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SIMULTANEOUS MICRO-DETERMINATION OF NICOTINAMIDE AND ITS MAJOR METABOLITES, N^1-METHYL-2-PYRIDONE-5-CARBOXAMIDE AND N^1-METHYL-4-PYRIDONE-3-CARBOXAMIDE, BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simultaneous micro-determination of nicotinamide and its major metabolites, N^1-methyl-2-pyridone-5-carboxamide (2-py) and N^1-methyl-4-pyridone-3-carboxamide (4-py) by high-performance liquid chromatography is described. The method employs a 7-ODS-L (250 mm×4.6 mm I.D., particle size 7 μm) column eluted with 10 m*M* potassium dihydrogenphosphate–acetonitrile (96:4, v/v; pH adjusted to 3.0 by the addition of concentrated phosphoric acid) at a flow-rate of 1.0 ml/min. The UV detector was set at 260 nm. The detection limits for nicotinamide, 2-py and 4-py were 10 pmol (1.22 ng), 2 pmol (304 pg) and 2 pmol (304 pg), respectively, at a signal-to-noise ratio 5:1. Isonicotinamide was used as an internal standard. The technique was applied to the analysis of rat and human urines. The total analysis time was ca. 15 min.

INTRODUCTION

Urinary excretion of N^1-methylnicotinamide and N^1-methyl-2-pyridone-5-carboxamide (2-py) is considered as a biological index of niacin nutrition [1]. However, in many cases only the urinary excretion of N^1-methylnicotinamide is measured since the method for measuring 2-py is more complicated than that for N^1-methylnicotinamide [2–4]. Furthermore, Chang and Johnson [5] reported that urinary excretion of N^1-methyl-4-pyridone-3-carboxamide (4-py) cannot be

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ignored in assessing niacin nutrition. Thus the development of a simple determination method of 4-py is needed.

In the previous papers we have reported the micro-determination of 2-py in urine [6] and of nicotinamide in tissues and blood [7] by high-performance liquid chromatography (HPLC). In the clean-up step by extraction with diethyl ether, we thought that the isomeric metabolite 4-py, which was first isolated and identified as a normal major metabolite of nicotinic acid and nicotinamide in rats, humans and monkeys [5,8], could not be extracted, as previously reported [8], because 4-py is insoluble in diethyl ether. However, during the isolation of 4-py, we found that 4-py as well as 2-py and nicotinamide [6,7] are soluble in diethyl ether under alkaline conditions. Furthermore, the elution times of 2-py and 4-py were the same as under the HPLC conditions reported previously [6]. In this paper, we report an HPLC method for simultaneous micro-determination of nicotinamide, 2-py and 4-py in urine following effective clean-up step with diethyl ether.

EXPERIMENTAL

Materials

N^1-Methylnicotinamide was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). 2-Py was synthesized from N^1-methylnicotinamide by the method of Pullman and Colowick [9]. 4-Py was synthesized from N^1-methylnicotinamide using partially purified N^1-methylnicotinamide oxidase from rat liver and isolated as follows. Partially purified N^1-methylnicotinamide oxidase was prepared by the procedures of Quinn and Greengard [10]. The reaction mixture (114 ml) consisted of 9 ml of 0.5 *M* dipotassium hydrogenphosphate–potassium dihydrogenphosphate buffer (pH 7.0), 18 ml of 20 m*M* N^1-methylnicotinamide, 62 ml of water, 10 ml of 10 m*M* potassium ferricyanide and 15 ml of partially purified N^1-methylnicotinamide oxidase (45 mg as protein). This mixture was incubated at 37°C for 4 h, and the reaction was stopped by the addition of 1.5 ml of 70% perchloric acid. The precipitate was removed by centrifugation (10 000 *g*, 10 min). The supernatant was concentrated in vacuo at 60°C. The dried material was dissolved in 5 ml of water, 6 g of potassium carbonate and 30 ml of diethyl ether were added to this solution, and the mixture was mixed well for 5 min. After centrifugation (600 *g*, 1 min), the diethyl ether layer was removed and retained. This diethyl ether extraction was repeated three times. The pooled diethyl ether (ca. 90 ml) was evaporated at 50°C. The dried material was dissolved in 1 ml of water. The solution was spotted on high-performance thin-layer chromatographic (HPTLC) plates coated with cellulose F_{254S} (10 cm×10 cm, E. Merck, Darmstadt, F.R.G.) to separate 2-py and 4-py. The plates were developed with 2-propanol–concentrated hydrochloric acid–water (70:15:15, v/v/v) for 3 h at room temperature. 4-Py (R_F ca. 0.49) was scraped off the HPTLC plate and extracted with water. The UV spectra of the isolated material in water and 1 *M* hydrochloric acid are shown in Fig. 1. These UV spectra were consistent with those of the synthesized 4-py reported by Chang and Johnson [5,8]. The characteristic property of 4-py, the blue shift of the peak in 1 *M* hydrochloric acid,

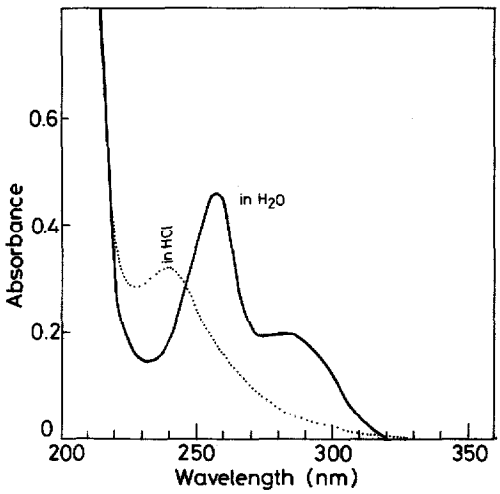

Fig. 1. UV spectra of N^1-methyl-4-pyridone-3-carboxamide in water and in 1 *M* hydrochloric acid.

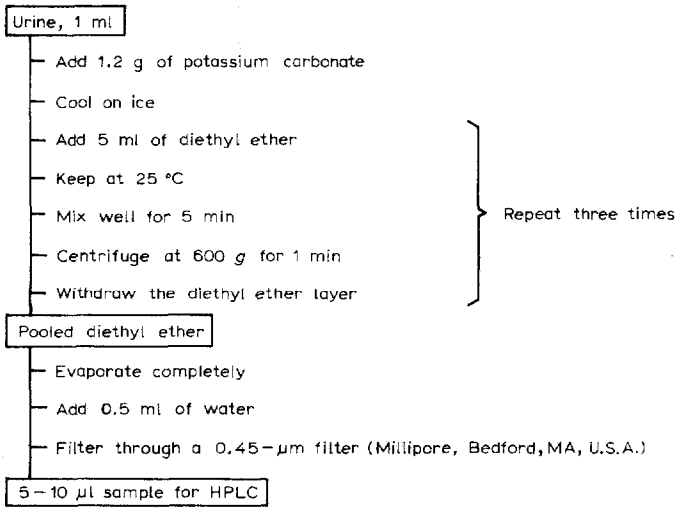

Fig. 2. Extraction of 2-py, 4-py and nicotinamide from urine.

was observed in the isolated material, as reported by Chang and Johnson [5,8]. Accordingly, the isolated material was identified as 4-py. This 4-py was 100% pure according to the HPLC determination. Isonicotinamide (used as the internal standard) and nicotinamide were obtained from Wako (Osaka, Japan). All other chemicals used were of the highest purity obtainable from commercial sources.

Liquid chromatography

The HPLC system consisted of an LC-4A liquid chromatograph (Shimadzu, Kyoto, Japan), a Model 7125 syringe-loading sample injector (Rheodyne, Cotati,

CA, U.S.A.), a 7-ODS-L column (250 mm×4.6 mm I.D., particle size 7 μm) (Chemco Scientific, Osaka, Japan), and SPD-2AS UV detector (Shimadzu) and Chromatopac C-R3A (Shimadzu). The mobile phase was 10 m*M* potassium dihydrogenphosphate–acetonitrile (96:4, v/v, pH adjusted by addition of concentrated phosphoric acid), the flow-rate was 1.0 ml/min, the detection wavelength was 260 nm, and the column temperature was 25 ± 1 °C.

Animal experiments and preparation of samples

Male rats of the Sprague–Dawley strain (six weeks old) were purchased from Clea Japan (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 was 22 ± 2 °C and the humidity was ca. 60%. Food, CE-2 (Clea Japan) 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 to the 16th–18th day and stored at −25 °C until use. The clean-up step of urine is shown in Fig. 2.

RESULTS AND DISCUSSION

Linear range and limit of detection

The concentration of isolated 4-py was calculated using the molar absorptivity of $1.35 \cdot 10^4$ at 260 nm [8]. The calibration curve for 4-py was linear in the range from 2 pmol (304 pg) to 5 nmol (760 ng) per injection, with a correlation coefficient of 0.999 (linear regression equation: 4-py (pmol) = integrated absorption area/174). The detection limit in 4-py and 2-py was 2 pmol (304 pg) at a signal-to-noise ratio of 5:1 [6]. Under the HPLC conditions, the linear regression equations of nicotinamide and 2-py were: nicotinamide (pmol) = integrated absorption area/52 and 2-py (pmol) = integrated absorption area/187. These equations are different from those previously reported [6,7] because the pH of the elution buffer is different.

Recovery of 4-py and isonicotinamide from urine

A known amount of the isolated 4-py was added to urine prior to extraction, and the 4-py was extracted as shown in Fig. 2. The recovery was calculated from the following equation: recovery (%) = [integrated absorption area of (endogenous 4-py + added 4-py)/10 μl] − (integrated absorption area of endogenous 4-py/10 μl) × 500 μl/10 μl × (1/integrated absorption area of added 4-py prior to the extraction) × 100. The resulting value for the recovery of 4-py from urine was 97.9 ± 3.6% (mean ± S.D., $n = 5$) at a concentration of ca. 200 nmol (30.4 μg) per ml of urine.

Isonicotinamide was used as the internal standard. When isonicotinamide was added to urine, its recovery was 99.6 ± 1.5% (mean ± S.D., $n = 5$).

Daily urinary excretion of 2-py and 4-py in rats and humans

Fig. 3 shows a typical chromatogram of reference isonicotinamide (internal standard), nicotinamide, 2-py and 4-py; the elution times were ca. 5.775, 6.725, 8.942 and 11.100 min, respectively. The total analysis time was 15 min. Fig. 4A and B show the elution profiles obtained when the extracts of rat urine and hu-

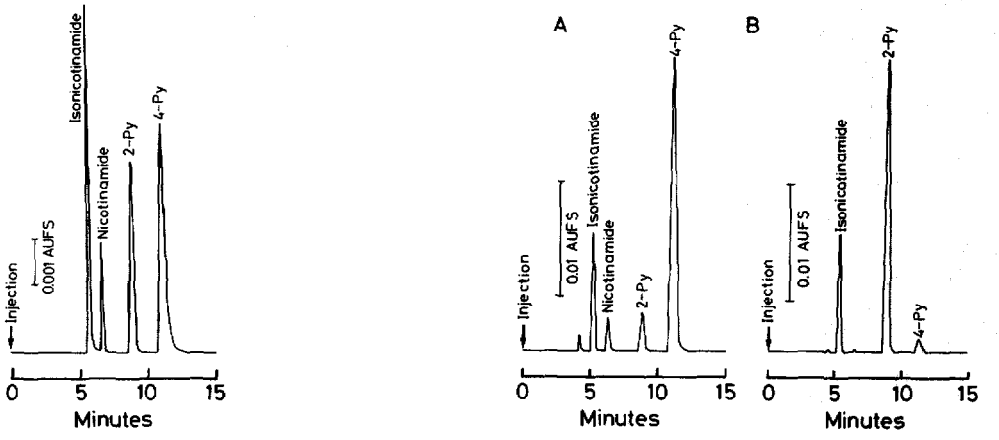

Fig. 3. Chromatogram of a reference mixture of isonicotinamide, nicotinamide, 2-py and 4-py. The conditions are described in Experimental. Amounts injected: isonicotinamide, 1.36 nmol; nicotinamide, 0.51 nmol; 2-py, 0.34 nmol; 4-py, 0.78 nmol.

Fig. 4. Chromatograms of extracts of rat urine (A) and human urine (B). (A) Amounts injected per 10 μl; isonicotinamide, 1.81 nmol; nicotinamide, 1.88 nmol; 2-py, 0.31 nmol; 4-py 4.27 nmol. (B) Amounts injected per 10 μl: isonicotinamide, 1.81 nmol; 2-py, 2.24 nmol; 4-py, 0.26 nmol.

man urine were applied to a 7-ODS-L column. All interfering peaks were removed by the clean-up step. The peaks were identified as nicotinamide, 2-py and 4-py on the basis of their entire UV spectra, compared with those of authentic nicotinamide, 2-py and 4-py, respectively. Daily urinary excretion of 2-py and 4-py in rats of the Sprague–Dawley strain fed a CE-2 diet, which contains 0.28 g of tryptophan and 12.2 mg of niacin per 100 g diet, were 1188.7 ± 473.7, 600.1 ± 149.1 and 3909.1 ± 1132.3 nmol, respectively, as shown in Table I. Urinary excretion of 4-py in these rats was found to be ca. seven-fold higher than that of 2-py.

Daily urinary excretion of 2-py and 4-py in healthy college women (21 years old) was measured. They consumed a self-selected food for three days. Urine was collected for 24 h. Food consumption was weighed. Niacin and protein intakes were calculated based on the "Tables of Food Composition in Japan" [12]. The niacin intakes from tryptophan were calculated by assuming that the tryptophan

TABLE I

DAILY URINARY EXCRETION OF NICOTINAMIDE, 2-PY AND 4-PY IN RATS OF THE SPRAGUE–DAWLEY STRAIN

Rat No.	Nicotinamide (nmol/day)	2-py (nmol/day)	4-py (nmol/day)	4-py/2-py ratio
1	610.6	562.4	3023.3	5.38
2	1529.8	805.6	2477.9	3.45
3	1635.6	563.1	5372.6	9.54
4	700.8	356.1	4547.5	12.77
5	1640.1	640.1	3308.0	5.17
6	1015.3	673.0	4725.5	7.02
Mean $\pm$ S.D.	1187.7 ± 473.7	600.1 ± 149.1	3909.1 ± 1132.3	7.22 ± 3.41

TABLE II

DAILY URINARY EXCRETION OF 2-PY AND 4-PY IN HEALTHY COLLEGE WOMEN

Subject No.	Niacin equivalent intake (μmol/day)	2-py (μmol/day)	4-py (μmol/day)	4-py/2-py ratio
1	259.3	96.66	11.05	0.114
2	199.4	66.11	7.82	0.118
3	176.1	66.11	7.82	0.118
4	172.9	73.85	7.36	0.100
5	149.6	36.47	3.72	0.102
6	149.6	39.68	4.46	0.112
7	149.5	32.01	4.85	0.152
8	141.1	46.02	5.25	0.114
9	94.9	23.55	2.53	0.107
10	84.9	22.07	2.37	0.107
Mean ± S.D.	157.7 ± 49.8	50.25 ± 24.43	5.72 ± 2.75	0.114 ± 0.015

content is 1% of protein intake and that 1 mg of niacin is converted from 60 mg of tryptophan. The total amount of niacin and the niacin converted from tryptophan was referred to "niacin equivalent". The subjects' niacin equivalent intake was 157.7 ± 49.8 μmol per day, and their daily urinary excretion of 2-py and 4-py was 50.25 ± 24.43 and 5.72 ± 2.75 μmol, respectively, as shown in Table II. Chang and Johnson [5] reported that the ratio of 4-py to 2-py was ca. 0.400 in males (range 0.237–0.694) and ca. 0.270 in females (range 0.192–0.481). In our results, obtained from Japanese females, this ratio was ca. 0.114 (range 0.100–0.152). This difference is attributed to differences of niacin intake and of genes. These results show that the measurement in urine of 4-py as well as 2-py and N^1-methylnicotinamide is of value as an index for niacin nutrition, as suggested by Chang and Johnson [5]. The level of nicotinamide in human urine was below the detection limit. This method can measure simultaneously 2-py, 4-py and nicotinamide without interference, and we believe that it will prove of help in assessing niacin nutrition.

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