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Journal of Chromatography A, 709 (1995) 203–208

JOURNAL OF CHROMATOGRAPHY A

High-performance liquid chromatographic and capillary electrophoretic determination of free nicotinic acid in human plasma and separation of its metabolites by capillary electrophoresis

P.K. Zarzycki, P. Kowalski, J. Nowakowska, H. Lamparczyk*

Medical Academy, Faculty of Pharmacy, Gen. J. Hallera 107, PL-80416 Gdańsk, Poland

Abstract

Two methods are described based on high-performance liquid chromatography and capillary electrophoresis that provide the selective and sensitive determination of nicotinic acid in human plasma. Moreover, the capillary electrophoresis system was used for the separation of nicotinic acid, nicotinamide, nicotinamide N-oxide, N'-methylnicotinamide, 6-hydroxynicontinic acid, nicotinuric acid and barbital (internal standard). The extraction procedure is simple; no gradient elution or derivatization is required. Both methods can be useful for clinical and biomedical investigations.

1. Introduction

Nicotinic acid and its derivatives are widely used in the treatment of hyperlipaemia. In order to determine its concentration in human plasma, spectrophotometric [1–3], paper chromatographic [4], thin-layer chromatographic [5], gas chromatographic [6,7] and high-performance liquid chromatographic (HPLC) [8–13] methods have been described, but some of these methods are currently rarely used. The spectrophotometric method is not specific enough. Generally, planar chromatographic methods are time consuming and are not precise enough. In gas chromatographic methods, nicotinic acid must be derivatized to volatile compounds [6,7]. HPLC methods require either a very long column (50 cm) packed with an ion-exchange stationary phase [9] or the use of an ion-pair chromatographic system [12,13]. Recently, the separation of a standard mixture of eight acids, including nicontinic acid, using electroosmotic flow capillary electrophoresis (CE) was described by Liu and Sheu [14].

The plasma concentration profiles of nicotinic acid and nicotinuric acid after oral administration of 500-mg doses of nicotinic acid was reported by Takikawa et al. [9]. The peak concentrations (C_{max}) of nicotinic acid and nicotinuric acid were ca. 9.2 and 3.0 μg/ml, respectively.

The aim of this study was to evaluate two methods, HPLC and CE, for the determination of nicotinic acid in human plasma.

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00247-2

2. Experimental

2.1. Reagents

Nicotinic acid, nicotinamide N-oxide, N'-methylnicotinamide, 6-hydroxynicotinic acid, nicotinuric acid, *n*-dodecylamine and 3-(N-morpholino)propanesulfonic acid (MOPS) were purchased from Sigma (St. Louis, MO, USA). Nicotinamide was obtained from Koch-Light Laboratories (Colnbrook, Bucks., UK). Barbital, anthranilic acid, sodium tetraborate decahydrate, potassium dihydrogenphosphate, sodium nitrate, phosphoric acid (85%) and hydrochloric acid were obtained from POCh (Gliwice, Poland). Acetonitrile (Merck, Darmstadt, Germany) was of HPLC grade. Methanol and acetone were purchased from POCh; acetone was purified by double distillation from above anhydrous $CaCl_2$. Water was purified by double distillation. Mobile phases and buffers were filtered through a 1.5-μm membrane prior to use. The structures of nicotinic acid metabolites and the internal standards are shown in Fig. 1.

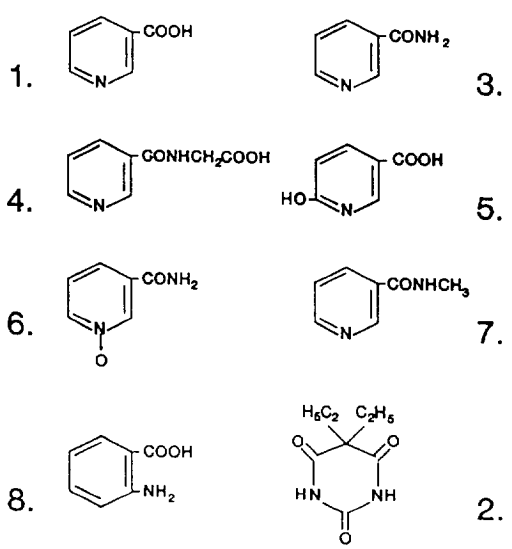

Fig. 1. Structures of nicotinic acid (1), nicotinamide (3), nicotinuric acid (4), 6-hydroxynicotinic acid (5), nicotinamide N-oxide (6), N'-methylnicotinamide (7) and the internal standards barbital (2) and anthranilic acid (8).

2.2. High-performance liquid chromatography

The method employed is based on two methods described by others [12,13]. The mobile phase used was a mixture of acetonitrile and MOPS buffer (10 m*M* MOPS, pH 5.4) (25:75, v/v). *n*-Dodecylamine was added to the mobile phase to give a final concentration of 1 m*M*.

Stock solutions of standards were prepared in acetonitrile at a concentration of 1 mg/ml. From these stock solutions, appropriate injection standard solutions were prepared by mixing the required volume of the stock solution and the chromatographic mobile phase. Typically, 20 μl of these standard solutions were injected.

An ODS-2 column (250 × 4 mm I.D., particle size 5 μm) was obtained from Knauer (Berlin, Germany). The liquid chromatograph consisted of an analytical solvent pump (Knauer A0307), a UV–Vis spectrophotometer (A0293) and a linear recorder (Knauer). A Rheodyne Model 7125 injection valve and a 20-μl loop were used for sample introduction. The UV detector was operated at 262 nm. The flow-rate was 1 ml/min. The column temperature (60 ± 0.1°C) was controlled by immersing the column in a stirred constant-temperature bath containing water used as a heat-exchange medium. The bath was connected to a thermostat. Additionally, the bottle containing the mobile phase was thermostated for 1 h before the experiment in order to obtain proper temperature equilibrium.

2.3. Capillary electrophoresis

A P/ACE 2100 capillary electrophoresis system, from Beckman, equipped with a UV detector operated at 254 nm, was used. The electrophoretic analyses were performed using an unmodified fused-silica capillary (51 cm from injector to detector, 57 cm total length, 50 μm I.D.). Samples were injected by a pneumatic system; the best overall separation was obtained with a 7-s injection. The voltage was maintained at 25 kV and the temperature was set at 25°C. Solutions of 10 m*M* sodium tetraborate (pH 9.36) or acetonitrile–buffer [10 m*M* potassium dihydrogenphosphate (pH = 2.50), titrated with phos-

phoric acid] (1:9, v/v) were used for electrophoretic experiments.

Stock solutions of standards were prepared in acetonitrile at a concentration of 1 mg/ml. From these solutions, appropriate injection solutions were prepared by mixing the required volume of the stock solution with water.

2.4. Sample preparation

The plasma samples (0.5 ml) were spiked with required amounts of nicotinic acid and also, in the case of CE, with nicotinamide. After that, internal standards i.e., 50 μl of anthranilic acid at a concentration of 100 μg/ml or 10 μl of barbital at a concentration 1000 μg/ml for HPLC and CE, respectively, were added. Subsequently, the samples were deproteinized with a 3 ml of acetone–water (2:1, v/v), centrifuged and evaporated to dryness. The residue was dissolved in 0.5 ml of 0.1 *M* concentrated hydrochloric acid, mixed with 1 ml of methanol and evaporated to dryness. Finally, the residue was dissolved in 200 μl of water or 350 μl of the HPLC mobile phase.

3. Results and discussion

3.1. Samples

Most of the experiments were performed on spiked drug-free plasma samples, although limited numbers of analyses were made using plasma samples from healthy volunteers, after ingestion of a 250-mg dose of nicotinic acid. From the analytical point of view, the results were similar. This can be easily explained, because the protein binding of nicotinic acid is very weak [15].

It is well known that the liquid–liquid extraction of nicotinic acid and some of its metabolites from water or plasma is not effective, because they are very hydrophilic and water soluble at all pH values [9]. In the described purification procedure, the plasma sample was deproteinized with acetone–water and the sample evaporated without extraction by water-immiscible solvent.

3.2. HPLC method

The mechanism of the separation of the acids on the HPLC column is based on the formation of ion pairs between nicotinic acid or anthranilic

Table 1
Numerical data for detection linearity in the HPLC method

Concentration of nicotinic acid (x) (μg/ml)	Peak height (y) (mm)
0.2	3
2.0	21
5.0	70
10.0	145
20.0	276
40.0	548
60.0	768

Regression equation: $y = 7.0\ (\pm 8.0) + 13.0\ (\pm 0.3)x$, $r = 0.9989$.

Table 2
Numerical data for calibration graph for nicotinic acid in the HPLC method with anthranilic acid as internal standard

Concentration of nicotinic acid (x) (μg/ml)	Peak-height ratio (y)
0.5	0.1250
1.0	0.1558
2.0	0.3765
4.0	0.7917
8.0	1.5500
12.0	2.3320

Regression equation: $y = 0.04\ (\pm 0.02) + 0.195\ (\pm 0.003)x$, $r = 0.9997$.

Table 3
Intra-assay precision for the HPLC method

Amount added (μg/ml)	Amount found ±S.D.[a] (μg/ml)	R.S.D.[a](%)
0.5	0.479 ± 0.07	15.2
1.0	1.068 ± 0.1	12.1
2.0	2.032 ± 0.1	6.3
4.0	3.914 ± 0.3	7.3
8.0	8.324 ± 0.2	2.7
12.0	12.611 ± 0.8	6.1

[a] $n = 5$.

acid and *n*-dodecylamine [12,13]. The detection limit of authentic samples for the HPLC method was 0.1 μg/ml and the detection curve was linear over a wide range of nicotinic acid concentration, from 0.2 to 60 μg/ml (Table 1). Numerical data for the calibration graph and its statistics are given in Table 2. The inter-assay variability was determined by analysing five replicates at each concentration in the range 0.5–12 μg/ml (Table 3). The chromatograms of a drug-free plasma sample and a plasma sample spiked with nicotinic acid and internal standard are shown in Fig. 2. The retention time of nicotinic acid, under these conditions, is 7.49 min. The separation between the nicotinic acid and internal standard peaks is excellent. As can be seen, these peaks do not interfere with the background peaks. However, it was not possible to obtain a satisfactory result with respect to sensitivity and separation for nicotinic acid metabolites from plasma using the HPLC method.

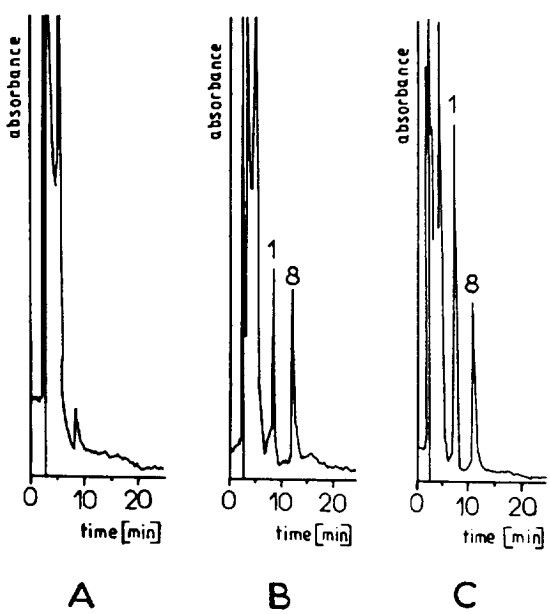

Fig. 2. (A) HPLC of drug-free plasma sample. (B) Chromatogram of a plasma sample containing nicotinic acid at 4 μg/ml and internal standard at 10 μg/ml. (C) Chromatogram of a plasma sample containing nicotinic acid at 8 μg/ml and internal standard at 10 μg/ml. Mobile phase: acetonitrile–10 m*M* MOPS buffer m*M* (pH = 5.4) (25:75, v/v), modified by the addition of 1 m*M* *n*-dodecylamine. Column, Knauer ODS-2 (250 × 4 mm I.D., particle size 5 μm) at 60°C, flow-rate, 1 ml/min; sample loop, 20 μl; UV detection at 262 nm. Peaks: 1 = nicotinic acid; 8 = internal standard (anthranilic acid).

3.3. CE method

The separation between nicotinamide, barbital and nicotinic acid by CE with UV absorbance detection at 254 nm is shown in Fig. 3A. The standards were well separated and their migration times were 2.97, 4.61 and 5.45 min, respectively. Fig. 3B and C show typical electropherograms of a plasma blank and a plasma sample spiked with nicotinomide, barbital (internal standard) and nicotinic acid at concentrations of 12, 20 and 12 μg/ml, respectively. Using 10 m*M* borax solution as a background electrolyte, the separation between nicotinic acid, barbital, nicotinuric acid and 6-hydroxynicotinic acid is satisfactory. Fig. 4 shows that the nicotinic acid peak does not interfere with the investigated metabolites and internal standard peaks. However, under these conditions no separation is observed between nicotinamide, nicotinamide N-oxide and N′-methylnicotinamide.

The detection curve was linear over a wide range of nicotinic acid concentration, from 1 to 100 μg/ml (Table 4). Table 5 gives numerical data for the calibration together with the statistical evaluation. The inter-assay variability was determined by analysing five replicates at each concentration in the range 1–12 μg/ml (Table 6).

Fig. 5 shows an electropherogram of the investigated compounds using acetonitrile–buffer [10 m*M* potassium dihydrogenphosphate (pH 2.50), titrated with phosphoric acid] (1:9, v/v) as background electrolyte. The separation between nicotinic acid and its metabolites was greatly improved in comparison with the separation obtained with a borax electrolyte (Fig. 4). However, the migration order of the investigated compounds is changed and the migration times

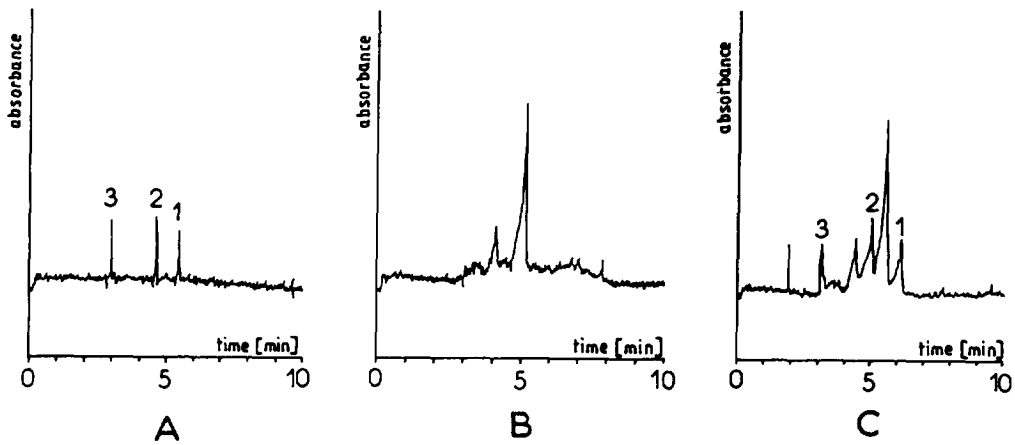

Fig. 3. (A) Electropherogram of a standard solution containing: (1) nicotinic acid, (2) barbital and (3) nicotinamide. (B) Electropherogram of drug-free plasma sample. (C) Electropherogram of a plasma containing (1) nicotinic acid at 12 μg/ml, (2) barbital (internal standard) at 20 μg/ml and (3) nicotinamide at 12 μg/ml. Conditions: applied voltage, 25 kV; 7-s pneumatic injection, unmodified fused-silica capillary (57 cm × 50 μm I.D.) at 25°C; buffer 10 m*M*, sodium tetraborate decahydrate (pH 9.36); UV detection at 254 nm.

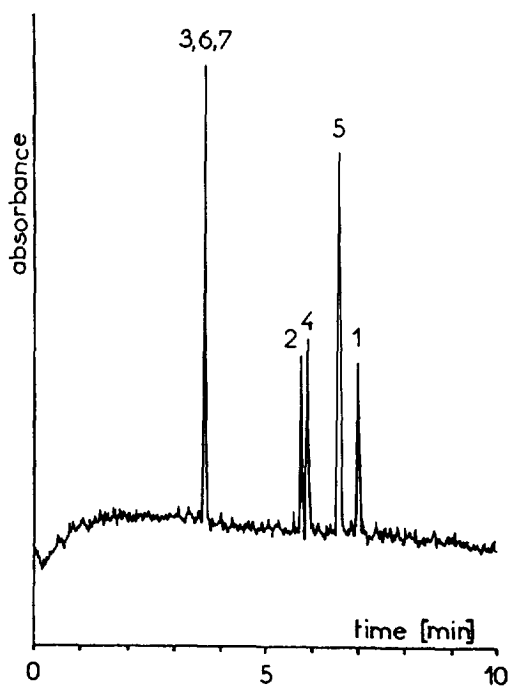

Fig. 4. Electropherogram of a standard solution containing (1) nicotinic acid, (2) barbital and (4) nicotinuric acid at concentrations of 20 μg/ml and (3) nicotinamide, (5) 6-hydroxynicotinic acid, (6) nicotinamide N-oxide and (7) N'-methylnicotinamide at concentrations of 10 μg/ml. Conditions as in Fig. 3.

Table 4
Numerical data for detection linearity in the CE method

Concentration of nicotinic acid (x) (μg/ml)	Peak height (y) (mm)
1	8
2	10
5	19
10	32
20	67
40	136
60	206
100	316

Regression equation: $y = 4.0\ (\pm 3.0) + 3.18\ (\pm 0.06)x$, $r = 0.9989$.

of nicotinamide N-oxide and 6-hydroxynicotinic acid are longer (21.08 and 26.99 min, respectively).

In conclusion, it can be stated that both methods are sensitive, specific and reproducible enough for therapeutic drug monitoring. The main advantage in the application of CE for the determination of nicotinic acid is the low cost of the reagents used in the mobile phase. Moreover, CE gives the possibility of determining nicotinic acid and its metabolites simultaneously.

Table 5
Numerical data for calibration graph for nicotinic acid in the CE method with barbital as internal standard

Concentration of nicotinic acid (x) (μg/ml)	Peak-height ratio (y)
1	0.1110
2	0.1710
4	0.2637
6	0.4075
8	0.5144
10	0.6342
12	0.7270

Regression equation: $y = 0.053\ (\pm 0.009) + 0.057\ (\pm 0.001)x$, $r = 0.9982$.

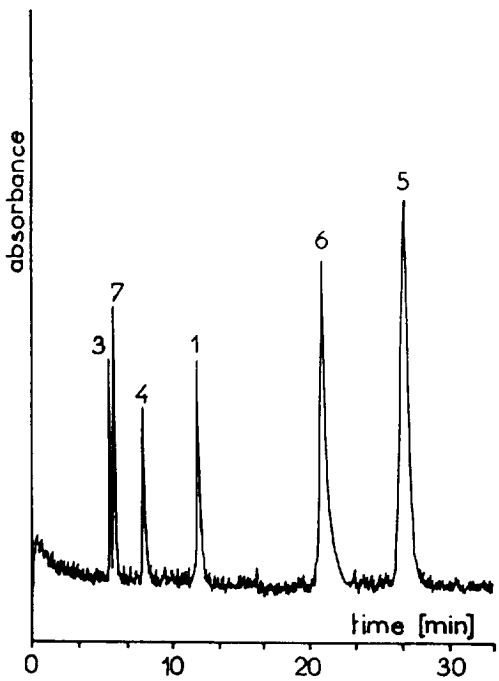

Fig. 5. Electropherogram of a standard solution containing (1) nicotinic acid, (3) nicotinamide, (4) nicotinuric acid (5) 6-hydroxynicotinic acid, (6) nicotinamide N-oxide and (7) N'-methylnicotinamide at concentrations of 20 μg/ml. Electrolyte, acetonitrile–buffer [10 mM potassium dihydrogenphosphate (pH 2.50), titrated with phosphoric acid]. Other conditions as in Fig. 3.

Table 6
Intra-assay precision for the CE method.

Amount added (μg/ml)	Amount found ±S.D.[a] (μg/ml)	R.S.D.[a](%)
1	1.008 ± 0.1	11.5
2	2.059 ± 0.2	9.8
4	3.683 ± 0.4	9.9
6	6.203 ± 0.4	7.1
8	8.074 ± 0.7	9.0
10	10.174 ± 0.7	6.8
12	11.799 ± 1.0	8.3

[a] $n = 5$.

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