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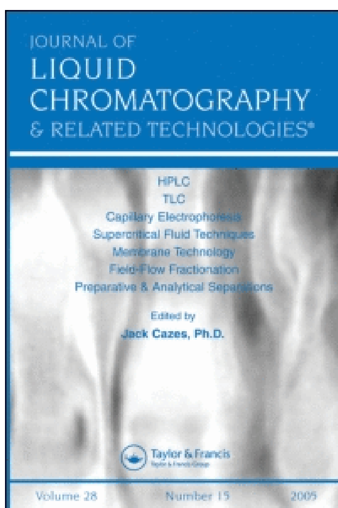

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AN IMPROVED METHOD FOR THE DETERMINATION OF NICOTINIC ACID IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple, specific, and sensitive HPLC method was developed for the determination of nicotinic acid in human plasma. Plasma is deproteinized with acetonitrile and centrifuged. The supernatant solution is evaporated and reconstituted in mobile phase. Separation is achieved using an IB-SIL CN column with a mobile phase composed of acetonitrile:methanol:water:acetic acid (700:150:150:1, v/v/v/v). Detection is by ultraviolet absorbance at 263 nm. The retention times of nicotinic acid and 6-methyl nicotinic acid (internal standard) are approximately 3.8 and 8.8 minutes, respectively. The assay is linear in concentration ranges of 100 to 10,000 ng/mL (original method) and 20 to 2,000 ng/mL (high-sensitivity method). The analysis of pooled quality controls (150, 1,250, and 7,530 ng/mL) demonstrates excellent precision with relative standard deviations (RSD) (n=18) of 3.4%, 2.7%, and 2.8%, respectively. For the high sensitivity method, quality control pools prepared at 60, 150, and 1,250 ng/mL had RSDs (n=18) of 5.8%, 2.9%, and 2.8%, respectively. The method is accurate with all intraday (n=6) and overall (n=18) mean values being less than 9% from theoretical at all control concentrations.

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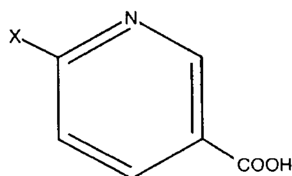

Nicotinic acid x = H

6-Methyl nicotinic acid x = CH_3

Figure 1. Molecular Structures of Nicotinic Acid and Internal Standard

INTRODUCTION

Nicotinic acid (niacin) (Figure 1) is part of the vitamin B complex and is essential for the prevention of pellagra [1]. It also has clinical use as a vasodilator and lipolytic agent when used in therapeutic doses [2]. A sensitive and specific assay for nicotinic acid in plasma was needed to support a clinical trial. Nicotinic acid has been extracted from physiological fluids using an acetone/chloroform extraction [3,4,5] or protein precipitation [6,7] prior to quantitation. High-performance liquid chromatography (HPLC) methods have utilized ion exchange [3,4] or ion-pairing [5,6,7], prior to ultraviolet (UV) absorbance detection. The method described herein uses acetonitrile for deproteinization as described by Altmayer and Garret [6] followed by reversed-phase separation with a cyano-functional (CN) column and UV detection. The method is rapid, sensitive, and selective. The original method was validated with a limit of quantitation (LOQ) of 100 ng/mL nicotinic acid in plasma, but a higher sensitivity method was also validated, having an LOQ of 20 ng/mL.

MATERIALS & METHODS

Reagents and Test Materials

Nicotinic acid was purchased as a reference substance from USP (Rockville, MD, USA). The internal standard (ISTD), 6-methyl nicotinic acid, purity 99+%, was purchased from Aldrich (Milwaukee, WI, USA). Heparinized human plasma was purchased from Interstate Blood Bank (Gilbertsville, PA, USA). Phosphoric acid (85%), methanol, and acetonitrile were

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all HPLC grade (Fisher Scientific, Pittsburgh, PA, USA). All water was passed through a Milli-Q apparatus (Millipore, Bedford, MA, USA).

Chromatographic System

The HPLC system consisted of a Perkin-Elmer Series 10 pump and an ISS-100 auto-injector (Norwalk, CT, USA), and an ABI/Kratos Model 783A programmable absorbance detector (Foster City, CA, USA) set at 263 nm (0.005 AUFS). Data collection and calculations were on a HP1000 computer with a Hewlett-Packard 3350A Laboratory Automation System (Palo Alto, CA, USA). The analytical column was an IB-SIL CN (150 mm x 4.6 mm, 10 μm particle size, Phenomenex, Torrance, CA, USA). The mobile phase was acetonitrile:methanol: water:acetic acid (700:150:150:1, v/v/v/v) with a flow rate of 1.5 mL/minute. The mobile phase proportions were changed to (700:190:
110:1) for the high-sensitivity method.

Standard and Control Preparation

Stock solutions (1 mg/mL) of nicotinic acid and ISTD were prepared in water and were stable for 2 months when stored at 5°C. Working solutions of nicotinic acid (0.1 to 50 μg/mL) and ISTD (20 or 30 μg/mL) were diluted weekly from the stock solutions and stored at 5°C to prevent bacterial growth.

Pooled quality control samples (controls) were prepared to determine the precision and accuracy of the method and to evaluate the stability of samples. Plasma control pools (60, 150, 1,250, and 7,500 ng/mL) were prepared by diluting 0.6 and 1.5 mL of
10 μg/mL nicotinic acid, and 125 and 750 μL of 1 mg/mL nicotinic acid, respectively, to 100 mL with blank human plasma. Controls pools were aliquoted into glass vials with PTFE-lined caps and stored at -20°C.

Sample Preparation

Samples were prepared by adding 200 μL of human plasma, 25 μL of ISTD (30 μg/mL), and 1 mL of acetonitrile to glass test tubes. Calibration standards (100 to 10,000 ng/mL) were prepared by using
200 μL of blank plasma and 40 μL of the appropriate nicotinic acid working solutions (0.5 to 50 μg/mL). The tubes were vortexed, allowed to stand 10 minutes, and then vortexed again. The tubes were centrifuged and the supernatant solution was decanted into clean tubes. The

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solution was evaporated to dryness at 40°C under nitrogen. The residue was reconstituted in 1 mL of mobile phase and 100 μL was injected into the HPLC system.

Recovery standards (used to determine the absolute recovery of nicotinic acid) were prepared by extracting blank plasma through the evaporation step, spiking the residue with nicotinic acid and ISTD solutions, evaporating to dryness again, and reconstituting in mobile phase.

Sample Preparation for the High-Sensitivity Method

Sample preparation for the high-sensitivity method has the following modifications. The ISTD concentration was 20 μg/mL and the injection volume was 150 μL. Calibration standards (20 to 2,000 ng/mL) were prepared by using 200 μL of blank plasma and 40 μL of the appropriate nicotinic acid working solutions (0.1 to 10 μg/mL).

Validation

Duplicate calibration curves were analyzed on each of three days. Triplicate controls at each concentration and blank plasma were included with each calibration curve. Calculations were by weighted (1/concentration) least squares linear regression analysis of the peak height ratios of nicotinic acid/ISTD versus the concentration of nicotinic acid.

RESULTS AND DISCUSSION

Reversed-phased chromatography using a CN column provided the selectivity, sensitivity and ruggedness required to determine the nicotinic acid concentrations of a large group of clinical specimens. Resolution of the nicotinic acid and the endogenous compounds could not be maintained using reversed-phase ion-pair chromatography on a C18 column. The CN column did produce chromatography with acceptable peak shape, retention on column, and resolution from interferences (Figure 2). The retention times of nicotinic acid and the ISTD were approximately 3.8 and 8.8 minutes, respectively. A tertiary mobile phase was required with the CN column to separate the internal standard from a late-eluting peak. The chromatography of the CN column was sensitive to the composition of injected solutions, but this was overcome by reconstitution in mobile phase. It was necessary to dry the extracts thoroughly before reconstitution, since residual moisture was found to impair resolution of the internal standard and matrix components. Column "aging" was evident as retention times decreased over time. Minor modifications to the mobile phase were made to compensate for

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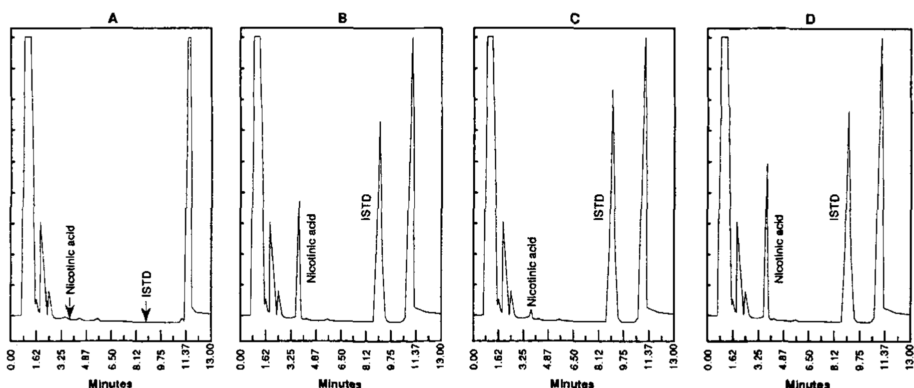

Figure 2. Chromatograms (High Sensistivity Method) of (A) Blank Human Plasma, (B) 1000 ng/mL calibration standard, (C) 60.0 ng/mL control, (D) 1250 ng/mL control

TABLE 1

Calibration Curve Parameters for Nicotinic Acid in Human Plasma
(Results of validation study over a 3-day period)

		Original Method			High-Sensitivity Method		
Day	Curve	Slope	Y Intercept	Correlation coefficient	Slope	Y Intercept	Correlation coefficient
1	1	0.0003	-0.0018	1.0000	0.0003	0.0023	0.9999
	2	0.0003	-0.0008	0.9996	0.0003	0.0020	0.9996
2	3	0.0004	0.0036	0.9998	0.0004	0.0030	0.9999
	4	0.0004	0.0049	0.9999	0.0004	0.0031	0.9993
3	5	0.0003	0.0021	0.9999	0.0003	0.0026	0.9998
	6	0.0003	0.0030	0.9998	0.0003	0.0011	0.9999

changes in retention, so each CN column performed well for over a thousand injections. The susceptibility of the chromatography to matrix effects was also noted while developing the high-sensitivity method. Attempts to increase the sensitivity by increasing the volume of plasma extracted or to reduce the final reconstitution volume resulted in co-elution of the internal standard with the late-eluting peak. The injection volume could not exceed 150 μL for the same reason.

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TABLE 2

Calibration Curve Data for Nicotinic Acid in Human Plasma
(Results of validation study over a 3-day period.)

Original Method				High-Sensitivity Method			
Theo-retical conc. (ng/ml)	Calculated concentration (ng/µl) Mean ± SD (n=6)	RSD (%)	Dev (%)	Theo-retical conc. (ng/ml)	Calculated concentration (ng/µl) Mean ± SD (n=6)	RSD (%)	Dev (%)
100	101 ± 4.0	3.9	1.5	20	20.6 ± 1.00	4.9	3.0
201	194 ± 6.0	3.1	-3.4	40	40.3 ± 1.24	3.1	0.6
482	480 ± 13.0	2.7	-0.4	100	99.4 ± 3.32	3.3	-0.6
1000	1010 ± 28	2.8	1.0	200	200 ± 3.0	1.5	0.0
2010	2020 ± 40	2.0	0.5	500	476 ± 20.0	4.2	-4.8
4820	4870 ± 103	2.1	1.0	1000	1000 ± 15	1.5	0.0
9640	9560 ± 61	0.6	-0.8	2000	2020 ± 13	0.6	1.0

TABLE 3

Precision and Accuracy of the Assay for Nicotinic Acid in Human Plasma
(Results of validation study over a 3-day period.)

	Control Conc. (ng/mL)	Calculated Concentration (ng/mL) Within Day Mean ± SD (n=6)	RSD (%)	Dev (%)	Overall Mean ± SD (n=18)	RSD (%)	Dev (%)
Original Method	150	156 ± 4.3	2.8	4.0	157 ± 5.3	3.4	4.7
		158 ± 7.2	4.6	5.0			
		158 ± 4.8	3.0	5.1			
	1250	1190 ± 23	2.0	-4.8	1160 ± 29	2.5	-7.2
		1140 ± 25	2.2	-8.8			
		1160 ± 29	2.5	-7.2			
	7530	7400 ± 240	3.2	-1.7	7300 ± 202	2.8	-3.1
		7170 ± 77	1.1	-4.8			
		7320 ± 201	2.7	-2.8			
High-Sensitivity Method	60	62.1 ± 1.00	1.6	3.5	58.6 ± 3.41	5.8	-2.3
		56.3 ± 2.96	5.3	-6.2			
		57.4 ± 2.70	4.7	-4.3			
	150	151 ± 4.8	3.2	0.7	149 ± 4.3	2.9	-0.9
		149 ± 1.8	1.2	-1.0			
		146 ± 4.8	3.3	-2.4			
	1250	1260 ± 11	0.9	0.8	1240 ± 26	2.1	-0.8
		1230 ± 21	1.7	-1.6			
		1240 ± 34	2.8	-0.8			

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TABLE 4

Precision and Accuracy of the Assay for Nicotinic Acid in Human Plasma
(Results of quality control samples during analysis of clinical samples.)

	Control Conc. (ng/mL)	Number of controls (n)	Mean ± SD	RSD (%)	Dev (%)
Original Method	150	56	153 ± 11.2	7.3	2.1
	1250	77	1190 ± 49	4.1	-4.8
	7530	76	7290 ± 336	4.6	-3.2
High-Sensitivity Method	60	42	58.7 ± 4.30	7.3	-2.2
	150	31	148 ± 7.5	5.1	-1.3
	1250	26	1240 ± 67	5.4	-0.8

Representative chromatograms (Figure 2) of the calibration standards and controls demonstrate excellent peak shape and separation between nicotinic acid and the ISTD (6-methyl nicotinic acid). The specificity of the assay is shown in chromatograms of blank plasma (Figure 2). The nicotinic acid and ISTD regions of the chromatograms were free from interferences for six lots of plasma.

Recoveries were calculated by comparing the peak heights of duplicate extracted calibrations standards with duplicate recovery standards at the same theoretical concentrations. Recoveries were approximately 80% at all calibration levels for both nicotinic acid and the ISTD in the original and high-sensitivity method.

Calibration curve parameters for the original and high-sensitivity methods are in Table 1. The curves were linear from 100 to 10,000 ng/mL and from 20 to 2,000 ng/mL with all correlation coefficients greater than 0.999. Calculated values for the calibrations standard are in Table 2. The mean (n=6) back-calculated values of the calibration standards had relative standard deviations (RSD) less than 5%, and deviated less than 5% from theoretical.

The precision of the original and high-sensitivity methods as measured by the RSDs of the daily (n=6) and overall (n=18) mean values of the human plasma controls was better than 6% at all concentrations (Table 3). The accuracy of the methods was measured by comparing the means of the measured concentrations of the controls with their theoretical concentrations. All of the daily (n=6) and overall (n=18) mean values were within 9% of their theoretical values (Table 3). These results show that the method is accurate and precise.

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This method was used to analyze several thousand clinical samples with very good results. Controls assayed during the course of this analysis had excellent precision and accuracy (Table 4), which demonstrated the ruggedness of the assay.

The method presented here is simple, sensitive, and specific, with sufficient precision and accuracy to permit the routine determination of nicotinic acid in human plasma down to a concentration of 20 ng/mL.

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