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## Note

# High-performance liquid chromatographic determination of nicotinic acid and its metabolites, nicotinuric acid and nicotinamide, in plasma

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Nicotinic acid has been used for the treatment of hyperlipemia. It is pharmacologically active at very low plasma concentrations of 0.1—0.4 μg/ml [1, 2]. Nevertheless, a large dose (1.5—6 g/day) of nicotinic acid is necessary to maintain the pharmacologically effective plasma concentration because of its rapid elimination from blood [3]. In order to prevent the rapid and excessive increase of plasma concentration and the subsequent side-effects, several prodrugs have been examined and used [4—7].

Though the plasma concentration of nicotinic acid is a significant index of its pharmacological effects and side-effects [1, 2], no sensitive, specific, precise, and accurate method by which such low effective concentrations can be determined has been developed. Carlson's method [8] has been widely used for monitoring plasma concentrations of nicotinic acid after administration of nicotinic acid and its prodrugs. This method is based on extraction of nicotinic acid from plasma followed by a colorimetric determination with the modified Kõnig reaction [9]. However, this method is not necessarily specific only for nicotinic acid, but also for nicotinamide and other metabolites to some extent. With this method, furthermore, a large amount of plasma (3 ml) is necessary for measuring low concentrations (less than 1 μg/ml nicotinic acid). Blank extinction equivalent to 0.5—0.8 μg/ml of nicotinic acid was obtained [1]. And this blank extinction is not ascribed to endogenous nicotinic acid or nico-

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tinamide because normal plasma contains only minute amounts of these (less than 0.05 μg/ml) [1, 10, 11].

Some other methods have been reported for the assay of nicotinic acid in plasma. With the microbiological assay, the total concentration of nitotinic acid and nicotinamide is determined [12]. Preliminary isolation of nicotinic acid by thin-layer chromatography followed by quantitative analysis is time-comsuming and losses are great [8]. The spectrophotometric assay developed by Diab [13] was not sensitive enough to assay low effective plasma concentrations of nicotinic acid. In this study, we developed a selective and sensitive high-performance liquid chromatographic (HPLC) assay method for nicotinic acid in plasma by cation-exchange chromatography.

## EXPERIMENTAL

### *Materials*

Nicotinic acid and nicotinuric acid were purchased from Kishida, Osaka, Japan, and Sigma, St. Louis, MO, U.S.A., respectively. Nicotinamide and quinaldic acid were from Wako Pure Chemicals, Osaka, Japan. All the chemicals were of reagent grade and used without further purification.

### *Procedures*

One half milliliter of distilled water and 3 ml of acetone were added to 0.5 ml of plasma in a test tube, and agitated with a Vortex mixer. After centrifugation at 1500 *g* for 10 min, 3 ml of the supernate were transferred into a glass-stoppered test tube containing 3 ml of chloroform. The mixture was shaken for 5 min and then centrifuged at 1500 *g* for 5 min. One half milliliter of the aqueous layer was acidified with 0.1 ml of 0.1 *N* hydrochloric acid, and dried up with a centrifugal evaporator (Model RD-21, Yamato Scientific Co., Tokyo, Japan) at 60°C for 30 min. After addition of 200 μl of methanol to the residue, the solvent was evaporated in vacuo. Then, 200 μl of the internal standard (quinaldic acid) in acetone (4 μg/ml) were added. After centrifugation at 3500 *g* for 10 min, 150 μl of the supernate were taken to dryness in vacuo. The residue was dissolved in 50 μl of redistilled water and 20 μl of the solution were injected into the HPLC system. Plasma samples containing more than 2 μg/ml nicotinic acid were appropriately diluted with distilled water before being processed as above.

### *Chromatographic conditions*

A liquid chromatograph (Hitachi 635A) equipped with a high-pressure sampling valve (635-0650, 1 μl to 2.0 ml) and multiwavelength UV detector (Hitachi 635M) was used. For the stationary phase, a cation-exchange column (Zipax SCX, 25—37 μm, 50 cm × 2.1 mm I.D., E.I. Du Pont de Nemours & Co., Wilmington, DE, U.S.A.) was used, and the column was warmed at 45°C using a constant-temperature water bath circulator. The mobile phase consisted of 0.02 *M* phosphate buffer solution [$NaH_2PO_4$—$H_3PO_4$, 65 : 35 (pH 2.6) and 78 : 22 (pH 2.8) for nicotinic acid assay and metabolites assay, respectively]. The flow-rate was 1.0 ml/min and the pressure was approximately 30 kg/cm$^2$.

The wavelength and absorbance units full scale were 260 nm and 0.005, respectively.

*Calibration graph*

Standard solutions containing 0.1, 0.2, 0.4, 0.8, 1.2, 1.6, and 2.0 μg/ml nicotinic acid in distilled water were prepared. Instead of 0.5 ml of distilled water, 0.5 ml of each standard solution was added to 0.5 ml of drug-free plasma and the samples were processed as described above. The ratios of the peak height of nicotinic acid to that of quinaldic acid (internal standard) were used to construct a calibration graph.

*Monitoring of plasma concentrations*

The experiment was performed on a healthy female subject aged 27 years, weighing 47 kg. The basal blood sample was first taken after overnight fasting. Then 500 mg of nicotinic acid were administered orally with 100 ml of tap water. Blood samples were drawn through an indwelling venous catheter at 0.25, 0.5, 1, 2, 3, and 4 h. Food and beverages were restricted for 4 h after administration.

## RESULTS AND DISCUSSION

*Sample preparation*

Since nicotinic acid is water-soluble at all pH values, it can not be extracted from aqueous phase by organic solvents. Therefore, it is not easy to remove the interfering endogenous substances in plasma. Deproteinization by acetone and subsequent chloroform extraction have been used to reduce such substances [8, 14]. However, the aqueous layer obtained by these procedures was not sufficiently cleaned-up for the HPLC analysis of nicotinic acid. For further clean-up, the aqueous layer was acidified to make the carboxyl group of nicotinic acid non-ionic in form and the residue was dissolved in acetone. Under this condition, the solubilities of the cationic endogenous substances which have an affinity for the cation-exchange column were restricted although nicotinic acid can be dissolved.

The steps of addition and subsequent removal of methanol before addition of the internal standard acetone solution are necessary. Without these steps, the recovery and reproducibility became poor. Although the mechanism is unknown, it is likely related to the dissolution rate of nicotinic acid in acetone.

*Stationary phase*

Recently, in place of ion exchange, an ion-pair reversed-phase system has been preferably used for the HPLC assay of ionic or ionizable compounds because of its high column efficiency [11,15—17]. Hengen et al. [14] described an assay method for nicotinic acid by ion-pair reversed-phase chromatography. However, we could not obtain a satisfactory result in respect of sensitivity and separation from plasma blank with this method. Cation-exchange chromatography was preferable to ion-pair reversed-phase chromatography in our study of assay of nicotinic acid in plasma. The mobile phase was examined according to the investigation by Williams et al. [18].

*Selectivity*

Fig. 1 shows the chromatogram of plasma sample spiked with 1 μg/ml nicotinic acid, 4 μg/ml nicotinuric acid, and 8 μg/ml nicotinamide compared to plasma blank. Nicotinic acid and internal standard were well separated from endogenous substances and metabolites (nicotinuric acid and nicotinamide). No unexpected metabolite interfered in the chromatogram obtained from plasma sample after oral administration of nicotinic acid.

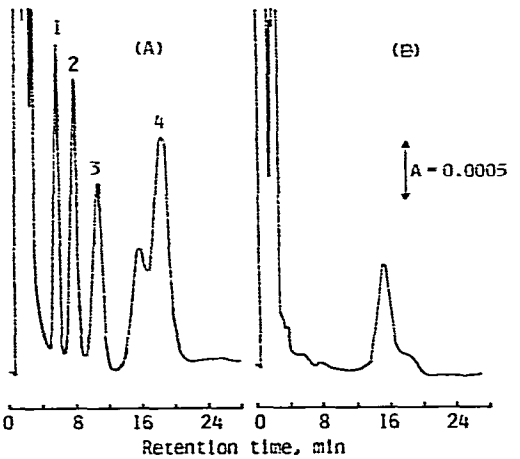


Fig. 1. High-performance liquid chromatograms of plasma sample spiked with 1 μg/ml nicotinic acid, 4 μg/ml nicotinuric acid, and 8 μg/ml nicotinamide (A), and of plasma blank (B). Mobile phase: $NaH_2PO_4$—$H_3PO_4$ (65 : 35). Peaks: 1 = nicotinic acid, 2 = internal standard, 3 = nicotinuric acid, 4 = nicotinamide.

The calibration curve of peak height ratio was linear with a correlation coefficient of 0.9997. The coefficient of variation at 0.5 μg/ml was 3.05% (n = 7).

The relative recovery of nicotinic acid from plasma sample containing 0.5 μg/ml was estimated in comparison with the assay of an aqueous solution, and 92 ± 2.8% (mean ± S.D., $n$ = 7) was accounted for. Nicotinic acid was spiked by the same procedure as described for the calibration graph.

The basal value of nicotinic acid (endogenous nicotinic acid) in six healthy adults was not determinable (signal-to-noise ratio = 2—3). The signal-to-noise ratio of the peak height response of 0.1 μg/ml which was the lowest concentration in the calibration curve examined was approximately 10. In this experiment "not determinable" means less than 0.05 μg/ml, though this method is sensitive enough to detect less than 0.05 μg/ml.

*Determination of metabolites*

The assay method for two major metabolites of nicotinic acid, nicotinuric acid (glycine conjugate) and nicotinamide, in plasma was also investigated. They could be assayed simultaneously by using the same sample solution pre-

pared for the nicotinic acid assay by HPLC; the only change was in the mobile phase (Fig. 2). Standard solutions containing 0.5, 1, 2, 3, 4, and 5 μg/ml nicotinuric acid, and 1, 2, 4, 6, 8, and 10 μg/ml nicotinamide were prepared. The calibration graphs were constructed by the same method as that of nicotinic acid, and they were linear with correlation coefficients of 0.9992 and 0.9985 for nicotinuric acid and nicotinamide, respectively. As the procedure for sample preparation was developed focusing on nicotinic acid, it is not necessarily satisfactory for each metabolite. However, without an additional volume of plasma and newly prepared samples by the other methods, they can be assayed using the sample solution prepared for nicotinic acid assay under the same HPLC conditions except for the change in the mobile phase.

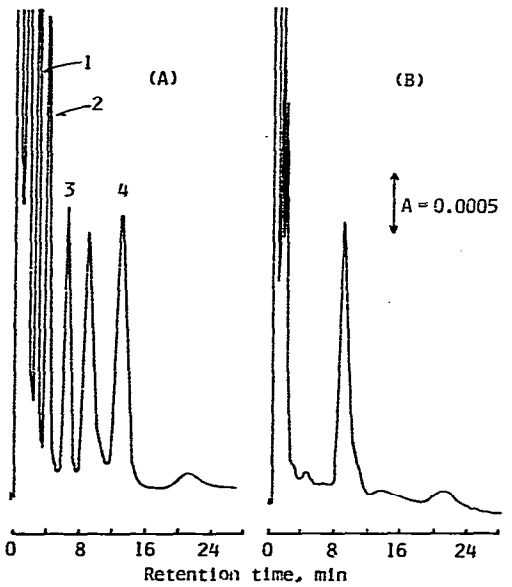


Fig. 2. High-performance liquid chromatograms of plasma sample spiked with 1 μg/ml nicotinic acid, 4 μg/ml nicotinuric acid, and 8 μg/ml nicotinamide (A), and of plasma blank (B). Mobile phase: $NaH_2PO_4$—$H_3PO_4$ (78 : 22). Peaks: 1 = nicotinic acid, 2 = internal standard, 3 = nicotinuric acid, 4 = nicotinamide.

*Plasma concentration profiles*

By using the newly developed assay method, the monitoring of plasma concentrations of nicotinic acid and its metabolites was performed. The plasma concentration profiles are shown in Fig. 3. After the rapid increase of nicotinic acid in large excess, it was rapidly eliminated from plasma. Nicotinuric acid, on the other hand, increased with the increase of the concentration of nicotinic acid and was gradually eliminated. The concentrations of nicotinamide were non-determinable (< 0.5 μg/ml) at all sampling times.

It is possible to determine the low plasma concentrations of nicotinic acid specifically and sensitively by the method described in this report. Our results suggest that the method is useful for monitoring nicotinic acid which is a significant index of its pharmacological effects and side-effects.

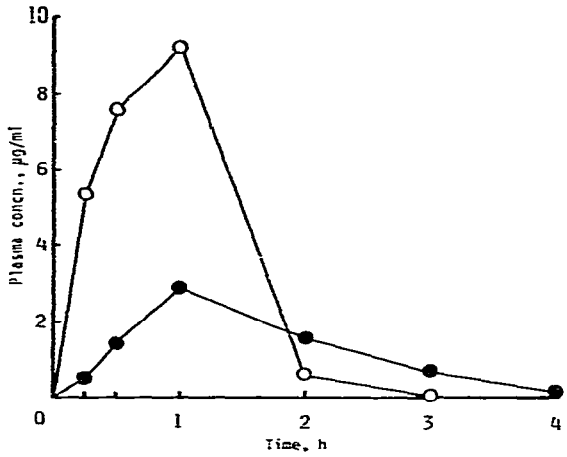


Fig. 3. Plasma concentration profiles of nicotinic acid (○) and nicotinuric acid (●) after oral administrations of 500 mg of nicotinic acid.

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