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Journal of Chromatography B, 661 (1994) 154–158

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Simultaneous measurement of nicotinic acid and its major metabolite, nicotinuric acid in urine using high-performance liquid chromatography: application of solid–liquid extraction

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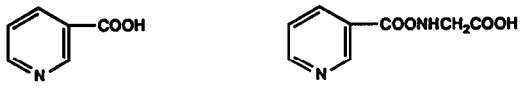
First received 3 May 1994; revised manuscript received 18 July 1994

Abstract

The concentrations of nicotinic acid (NiAc) and nicotinuric acid (NiUAc), a major metabolite of NiAc, were simultaneously determined in urine using solid-phase extraction (cation-exchange extraction) and reversed-phase high-performance liquid chromatography with ultraviolet detection. The intra- and inter-day precision studies showed good reproducibilities: the coefficients of variations were less than 8.1% for NiAc and 8.8% for NiUAc. The calibration curves were linear ($r^2 > 0.9934$) in the concentration range 10–1000 μg/ml. The removal of endogenous interferences in urine by solid-phase extraction presented here is superior to the pretreatment protocols reported previously by other workers. The method was used in a preliminary pharmacokinetic study in rats after intravenous administration of NiAc (5 and 15 mg/kg).

1. Introduction

Nicotinic acid (Niacin, NiAc), a constituent of the vitamin B complex, shows hypolipemic activity at high doses [1–3] and has been used in the treatment of hyperlipemia for many years. However, its side-effects, flushing and hepatitis, limit its use. NiAc has two major metabolic pathways: one is conjugation with glycine to produce nicotinuric acid (NiUAc) and the other is the formation of pyridone derivatives through nicotinamide (for structures of both compounds see Fig. 1). The former is a quantitatively important metabolite after a high dose of NiAc [4–6]. Since nicotinamide does not show the flushing side-effect, NiUAc formation and/or NiAc itself may be the reason for flushing. Recently, Hengen et al. [7] reported that NiAc shows non-linear pharmacokinetics in man. However, little information regarding the pharmacokinetic behavior of NiAc and NiUAc is available. Consequently, the detailed pharmacokinetics of both NiAc and NiUAc have to be elucidated. To study the pharmacokinetics and



Nicotinic acid Nicotinuric acid

Fig. 1. Structures of nicotinic acid and nicotinuric acid.

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0378-4347/94/$07.00
SSDI 0378-4347(94)00332-7

pharmacodynamics of NiAc, it is essential to establish a practical method to measure NiAc and its major metabolite NiUAc in urine as well as in plasma (serum). Hengen et al. [7] measured NiAc and NiUAc in plasma and urine with HPLC. For urine samples, however, their method requires long pretreatment procedures (e.g. overnight volatilization) and two columns in tandem are necessary to avoid overlapping peaks. Shibata [8] reported a method to determine NiAc and NiUAc using treatment of plasma and urine with 0.6 *M* perchloric acid to precipitate proteins. Although the method is simple and easy, our experience shows that the prepared sample may include many interferences especially in the assay of urine samples.

As other researchers have pointed out, solvent extraction with organic solvents is not suitable for the assay of the polar compounds NiAc and NiUAc. We have developed a reversed-phase HPLC method for the simultaneous measurement of NiAc and NiUAc in urine with cation-exchange solid–liquid extraction. The present method is simple, reproducible and specific, and applicable to pharmacokinetic studies on NiAc and NiUAc. The method is certainly applicable to plasma (serum) samples.

2. Experimental

2.1. Materials

Nicotinic acid (NiAc) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Nicotinuric acid (NiUAc) and 6-methyl nicotinic acid were purchased from Sigma (St. Louis, MO, USA). Tetra-*n*-butylammonium phosphate (ion-pair chromatograph grade) was obtained from Nacalai Tesque (Tokyo, Japan). All other chemicals used were of special or HPLC grade. Solid-phase extraction was performed using a solid-phase extraction manifold (Vac-Elute, Varian Instruments, Harbor City, CA, USA) and a Bond Elut SCX column (Varian). Urine control was obtained from non-treated rats housed in metabolic cages, and was stored at −40°C until use.

2.2. High-performance liquid chromatography

The HPLC system employed consisted of an LC-2A liquid chromatograph (Shimadzu, Kyoto, Japan), a Model 7125 syringe loading sample injector (Rheodyne, Cotati, CA, USA), an Inertsil ODS-2 column, 250 × 4.6 mm I.D., 5 μm (GL Sciences, Tokyo, Japan), a SPD-2AS UV detector (Shimadzu) and a Chromatopac CR-3A integrator (Shimadzu). The mobile phase was 10 m*M* potassium phosphate buffer [containing 5 m*M* tetra-*n*-butylammonium phosphate (pH 7.0)]–acetonitrile (90:10, v/v); the flow-rate was 1.0 ml/min, the detection wavelength 254 nm, and the column oven temperature was 35°C.

2.3. Standard solutions

Solutions (1.00 mg/ml) of NiAc and NiUAc in purified water were prepared. The stock solutions were used to prepare 10, 50, 100, 200, 500, 750, and 1000 μg/ml standards of NiAc and NiUAc in stock drug-free rat urine. A 20 μg/ml solution of 6-methyl nicotinic acid (internal standard) in purified water was prepared from a 1.00 mg/ml stock solution. All standards were stored at 4°C until used.

2.4. Animal experiment

Male Wistar rats (250–350 g body weight) had free access to food (MF diet, Oriental Yeast, Tokyo, Japan) and water. A cannula was implanted into the right jugular vein for drug administration under pentobarbital anesthesia (50 mg/kg, intraperitoneally) one day before the experiment. Each animal was placed in an individual cage until it had fully recovered from the implantation procedure. Animals were fixed individually in a Boulman cage and received nicotinic acid (5 or 15 mg/kg) intravenously through the cannulation tubing. Urine was col-

lected up to 6 h after administration in a test tube through a cut-chip attached to the penis and stored at −40°C until assay.

2.5. Extraction procedure

The Bond Elut column, set to a Vac-Elut, was pretreated by rinsing with 3 ml of methanol and 3 ml of water. To 300 μl of the urine sample or calibration standard diluted with 2 volumes of purified water, 10 μl of a 20 μg/ml solution of 6-methyl nicotinic acid (internal standard) was added. The mixture was loaded onto the pretreated Bond Elut column under low vacuum. After washing with subsequently 3 ml of water and 3 ml of 40% methanol, the column was dried for 5 min by air under high vacuum. The compounds were eluted with 2.5 ml of 2% (v/v) NH_4OH–methanol under low vacuum. The eluent was evaporated in vacuo, and the residue was reconstituted with 200 μl of the HPLC mobile phase.

2.6. Recovery from urine

The extraction recoveries of NiAc and NiUAc from rat urine were obtained by the absolute determination method. Spiked samples were prepared by adding known amounts of NiAc and NiUAc to blank urine, and analyzed by the procedures described above. The concentrations of the samples were 10, 50 and 200 μg/ml. The peak areas obtained were compared with those obtained by direct injection of each substance without extraction.

2.7. Calibration curve

For calibration standards, spiked samples were prepared as described above. The calibration standards were analyzed, and calibration curves were constructed by plotting the peak-area ratio to the internal standard against the known concentrations, using a least-squares regression program (Delta Graph Professional, Delta Point, Monterey, CA, USA) on a Macintosh computer.

2.8. Intra-day and inter-day precision

Spiked samples were prepared at concentrations of 50, 100, 200 and 500 μg/ml for both NiAc and NiUAc. The spiked samples were stored at −40°C until assay. The reproducibility and accuracy for each compound were determined by sample analysis. The inter-day study was performed over a one-week period.

3. Results and discussion

In the present study, our aim was to develop a selective method for the simultaneous determination of NiAc and NiUAc in urine. We focused in particular on the pretreatment protocol of the sample, since urine samples include many polar compounds which are difficult to remove and which interfere with the detection of the polar compounds NiAc and NiUAc. The HPLC conditions reported here are similar to those employed by Hengen et al. [7] and Shibata [8], although the sample pretreatment protocol is completely different. Previously published methods did not satisfactorily remove the interferences or they needed long columns (or a couple of columns) to prevent their interferences. The clean-up procedure using cation ion-exchange solid–liquid extraction presented in this study performed well compared with those used in previous methods.

The extraction protocol presented here employed two washes, one with 3 ml of water and the second with 3 ml of 40% methanol. The extraction recoveries using Bond Elut SCX were higher than 80.3% for NiAc and 80.8% for NiUAc, yielding a consistently good recovery. Without the 40% methanol wash, there were many interferences in the 2% ammonium–methanol eluent. On the other hand, with a washing volume of more than 5 ml of 40% methanol, the recoveries of both NiAc and NiUAc decreased. The coefficient of variation

Table 1
Extraction recovery study

Compound	Concentration (μg/ml)	Recovery (%)	C.V. (%)
NiAc	10	85.4 ± 9.4	11.0
	50	81.0 ± 4.5	5.6
	200	80.3 ± 6.2	7.7
NiUAc	10	84.2 ± 11.2	13.3
	50	80.8 ± 6.4	7.9
	200	81.6 ± 5.9	7.2

Each value represents mean ± S.D. ($n = 3$).

(C.V.) for the mean peak area was 8.1% for NiAc and 9.4% for NiUAc, indicating fairly good reproducibilities (Table 1).

Typical chromatograms of rat blank urine spiked with both NiAc and NiUAc, and of urine collected from a NiAc-treated rat (15 mg/kg, intravenously) over a 2–3 h period after administration are shown in Fig. 2. Chromatograms of blank urine treated according to the described procedure show no interfering peaks from endogenous compounds. The retention times of NiAc, NiUAc and 6-methyl nicotinic acid were 7.2, 8.2 and 13.1 min, respectively.

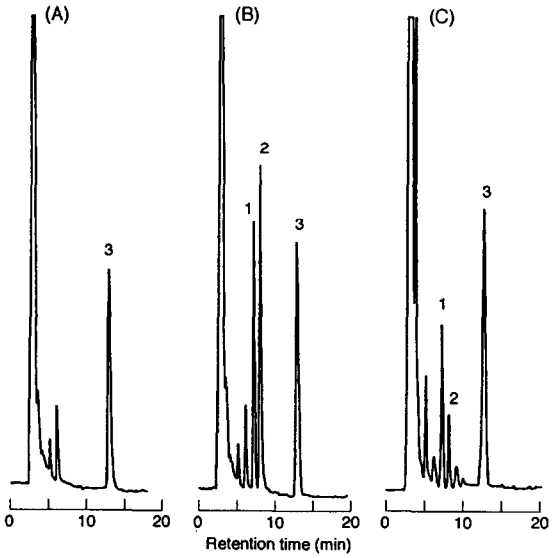

Fig. 2. Typical chromatograms of drug-free urine (A), urine spiked with 100 μg/ml of nicotinic acid and nicotinuric acid (B), and urine from a rat treated with 15 mg/kg of nicotinic acid 2–3 h after administration. Peaks: 1 = nicotinuric acid; 2 = nicotinic acid; 3 = 6-methyl nicotinic acid (internal standard).

The intra-day ($n = 5$) and inter-day ($n = 4$) reproducibility and accuracy were checked at concentrations of 50, 100, 200 and 500 μg/ml for each compound. The C.V. of the determined values was less than 8.1% (mean 5.9%, intra-day) and 8.0% (mean 5.8%, inter-day) for NiAc and 8.8% (mean 6.6%, intra-day) and 8.8% (mean 7.0%, inter-day) for NiUAc. In addition, the accuracy was estimated to be 95.2–105.2% (Table 2). Calibration curves for each compound showed good linearity over the concentration range 10.0–1000 μg/ml urine ($y = 0.00637x - 0.0257$ for NiAc and $y = 0.00804x + 0.0107$ for NiUAc). The correlation coefficient (r^2) was higher than 0.9934 for NiAc and 0.9998 for NiUAc. The present method of analysis showed good reproducibility and accuracy. The limit of detection of each compound in urine was ca. 5 μg/ml, representing approximately 40 ng injected. The limit of detection can be improved by using a larger sample size.

The present method was applied to the measurement of the urinary excretion of NiAc and its main metabolite, NiUAc in rats. Fig. 3 shows the cumulative urinary excretion of the unchanged NiAc and the metabolite NiUAc after NiAc injection. After intravenous administration of NiAc (5 and 15 mg/kg), almost the total amount of NiAc and NiUAc excreted in urine was recovered within one hour after administration and the cumulative urinary excretion curves for both compounds reached a plateau 2 h after administration. Seventy to eighty percent of the dose was recovered in urine as NiAc and NiUAc. However, the ratio of the excreted amounts of NiUAc and NiAc after administration of the 15 mg/kg dose [0.85 ± 0.09 ($n = 3$)] was significantly lower than that obtained after administration of the 5 mg/kg dose [3.1 ± 0.8 ($n = 3$)]. This result suggests a saturable metabolisation of NiAc to NiUAc. The detailed urinary excretion data obtained in a pharmacokinetic study on NiAc will be reported in the future.

Table 2
Intra-day and inter-day reproducibilities for nicotinic acid and nicotinuric acid

Compound	Spiked concentration (μg/ml)	Intra-day ($n = 5$)			Inter-day ($n = 4$)		
		Determined concentration (mean ± S.D.) (μg/ml)	C.V. (%)	Accuracy (%)	Determined concentration (mean ± S.D.) (μg/ml)	C.V. (%)	Accuracy (%)
NiAc	50	51.8 ± 4.2	8.1	103.6	50.0 ± 4.0	8.0	100.0
	100	105.2 ± 6.2	5.9	105.2	102.3 ± 5.9	5.8	102.3
	200	190.4 ± 13.1	6.9	95.2	197.0 ± 13.6	6.9	98.5
	500	502.6 ± 13.9	2.8	100.5	500.7 ± 11.5	2.3	100.1
NiUAc	50	50.0 ± 1.8	3.6	99.9	51.8 ± 1.9	3.7	103.6
	100	102.3 ± 9.0	8.8	102.3	105.2 ± 9.3	8.8	105.2
	200	197.0 ± 14.2	7.2	98.5	190.5 ± 16.6	8.7	95.3
	500	500.7 ± 34.1	6.8	100.1	502.6 ± 34.2	6.8	100.5

4. Conclusions

In the present study a convenient HPLC method is described for the simultaneous determination of NiAc and its main metabolite NiUAc, with no urinary interferences. Because of its simplicity, specificity and good reproducibility, this method is applicable to pharmacokinetic studies on NiAc and NiUAc using urine samples.

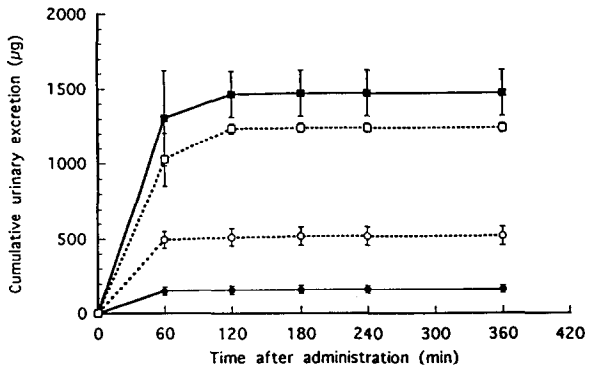

Fig. 3. Cumulative urinary excretion curves of nicotinic acid and nicotinuric acid in rats after intravenous administration of 5 mg/kg (●, ○) and 15 mg/kg (■, □) of nicotinic acid. Closed symbols are nicotinic acid and opened symbols are nicotinuric acid. Each point represents mean ± S.D. ($n = 3$).

Acknowledgement

A part of this research was supported by a Grant-in-Aid from Kinki University.

References

[1] L.A. Carlson, L. Orö and J. Östman, *Acta Med. Scand.*, 183 (1968) 457.
[2] W.B. Parsons and J.H. Flinn, *Arch. Intern. Med.*, 103 (1955) 783.
[3] N. Svedmyr, L. Harthon and L. Lundholm, *Clin. Pharmacol. Ther.*, 1969 (1969) 10.
[4] L. Mainaldi, *Acta Vitaminol.*, 14 (1960) 261.
[5] O.N. Miller, J.G. Hamilton and G.A. Goldsmith, *Am. J. Clin. Nutr.*, 10 (1962) 285.
[6] K.K. Reddi and E. Kodicek, *Biochem. J.*, 53 (1959) 286.
[7] N. Hengen, V. Seiberth and M. Hengen, *Clin. Chem.*, 24 (1978) 1740-1743.
[8] K. Shibata, *Agric. Biol. Chem.*, 52 (1988) 2873-2976.