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Simultaneous determination of nicotinic acid and its metabolites in rat urine by micellar electrokinetic chromatography with photodiode array detection

Masahiro Iwaki, Etsuko Murakami, Masako Kikuchi, Akinori Wada, Taro Ogiso, Yasuo Oda, Kanenobu Kubo, Kazuaki Kakehi*

Faculty of Pharmaceutical Sciences, Kinki University, Kowakae 3-4-1, Higashi-osaka 577, Japan

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Abstract

Nicotinic acid, nicotinamide and their possible metabolites were successfully separated within 17 min by micellar electrokinetic chromatography using 50 m*M* borate buffer (pH 9.0) containing 150 m*M* sodium dodecyl sulfate as the running buffer. Calibration curves for all compounds showed good linearity in a range of 5 μg/ml and 250 μg/ml with good correlation. The present method did not require any clean-up procedures and made it possible to determine all metabolites without interference on a photodiode array detector. Urine samples collected from Wistar male rats were analyzed after high-dose oral or intravenous administration of nicotinic acid or nicotinamide. Metabolic pathways of nicotinic acid in male Wistar rats are also discussed.

Keywords: Nicotinic acid; Nicotinamide

1. Introduction

Nicotinic acid (NiAc) and its derivatives show hypolipemic activity at high doses and have been used in the treatment of hyperlipemia [1–3]. However, the side effects of flushing and hepatitis limit its use. It is necessary to study the pharmacokinetics and pharmacodynamics of NiAc for elucidation of the mechanism of these side effects. The reported metabolic pathway of NiAc is shown in Fig. 1 [4].

NiAc is metabolized to nicotinuric acid (NiUAc) through conjugation with glycine and to pyridone derivatives through nicotinamide (NiNH2).

High hydrophilicity of NiAc and its metabolites has made it laborious to determine these compounds in biological fluids simultaneously, due to difficulties in the clean-up procedure [5]. The authors have developed a method for simultaneous measurement of NiAc and NiUAc in urine using high-performance liquid chromatography (HPLC) [6]. The method includes cation-exchange solid–liquid extraction procedures. However, these methods could not be applied to the analysis of all metabolites due to extreme differences in recovery efficiency for each metabolite.

Capillary electrophoresis (CE) has demonstrated its usefulness in the determination of drugs in pharmaceutical formulations [7,8]. Several research

*Corresponding author.

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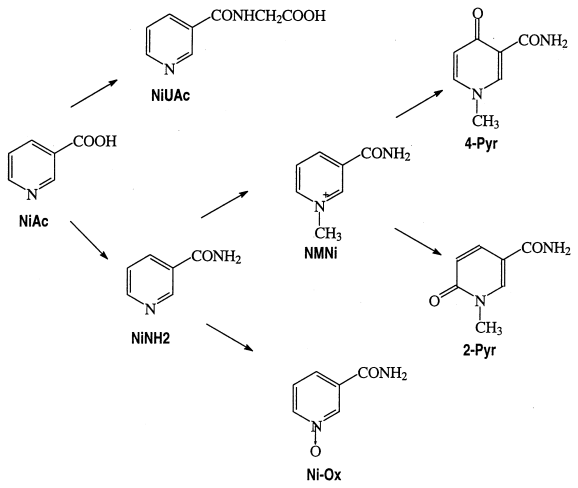

Fig. 1. Possible metabolic pathways of nicotinic acid.

groups have reported the determination of drugs in biological fluids by CE and its application to pharmacokinetic studies [9–12]. Lamparczyk et al. also described HPLC and CE of nicotinic acid and its metabolites spiked in blood [13].

In the present paper, we introduce a method to analyze NiAc and its possible metabolites in rat urine after high-dose administration of NiAc and NiNH2. The present method allows simultaneous and direct determination of NiAc and its possible metabolites in a diluted urine sample by using micellar electrokinetic chromatography (MEKC) with photodiode array detection (PAD). By monitoring the absorbance at 214 and 260 nm, NiAc and other metabolites are determined simultaneously without the interference of contaminant materials in the urine.

2. Experimental

2.1. Reagents

NiAc and 2-hydroxynicotinic acid (2OH-Ni) were obtained from Wako Pure Chemicals (Dosho-machi, Osaka, Japan). N^1-Methyl nicotinamide (NMNi), nicotinuric acid (NiUAc) and 6- hydroxynicotinic acid (6OH-Ni) were purchased from Sigma–Aldrich Japan K.K. (Chuo-ku, Tokyo, Japan). Nicotinamide N-oxide (Ni-Ox) was from Nacalai Tesque (Nakagyo-ku, Kyoto, Japan). Samples of 3- carboxy-1-methylpyridinium inner salt (trigonelline) and 6-methyl nicotinic acid (6Me-Ni) as the internal standards were from Tokyo Kasai Kogyo (Chuo-ku, Tokyo, Japan) and Sigma, respectively. Butyl acrylate–butyl methacrylate–methacrylic acid copolymer sodium salts (BBMA) was a gift from Drs. Terabe and Otsuka of Himeji Institute of Technology. Sodium dodecyl sulfate (SDS) was the electrophoresis grade and was obtained from Kishida (Kadoma, Osaka, Japan). Other reagents and solvents were of the highest grade commercially available and were used without further purification. All aqueous solutions were prepared using water purified with a Milli-Q purified system (Millipore Japan, Shinagawa, Tokyo).

N^1-Methyl-5-carboxamide-2-pyridone (2-Pyr) was prepared from trigonelline according to the method described by Huff [14]. The physicochemical data of 2-Pyr were as follows. ^{1}H Nuclear magnetic resonance (NMR) (D_2O, δ ppm): H-2, 8.353 (d, $J_{2,4}$=2.1 Hz); H-4, 7.985 (dd, $J_{4,2}$=2.1 Hz, $J_{4,5}$=9.2 Hz); H-5, 6.688 (d, $J_{5,4}$=9.4 Hz); N-CH_3, 3.669 (s). The ultraviolet absorption spectra were similar to those reported by Bernofsky [15].

2.2. Apparatus

^{1}H NMR spectra of 2-Pyr were measured on a JEOL JNM GX-500 spectrometer operating at 500 MHz in D_2O solution. The chemical shift was expressed as δ-scale (ppm) by using acetone as the external standard (2.133 ppm). The UV absorption spectra were measured on a Shimadzu UV-1600 spectrophotometer with a 1-cm quartz cell.

A CAPI 3000 CE system (Otsuka Electronics, Hirakata, Osaka, Japan), equipped with a PAD system operating in a range of 200–270 nm was employed. The untreated capillary [72 cm (effective length 60 cm)×75 μm I.D., Waters, Shinagawa, Tokyo, Japan) used in the present work was conditioned prior to use with a 10-min rinse of 0.1 *M* NaOH, a 3-min rinse with water and a 5-min rinse with the running buffer. However, the washing step with 0.1 *M* NaOH was omitted in each run. The applied voltage was 15 kV and the operation temperature was maintained at 37°C. The sample solution was introduced to the capillary by hydrostatic method (30 s). All compounds except for Ni-Ox

were monitored at 214 nm. Ni-Ox was monitored at 260 nm to eliminate interference by a co-migrating substance in the urine. High-performance CE-grade sodium tetraborate buffer (50 m*M*, pH 9.0), sodium phosphate buffer (50 m*M*, pH 7.0) and sodium phosphate buffer (50 m*M*, pH 2.5) were provided by Merck (Darmstadt, Germany). For examination of the optimum pH of the buffer solution, the pH of an aqueous solution of boric acid (100 m*M*) was adjusted with 1 *M* NaOH and diluted to a concentration of 50 m*M* of borate ion. The buffer solution was filtered through a 0.45-μm membrane filter before use.

2.3. Sample preparation

The standard solutions of NiAc, NiNH2 and their possible metabolites were prepared by dissolving each sample (1.0 mg) in 1.0 ml water. A 200-μl portion of the internal standard solution (trigonelline, 1.0 mg/ml) was added to the mixture of the diluted standard solution. All standard solutions were stored at 4°C until use.

2.4. Animal experiment

Male Wistar rats (Japan SLC, Shizuoka, Japan) weighing 200–290 g, were fasted for 16 h before the experiment. Animals were kept individually in a Boulman cage and received NiAc or NiNH2 (45 or 135 mg/kg) intravenously through the cannulation tubing or orally. Urine was collected in a test tube up to 10 h after administration, through a cut-chip attached to the penis and was stored at −40°C until analysis.

2.5. Analysis of urine samples

A 100-μl portion of a urine sample was diluted to 1.0 ml with water. A solution of the internal standard (1 mg/ml, 200 μl) was added to the diluted sample. A portion was analyzed by CE.

2.6. Preparation of calibration curves

For preparation of calibration curves, a solution (100 μl) containing the known amount of the compounds was spiked with a 10-times diluted blank urine sample (1.0 ml). After addition of the internal standard solution (200 μl), a portion was analyzed by the procedures above.

2.7. Intra-day and inter-day precision

Spiked samples were prepared by adding known amount of NiAc, NiNH2 and NiUAc to blank urine, and analyzed by the procedures described above. The spiked samples were stored at −40°C until assay. The inter-day study was performed over a one-week period.

3. Results and discussion

3.1. Selection of buffer and surfactant for electrophoresis or MEKC

Two strategies are considered useful for the analysis of NiAc and its possible metabolites. One is a simple electrophoretic mode, and differences in basicity of the nitrogen atom in the pyridine ring of NiAc and its metabolites may be a driving force for separation. The other is MEKC where hydrophobic characters are more important for separation. Fig. 2 shows an example of the separation of NiAc and seven other possible metabolites in an acidic buffer (50 m*M* phosphate buffer, pH 2.5).

The separation was completed within 30 min, but resolution among Ni-Ox, 2-Pyr and 2OH-Ni was not performed. Though the presence of 2OH-Ni and 6OH-Ni has not been reported in metabolites after administration of NiAc or NiNH2 in the rat, we attempted to separate eight compounds including these compounds to demonstrate the excellent separation.

In the second mode of separation using MEKC, we examined SDS and a polymer surfactant (BBMA) as the additive. Use of the buffer containing BBMA at a concentration of 1.0% (w/v) showed an interesting migration profile. However, resolution among NMNi, 2-Pyr and NiNH2 was not observed in 50 m*M* borate buffer (pH 9.0, data not shown). MEKC in alkaline borate buffer containing SDS allowed better resolution (see Section 3.2).

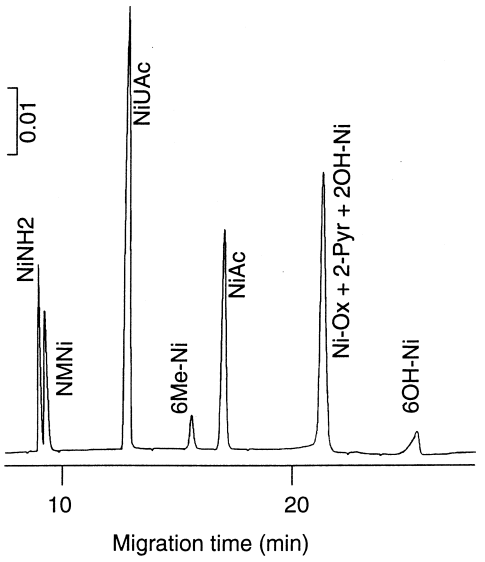

Fig. 2. CE of nicotinic acid and its possible metabolites in an acidic buffer. NiNH2=Nicotinamide; NMNi=N1-methyl-nicotinamide; NiUAc=nicotinuric acid; MeNiAc=methyl nicotinuric acid; NiAc=nicotinic acid; Ni-Ox=nicotinamide N-oxide; 2-Pyr=N1-methy-5-carboxamide-2-pyridone; 2OH-Ni=2-hydroxy nicotinic acid; 6OH-Ni=6-hydroxy nicotinic acid. Sample concentration, 100 mg/ml each. Analytical conditions: capillary, fused-silica 72 cm (effective length 60 cm)×75 μm I.D.; running buffer, 50 m*M* sodium phosphate buffer (pH 2.5); applied voltage, 15 kV; detection, UV absorbance at 214 nm; injection, 30 s (hydrostatic method); temperature, 37°C.

3.2. Effect of pH and SDS concentration on separation of NiAc and other metabolites

Fig. 3 shows the optimization studies on separation of NiAc and its possible metabolites. We found that trigonelline (the internal standard, I.S.) was observed almost at the same migration times of 7.5±0.1 min under all the conditions examined in the present study.

By using relative migration times to that of I.S., confirmation of the peaks was easy in a complex mixture observed for the analysis of the diluted urine sample. Therefore, relative migration times to I.S. were also used in the optimization studies.

Relative migration times of NiAc, 2OH-Ni, 6OH-Ni and NiUAc increased with the pH values of the buffer solutions. Those of other metabolites (NMNi, NiNH2, 2-Pyr and Ni-Ox) including I.S. were little affected by the changes of pH of the borate buffer. The electric current showed larger values than 80 μA at pH greater than 9.3. Fig. 3B shows the effect of the concentration of SDS on migration times. Relative migration times of all compounds except for Ni-Ox clearly increased. From these data, we decided to employ 50 m*M* borate buffer (pH 9.0) containing SDS to a concentration of 150 m*M* as the running buffer. In the present study, higher ionic

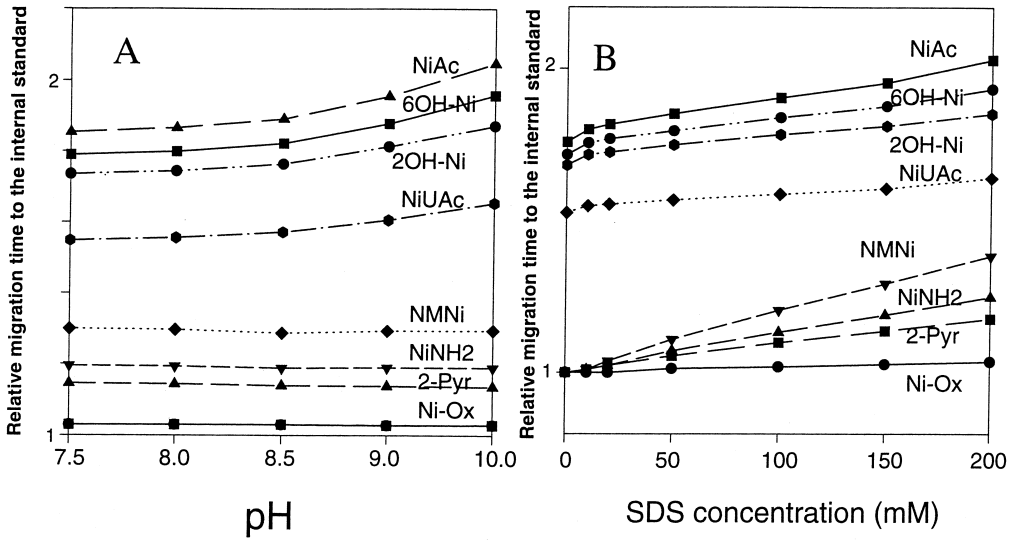

Fig. 3. Optimization study of analytical conditions in MEKC. (A) Effect of pH. (B) Effect of SDS concentration. Analytical conditions: running buffer, 50 m*M* sodium tetraborate buffer. Other conditions as in Fig. 2.

concentration than 50 m*M* caused large current values than 60 μA at 15 kV and the increase of noise in electropherograms.

Separation of a mixture of nicotinic acid and seven other possible metabolites at the optimized conditions are shown in Fig. 4.

Analysis was completed within 17 min. *N*-Methylated compounds (I.S., Ni-Ox, 2-Pyr, NiNH2 and NMNi) were observed earlier, because these compounds have a quaternary ammonium ion or a carboxamide group and are considered less charged than the later migrated compounds (NiUAc, 2OH-Ni, 6OH-Ni and NiAc). Hence, these earlier migrated compounds moved with electroosmosis, and were separated mainly based on partition between the buffer and SDS micelles. In contrast, later eluted compounds (NiUAc, 2OH-Ni, 6-OHNi and NiAc) were present as carboxylate anions, and migrated against electroosmotic flow at a different velocity based on their charge. Bernofsky reported the separation of nicotinic acid and its metabolites using anion-exchange chromatography on a column of Dowex x1 (formate form) [15]. The separation showed the similar elution order with the present results and indicated that the discussions described above were germane.

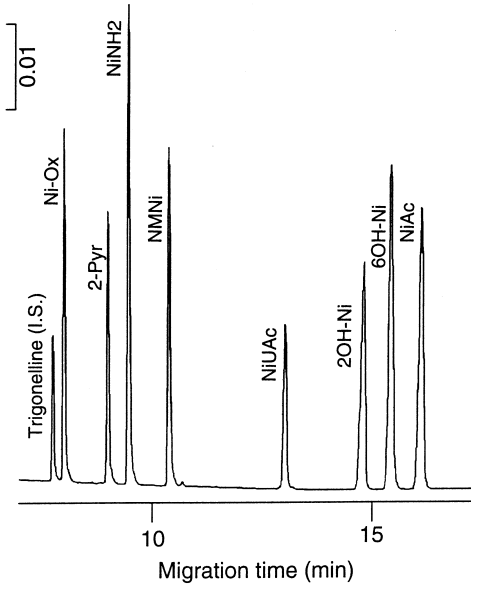

Fig. 4. Separation of a mixture of nicotinic acid and its possible metabolites by MEKC. Analytical conditions; running buffer, 50 m*M* sodium tetraborate buffer containing 150 m*M* SDS. Other conditions as in Fig. 2.

3.3. Interference by endogenous components in urine

Fig. 5A shows a typical electropherogram of a blank urine sample. The large peak (peak 1) observed at 8.05 min was that of the internal standard.

Spectrophotometric characteristics of each peak are also shown in Fig. 5B. The peak (peak 2) at 8.3 min was not resolved with that of Ni-Ox. Elimination of the interference due to the co-migrating substance was performed by multi-wavelength detection. Ni-Ox was determined by monitoring the absorbance at 260 nm, because peak 2 in blank urine had no absorption at 260 nm as shown in Fig. 5B. Other endogenous peaks observed in rat urine did not interfere with the determination of other metabolites. Therefore, metabolites other than Ni-Ox were determined by monitoring the absorbance at 214 nm to improve sensitivity.

3.4. Calibration curves and reproducibility

Under the conditions described in Section 3.2, calibration curves for all compounds showed good linearity at least in the range of 5–250 μg/ml, and the results are summarized in Table 1.

The present method showed excellent reproducibility, and the relative standard deviations (R.S.D.s) were within 3% for all compounds at 50 and 200 μg/ml levels, though the results observed for some metabolites showed larger values than 4% at the 20 μg/ml level. Recoveries of NiAc and its possible metabolites spiked in urine are shown in Table 2.

As the present method did not require the clean-up procedure, the recoveries of NiAc, NiNH2 and NiUAc were excellent. And the R.S.D.s were within 4% at 50 and 100 μg/ml levels, though R.S.D.s became larger at the 20 μg/ml level. A typical electropherogram is shown in Fig. 6 using a mixture at lower limit of detection (1.0 μg/ml).

All metabolites other than NiUAc were clearly distinguished even at this concentration. The present method did not allow micro-scale determination of NiAc and its possible metabolites below 2 μg/ml,

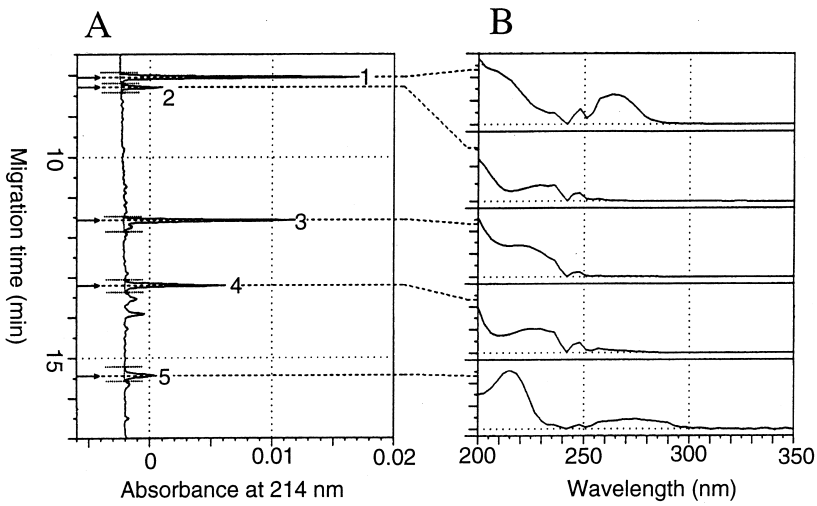

Fig. 5. MEKC of a diluted blank urine sample. A urine sample collected from a male Wistar rat under nestia was diluted 10-times with water. Peak 1 is due to trigonelline (the internal standard). Other peaks were due to endogenous components in urine and were not identified. Analytical conditions as in Fig. 4.

but may satisfy the routine analysis in high-dose administration such as in the treatment of hyperlipemia.

3.5. Application to measurement of the urinary excretion of NiAc and NiNH2 and their major metabolites in rat

NiAc in high-dose administration is used for the treatment of hyperlipemia, and often results in side effects such as flushing and hepatitis [6]. To confirm which compound(s) is responsible for these side effects, simultaneous determination of all metabolites is mandatory. Fig. 7 shows the cumulative urinary excretion of the unchanged drug and its metabolites after intravenous or oral administration at 45 mg/kg dosing. Figs. 7A and C show the curves after oral administration of NiAc and NiNH2, respectively. Both compounds were excreted as NiUAc with the parent drugs. Metabolites other than NiUAc were not determined for either compound. The curve observed in oral administration of NiAc reached a plateau 5 h

Table 1
Range of calibration curves and reproducibility in determination of nicotinic acid and its possible metabolites

Compound	Detection wavelength (nm)	Range of calibration curves (μg/ml)	Lower limit of detection (μg/ml)	Reproducibility in determination (%)[a,b]		
				20 μg/ml	50 μg/ml	200 μg/ml
Ni-Ox	260	10–250 (r=0.994)	2	7.3	0.41	0.62
2-Pyr	214	5–250 (r=0.997)	1	4.5	2.7	1.8
NiNH2	214	5–250 (r=0.999)	1	0.76	0.47	1.4
NMNi	214	5–250 (r=0.999)	1	0.76	0.32	1.7
NiUAc	214	10–250 (r=0.994)	5	3.5	2.2	1.7
2OH-Ni	214	5–250 (r=0.999)	1	3.7	2.6	1.9
6OH-Ni	214	5–250 (r=0.999)	1	4.5	2.7	1.4
NiAc	214	5–250 (r=0.999)	2	3.9	0.82	1.4

[a] The number of determinations was 6.
[b] Reproducibility is expressed as relative standard deviation.

Table 2
Intra-day and inter-day reproducibilities for NiAc, NiNH2 and NiUAc

Compound	Concentration (μg/ml)	Intra-day (n=6)		Inter-day (n=6)	
		Recovery (%)	R.S.D.[a] (%)	Recovery (%)	R.S.D. (%)
NiAc	20	103	5.4	101	5.1
	50	96	3.2	98	2.9
	100	98	2.1	99	1.9
NiNH2	20	103	4.8	104	5.1
	50	98	3.7	96	3.3
	100	99	1.2	97	1.8
NiUAc	20	106	6.6	104	6.3
	50	99	1.3	100	3.8
	100	100	1.5	96	2.4

[a] The number of determinations was 6.

after administration. However, the curves observed in oral administration of NiNH2 gradually increased even 10 h after administration. Ninety percent of the NiAc dose was recovered as NiAc and NiUAc in urine within 5 h after dosing, but the total recovery after NINH2 dosing was much lower due to slow excretion rate.

Similar results were also observed in intravenous administration as shown in Figs. 7B and D. However, NMNi and NiNH2 were found as minor metabolites after intravenous administration of NiAc. In contrast, only NMNi was found as the minor metabolite following NiNH2 injection. As indicated in Fig. 1, one metabolic pathway of NiAc forms Ni-Ox, 2-Pyr and 4-Pyr through formation of NiNH2. Though excretion of NMNi suggested the formation of NiNH2, 2-Pyr and 4-Pyr as final metabolites, these compounds could not be detected in the present study. Sugihara et al. [16] reported marked differences in different strains of rats in liver aldehyde oxygenase activity that strongly correlated with oxidation of NMNi, and Wistar rats showed

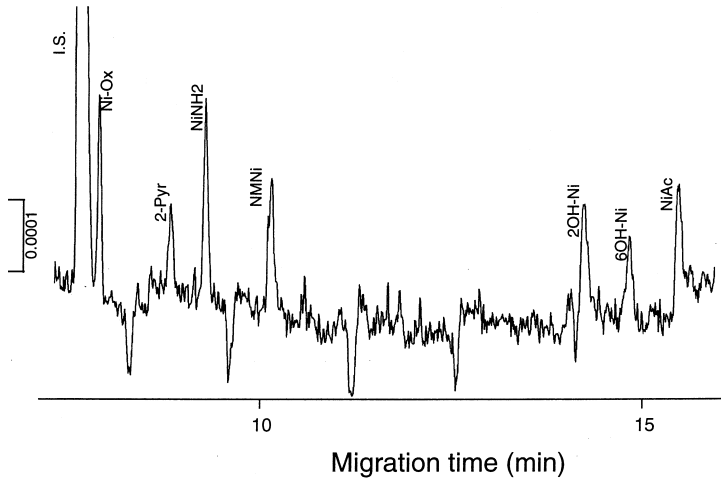

Fig. 6. Analysis of NiAc and its major metabolites at the lower limit of detection. The concentration of each compound was 1.0 μg/ml. Analytical conditions as in Fig. 4.

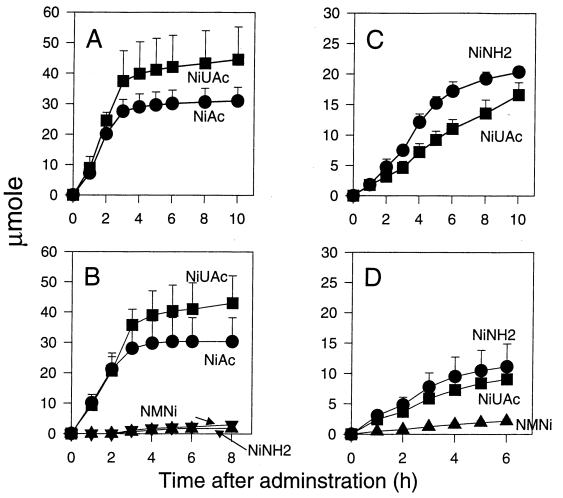

Fig. 7. Cumulative urinary excretion curves of nicotinic acid and nicotinuric acid in rats after intravenous and oral administration. (A) Oral administration of nicotinic acid at 45 mg/kg; (B) intravenous administration of nicotinic acid at 45 mg/kg; (C) oral administration of nicotinamide at 45 mg/kg; (D) intravenous administration of nicotinamide at 45 mg/kg.

significantly low enzyme activity in liver cytosols. Therefore, the pathway to NiUAc is the most predominant in Wistar rat used in the present study.

4. Conclusions

A method for the determination of nicotinic acid and nicotinamide and their six related metabolites was developed by using CE. The method allowed accurate determination of these compounds at least in the range of 5–250 mg/ml of all metabolites. Though one possible metabolite, nicotinamide *N*-oxide, was co-migrated with an endogenous component in urine, the interference could be eliminated by using multi-wavelength detection.

The method was applied to the determination of these compounds in rat urine after high-dose administration either orally or intravenously. The results indicated that the major metabolite was nicotinuric acid, and other possible metabolites were observed in very small amounts in Wistar rats.

References

[1] L.A. Carlson, L. Orö, J. Östman, Acta Med. Scand. 183 (1968) 457.
[2] W.B. Parsons, J.H. Flinn, Arch. Intern. Med. 109 (1955) 783.
[3] N. Svedmir, L. Harthon, L. Lundholm, Clin. Pharmacol. Ther. 196 (1969) 10.
[4] K. Shibata, T. Kawada, K. Iwai, J. Chromatogr. 424 (1988) 23–28.
[5] K. Yasuda, S. Shimada, Y. Itokawa, T. Takeuchi, M. Matsuoka, H. Otsuka, Vitamin 63 (1989) 259.
[6] M. Iwaki, T. Ogiso, H. Hayashi, E.T. Lin, L.Z. Benet, J. Chromatogr. B 661 (1994) 154–158.
[7] K.D. Altria, J. Chromatogr. 646 (1995) 495.
[8] S.R. Rabel, J.F. Stobaugh, Pharm. Res. 10 (1993) 171.
[9] D. Levèque, C.G. Renault, H. Monteil, F. Jehl, J. Chromatogr. B 697 (1997) 67–75.
[10] C.X. Zhang, Y. Aebi, W. Thormann, Clin. Chem. 42 (1996) 1805–1811.
[11] A. Schmutz, W. Thormann, Ther. Drug. Monit. 16 (1994) 483–490.
[12] G. Hempel, S. Haberland, P. Schulze-Westhoff, N. Mohling, G. Blaschke, J. Boos, J. Chromatogr. B 698 (1997) 287–292.
[13] P.K. Zarzycki, P. Kowalski, N. Nowakowska, H. Lamparczyk, J. Chromatogr. A 709 (1995) 203–208.
[14] J.W. Huff, J. Biol. Chem. 171 (1947) 639–640.
[15] C. Bernofsky, Anal. Biochem. 96 (1979) 189–200.
[16] K. Sugihara, K. Shimomiya, S. Kitamura, S. Ohta, K. Tatsumi, C.E. Green, C.A. Tyson, T. Ohara, S. Ninomiya, Xenobio. Metabol. Dispos. 12 (1997) S108–S109.