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DETERMINATION OF NICOTINIC ACID IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A sensitive method for the determination of nicotinic acid in serum is described which employs high-performance liquid chromatography with fluorescence detection. Nicotinic acid and 2-chloronicotinic acid as an internal standard in deproteinized serum are reacted with N,N'-dicyclohexyl-O-(7-methoxycoumarin-4-yl)methylisourea in acetone to give the corresponding fluorescent 4-hydroxymethyl-7-methoxycoumarin esters. The compounds are separated by reversed-phase chromatography on LiChrosorb RP-18 with isocratic elution using aqueous acetonitrile containing a small amount of sodium 1-hexanesulphonate as a mobile phase. The detection limit of nicotinic acid in serum was 0.2 nmol/ml. The method requires only 100 μl of serum.

INTRODUCTION

Nicotinic acid belongs to the vitamin B complex and it is a component of nicotinamide nucleotides. It shows pharmacological activities such as a vasodilator effect [1], and hypolipaemic action [2, 3], and it has a prophylactic effect in atherosclerosis [4]. Nicotinic acid has also been used for the treatment of pellagra [5]. These activities are dependent on the concentration of nicotinic acid in blood [6]. For the biomedical investigation of nicotinic acid

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and for the monitoring of the compound in blood during therapy, a sensitive and selective method is required.

Various methods have been reported for the determination of nicotinic acid in biological materials. Microbiological methods [7, 8] are sensitive but not selective; only the total amount of nicotinic acid and nicotinuric acid, a metabolite of nicotinic acid, can be determined in a narrow concentration range. The colorimetric method based on the König reaction [9] requires separation of nicotinic acid by thin-layer chromatography (TLC). The method is insensitive and requires 2—3 ml of serum. Liquid chromatography using an anion-exchange column [10] is time-consuming; reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [11] is much more sensitive, but still requires rather a large amount of serum (500 μl).

Recently, N,N′-dicyclohexyl-O-(7-methoxycoumarin-4-yl)methylisourea (DCCI) and N,N′-diisopropyl-O-(7-methoxycoumarin-4-yl)methylisourea (DICI) have been reported as fluorescent derivatization reagents of aliphatic and aromatic carboxylic acids [12,13]. These reagents give 4-hydroxymethyl-7-methoxycoumarin esters of the corresponding acids when reacted in organic solvent such as tetrahydrofuran or benzene at room temperature, but no ester is formed from nicotinic acid under these reaction conditions.

We found that both DCCI and DICI react with nicotinic acid in acetone in tightly closed test-tubes at a higher temperature to yield highly fluorescent esters which could be separated from the components of the reagent blanks by TLC or HPLC. On this basis we developed a sensitive method for the determination of nicotinic acid in a small amount of serum using reversed-phase HPLC with fluorescence detection. The fluorescence intensity of the reaction product with DCCI was greater than that with DICI and therefore DCCI was used in this method. Human serum fortified with nicotinic acid was used as a model to establish suitable conditions.

MATERIALS AND METHODS

Materials and apparatus

Nicotinic acid, 2-chloronicotinic acid, and silica gel TLC plates (Wakogel B-5, 5 × 10 cm, 250 μm thick) were purchased from Wako (Osaka, Japan). DCCI and DICI were kindly supplied by Dr. S. Goya (Faculty of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan) and were used without further purification. Sodium 1-hexanesulphonate was obtained from Tokyo-kasei (Tokyo, Japan). Other chemicals and solvents were of reagent grade. Normal sera were obtained from healthy volunteers (male, 21—52 years of age) of the Faculty of Pharmaceutical Sciences, Kyushu University, in the usual manner.

The HPLC system was a Yanaco L-200 liquid chromatograph (Yanagimoto, Kyoto, Japan) equipped with a Shimadzu FLD-1 fluorescence detector fitted with a coated mercury lamp and an EM-4 cut-off filter. A stainless-steel column (150 × 4.0 mm I.D.) was packed with LiChrosorb RP-18 (Merck Japan, Tokyo, Japan), particle size 5 μm, using a slurry technique [14]. Uncorrected fluorescence spectra were measured with a Hitachi MPF-4 spectrofluorometer in micro quartz cells (3 × 1.0 cm optical pathlength; 500 μl volume); spectral

bandwidths of 5 nm were used in both the excitation and emission sides of the monochromator.

Procedure

To a mixture of 100 μl of serum and 200 μl of aqueous 2-chloronicotinic acid (50 nmol/ml) as an internal standard placed in a 10-ml glass-stoppered centrifuge tube, 1.5 ml of acetone were added to precipitate the protein. The mixture was shaken for 10 min, followed by centrifugation (800 *g*, 5 min). The supernatant (1.3 ml) was transferred to a 10-ml glass-stoppered centrifuge tube and washed with 1.5 ml of chloroform. After centrifugation, 200 μl of the aqueous layer were transferred to a 10-ml glass-stoppered tube containing 100 μl of 0.05 *M* hydrochloric acid. The mixture was evaporated to dryness in vacuo at 25—30° C. To the residue, 100 μl of acetone were added and the resulting solution was shaken vigorously for 10 min, followed by centrifugation (800 *g*, 5 min). To 50 μl of the acetone solution (placed in a screw-capped 1.5-ml vial; Gasukuro Kogyo, Tokyo, Japan) 50 μl of 5m*M* DCCI solution in acetone were added. The tube was tightly closed and heated in a boiling water bath for 15 min. A 10-μl aliquot of the reaction mixture was injected into the chromatograph and eluted with acetonitrile—water (4:6, v/v) containing 5 m*M* sodium 1-hexanesulphonate. The flow-rate was 1.3 ml/min. The column temperature was ambient (approximately 25° C).

For the establishment of a calibration curve, a series of nicotinic acid standard solutions (5—200 nmol/ml) containing 2-chloronicotinic acid (50 nmol/ml) were prepared, and the mixtures of normal serum (100 μl) and standards (200 μl) were treated as described above. The peak height ratios of nicotinic acid and 2-chloronicotinic acid derivatives were plotted against the concentration of nicotinic acid.

TLC of the reaction mixture of nicotinic acid and 2-chloronicotinic acid with DCCI

A mixture (50 μl) of nicotinic acid and 2-chloronicotinic acid (each 100 nmol/ml) in acetone was treated with DCCI as described. The resulting mixture was applied on a silica gel thin-layer plate, and then developed with benzene—ethyl acetate (1:2, v/v) at approximately 25° C. The fluorescent bands corresponding to DCCI esters of nicotinic acid (R_F 0.41) and 2-chloronicotinic acid (R_F 0.68) were scraped off and the esters were extracted with acetone. The extraction solvent was removed in vacuo.

RESULTS AND DISCUSSION

HPLC conditions

Fig. 1 shows the chromatograms obtained with an acetone solution of nicotinic acid and 2-chloronicotinic acid, and with acetone alone as reagent blank. Although many peaks are observed in the reagent blank (Fig. 1b), which are probably due to decomposition products of DCCI under the conditions of the derivatization reaction, the peaks of the DCCI esters of nicotinic acid and 2-chloronicotinic acid are well separated (retention times 6.0 and 10.0 min, respectively) from the peaks of the reagent blank. The yields of both com-

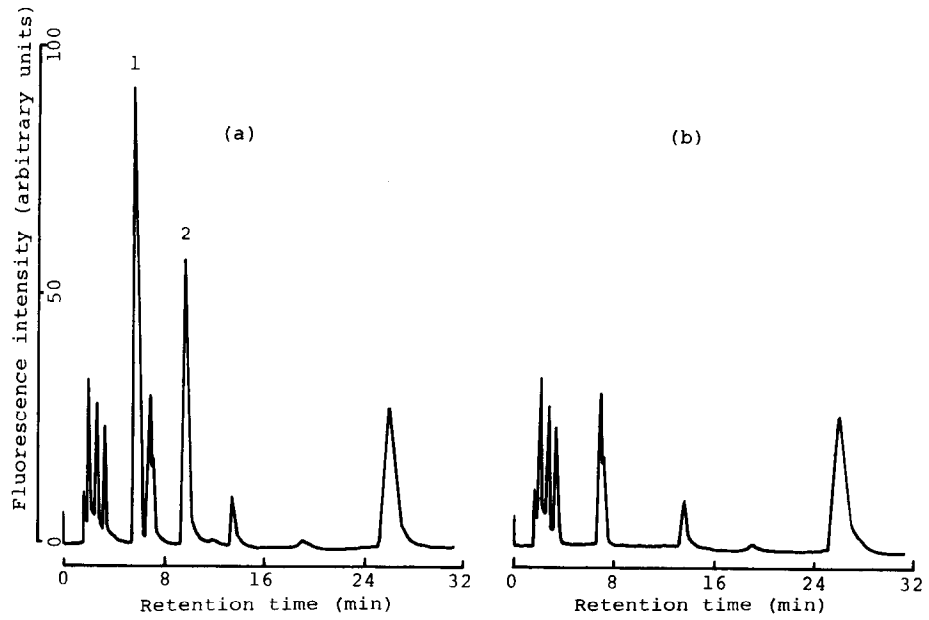

Fig. 1. Chromatograms of (a) DCCI esters of nicotinic acid and 2-chloronicotinic acid (internal standard), and (b) reagent blank. Aliquots (50 μl) of an acetone solution of nicotinic acid and 2-chloronicotinic acid (100 nmol/ml of each), and of acetone (blank) were subjected to the derivatization reaction. 1 = Nicotinic acid derivative; 2 = 2-chloronicotinic acid derivative.

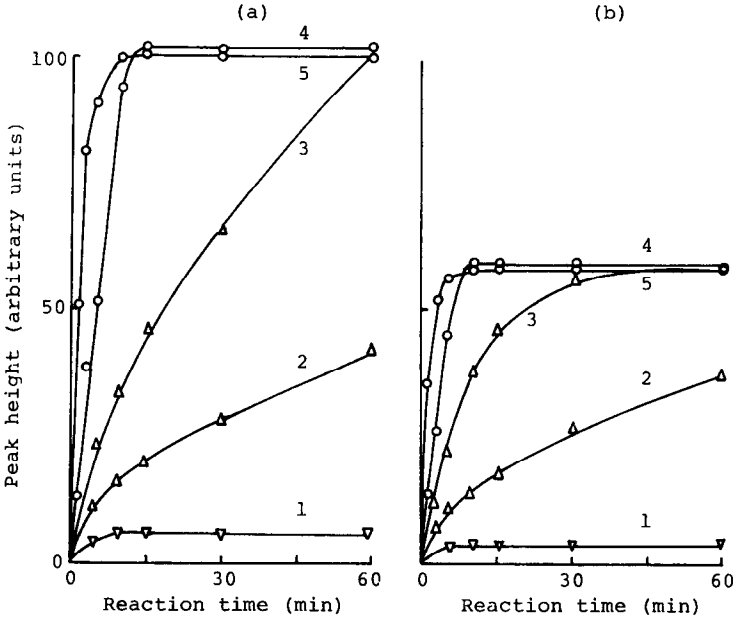

Fig. 2. Effect of reaction time and temperature on the peak heights of (a) nicotinic acid and (b) 2-chloronicotinic acid derivatives. Aliquots (50 μl) of an acetone solution of nicotinic acid and 2-chloronicotinic acid (100 nmol/ml of each) were treated with DCCI for various lengths of time at the following temperatures: 1, 20°C; 2, 40°C; 3, 60°C; 4, 80°C; 5, 100°C.

pounds are reproducible and there are linear relationships between peak heights and the amounts of nicotinic acid and 2-chloronicotinic acid esters. The eluates containing peak 1 and peak 2 (Fig. 1a) have fluorescence excitation maxima at 325 nm and 335 nm, respectively, and emission maxima at 395 and 390 nm, respectively.

The concentration of acetonitrile in the mobile phase affects the separation of the peaks. At concentrations above 50%, the peak for nicotinic acid overlaps with the nearest peak of the blank, while at a concentration of 35% or less there is a delay in the elution rate with broadening of the peaks. The presence of sodium 1-hexanesulphonate in the mobile phase does not affect the retention times of any of the peaks at concentrations of 0.5—10 m*M* but it sharpens the peaks at concentrations of 3 m*M* and above. In practice, 40% acetonitrile and 5 m*M* sodium 1-hexanesulphonate in the mobile pahse were used. When methanol was used in place of acetonitrile in the mobile phase, the peaks were broadened.

The formation of fluorescent derivatives of nicotinic acid and 2-chloronicotinic acid with DCCI is dependent on reaction time and temperature (Fig. 2). Maximum and constant peak heights for both compounds were obtained when the reaction was carried out at 80—100° C for approximately 10 min or longer. Heating at 100° C for 15 min was selected for convenience.

DCCI is soluble in polar and non-polar solvents, while nicotinic acid and 2-chloronicotinic acid are only soluble in polar solvents such as water, acetone and alcohols. Therefore, these solvents were examined for their usefulness in the derivatization reaction. Acetone gave the most intense and reproducible peaks with both acids; in contrast alcohols gave much smaller and unreproducible peaks (Table I). DCCI is decomposed to 4-hydroxymethyl-7-methoxycoumarin and other products in the presence of water under the conditions of the derivatization reaction. No derivative formation occurs in acetone if the reaction mixture contains water at 20% or higher.

The final reaction mixture in the procedure recommended in this work is stable for more than 2 h at room temperature with or without protection from light.

TABLE I

EFFECT OF SOLVENT ON THE DERIVATIZATION REACTION OF NICOTINIC ACID AND 2-CHLORONICOTINIC ACID WITH DCCI

Aliquots (50 μl) of a mixture of nicotinic acid and 2-chloronicotinic acid (100 nmol/ml of each) in the given solvent were subjected to the derivatization reaction.

Solvent	Relative peak height*	
	Nicotinic acid	2-Chloronicotinic acid
Acetone	100	60
Methylcellosolv	69	48
n-Propanol	72	54
Ethanol	56	32
Methanol	2	2

*Height of the peak for nicotinic acid obtained in the reaction in acetone was taken as 100.

Determination of nicotinic acid in serum

The deproteinization of serum can be achieved by adding acetone (final concentration approximately 83%) to serum diluted with an aqueous solution of the internal standard. Shaking for more than 5 min gives constant peak height ratios of nicotinic acid to 2-chloronicotinic acid, thus shaking for 10 min is recommended in the procedure. Washing of the deproteinized sample with chloroform serves to minimize the peaks ascribable to some substances occurring in serum.

Water should be removed from the deproteinized sample before derivatization for the reasons mentioned above. This can be done by adding diluted hydrochloric acid and then evaporating to dryness in vacuo. A very small amount of hydrochloric acid is required for the satisfactory extraction of nicotinic acid and 2-chloronicotinic acid with acetone from the resulting dried residue.

Fig. 3 shows typical chromatograms obtained from the serum of a healthy man who had not been administered nicotinic acid, and from the same serum fortified with nicotinic acid. Peaks for nicotinic acid and 2-chloronicotinic acid are separated by HPLC from all other peaks present in the serum sample and the reagent blank. Endogenous nicotinic acid in the serum produces only a small peak (Fig. 3a) because of its low concentration.

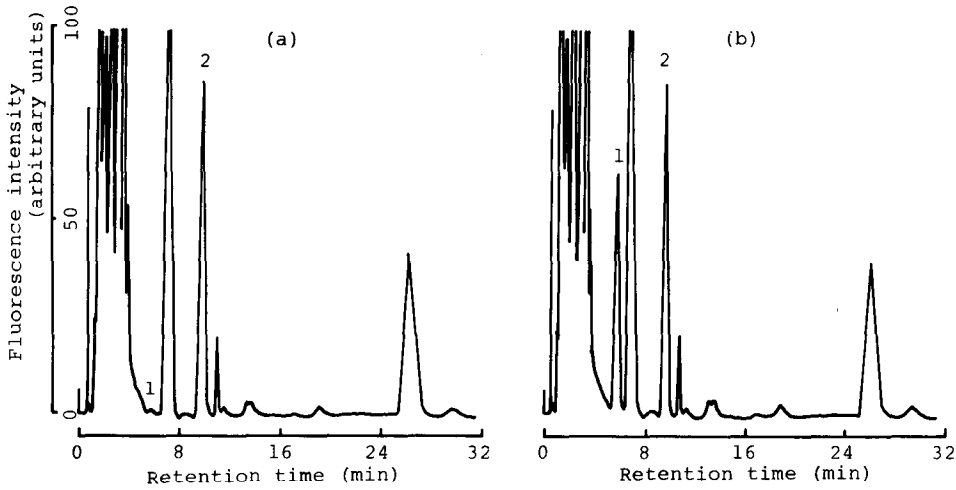

Fig. 3. Chromatograms from (a) normal serum and (b) serum fortified with nicotinic acid (50 nmol/ml). 1 = Nicotinic acid; 2 = 2-chloronicotinic acid.

The fluorescence excitation and emission spectra of peaks 1 and 2 in Fig. 3a or b, those of peaks 1 and 2 in Fig. 1a, and those of the DCCI esters of nicotinic acid and 2-chloronicotinic acid which were separated from other reaction products by TLC, were identical.

A linear relationship was observed between the peak height ratios of nicotinic acid to 2-chloronicotinic acid esters and the amounts of nicotinic acid added to serum in amounts between 100 and 400 nmol/ml. The recoveries of nicotinic acid and 2-chloronicotinic acid (100 nmol/ml of each) from serum were 81.5 ± 1.8% and 79.5 ± 1.5% (mean ± S.D., n = 10 for each), respectively.

The recoveries were calculated from the values obtained with the fortified serum samples and a nicotinic acid standard solution (50 nmol/ml) containing 2-chloronicotinic acid (50 nmol/ml).

The limit of detection for nicotinic acid in serum was 0.2 nmol/ml, at a signal-to-noise ratio of 2. The sensitivity is higher than that of the HPLC method with UV detection [11], but is not high enough to measure precisely endogenous levels of nicotinic acid in normal serum.

The precision was established by repeated determinations using normal serum to which 20 or 100 nmol/ml nicotinic acid was added. The coefficients of variation were 6.5 and 1.7% (n = 10 in each case), respectively. Nicotinamide and nicotinuric acid did not interfere with the nicotinic acid determinations.

This study provides the first HPLC method with fluorescence detection for the determination of nicotinic acid. The method may be applied in routine biomedical studies of nicotinic acid and for monitoring the drug during therapy.

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