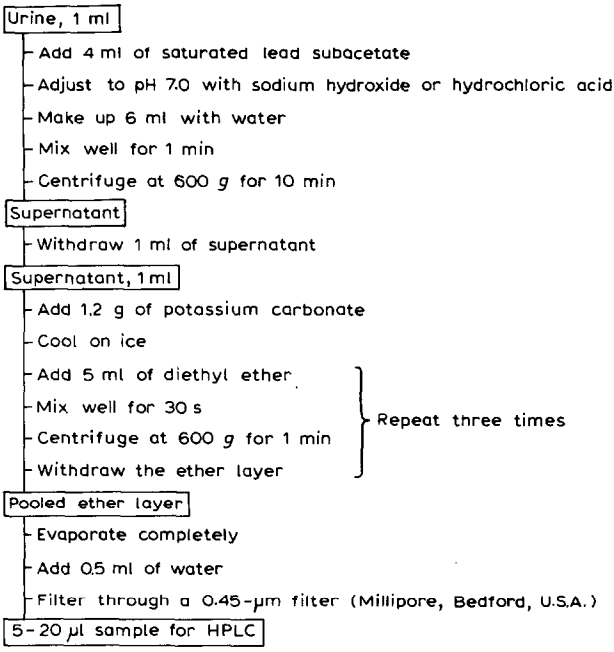

Fig. 1. Extraction of 2-Py from urine.

The preparation procedures for the determination of the pyridone are detailed in Fig. 1.

RESULTS AND DISCUSSION

UV spectrum and k' value of 2-Py

The UV spectrum of 2-Py in water is shown in Fig. 2. The substance was detected at 203, 258 and 294 nm, and the peak-heights ratio was 2.86:2.68:1. The absorbance was thus monitored at 260 nm.

The k' value of 2-Py decreased with an increase in the acetonitrile concentration in the mobile phase. We chose a mobile phase containing 4% acetonitrile for routine work. A typical chromatogram of reference 2-Py is shown in Fig. 3.

Linear range and limit of detection

The calibration curve for 2-Py was linear in the range from 2 pmol to 5 nmol per injection, with a correlation coefficient of 0.999 (linear regression equation: 2-Py (pmol) = integrated absorption area/225). The detection limit was 2 pmol (304 pg) at a signal-to-noise ratio of 5:1.

Recovery of 2-Py from urine

A known amount of the synthetic 2-Py was added to urine prior to extraction, and the 2-Py was extracted as shown in Fig. 1. The recovery was calculated from the following equation: recovery (%) = [integrated absorption area of (endogenous 2-Py + added 2-Py)/10 μl] − (integrated absorption area of endogenous 2-

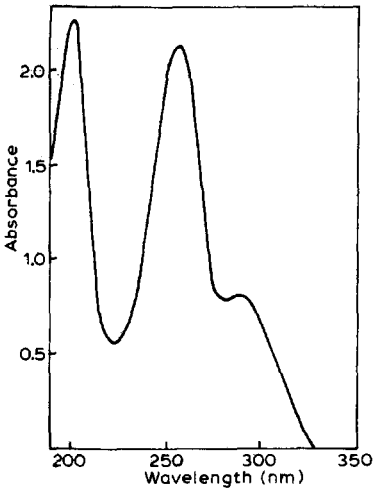

Fig. 2. UV spectrum of 2-Py in water. The concentration of 2-Py was $1.67 \cdot 10^{-4}$ *M*. The molar absorptivity of 2-Py in water at 260 nm was $1.26 \cdot 10^4$.

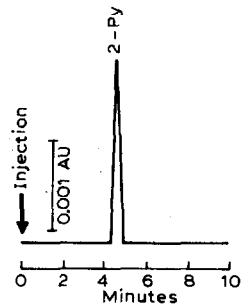

Fig. 3. Chromatogram of 2-Py. Conditions: column, 5-ODS-H (150 mm × 4.6 mm); mobile phase, 10 m*M* potassium dihydrogen phosphate–acetonitrile (96:4, v/v; pH 4.5); flow-rate, 1.0 ml/min; wavelength, 260 nm. Sample size: 182 pmol per 5 μl.

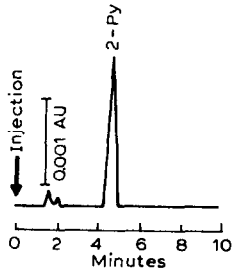

Fig. 4. Chromatogram of the extract of rat urine. The HPLC conditions were as in Fig. 2. Rats were fed the 20% casein diet. Sample size: 151 pmol per 10 μl.

Py/10 μl) × (500 μl/10 μl) × (5.7 ml/1 ml) × (1/integrated absorption area of added 2-Py prior to the extraction) × 100.

The resulting value for the recovery of 2-Py from urine was 104.5 ± 2.7% (mean ± S.E., $n = 5$). The isomeric metabolite N^1-methyl-4-pyridone-3-carbox-

amide (4-Py) could not be extracted by this procedure because, as previously reported [9], 4-Py is insoluble in diethyl ether.

Daily urinary excretion of 2-Py in rats fed a 20% casein diet with or without large amount of nicotinamide

Fig. 4 shows the elution profile obtained when the extract of rat urine was applied on a 5-ODS-H column. When urine was directly injected into the column, the 2-Py peak was obscured by other peaks. However, all interfering peaks were removed by the purification procedure shown in Fig. 1. The peak was identified as 2-Py on the basis of its entire UV spectrum compared with that of the synthetic 2-Py. In the rats fed a 20% casein diet, the daily urinary excretion of 2-Py was ca. 1 μmol (Table I). The excretion of 2-Py was increased ca. fourteen-fold by the feeding of the nicotinamide-containing diet (Table I).

This improved procedure for the measurement of 2-Py should be useful to many investigators in the field of niacin nutrition.

REFERENCES

1 W.E. Knox and W.I. Grossman, J. Biol. Chem., 166 (1946) 391.
2 W.E. Knox and W.I. Grossman, J. Biol. Chem., 168 (1947) 363.
3 J.W. Huff and W.A. Perlzweig, J. Biol. Chem., 167 (1947) 157.
4 F. Rosen, W.A. Perlzweig and I.G. Leder, J. Biol. Chem., 179 (1949) 157.
5 J.M. Price, J. Biol. Chem., 211 (1954) 117.
6 M.E. Pullman and S.P. Colowick, J. Biol. Chem., 206 (1954) 121.
7 A.E. Harper, J. Nutr., 68 (1959) 405.
8 T. Kawada, K.-I. Hagihara and K. Iwai, J. Nutr., 116 (1986) 1272.
9 M.L.W. Chang and B.C. Johnson, J. Biol. Chem., 234 (1959) 1817.