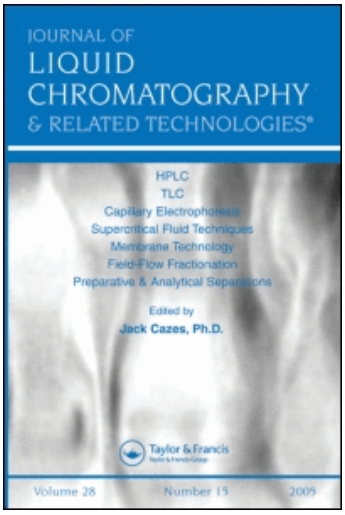

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A Simple High Performance Liquid Chromatography Procedure for the Determination of N¹-Methylnicotinamide in Urine

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A SIMPLE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY PROCEDURE FOR THE DETERMINATION OF N^1-METHYLNICOTINAMIDE IN URINE

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ABSTRACT

A simple high performance liquid chromatography procedure for the determination of N^1-methylnicotinamide in urine samples is described. The procedure eliminates the need for extraction or ion exchange clean-up of urine samples prior to their analysis. Human and rat urine samples can be analyzed for N^1-methylnicotinamide directly following a simple pH adjustment. The metabolite was separated and quantitated on a 5μ Ultrasphere ODS (C18) reverse-phase column. The mobile phase contained 10 mM K_2HPO_4 and 10 mM sodium 1-octanesulfonate in 8% acetonitrile at pH 7.0. The system has been used to conduct over 1000 determinations during a period of three months without reduction in performance or efficiency.

INTRODUCTION

Recently high performance liquid chromatography (HPLC) has been used to measure niacin and two metabolites important in assessing niacin status in humans, N^1-methylnicotinamide (N^1MN)

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and N^1-methyl-2-pyridone-5-carboxamide (2-PYR) (1,2). In 1981, Sandhu and Fraser (3) described an HPLC method for the measurement of niacin metabolites in rat urine. Samples were purified by a modified extraction procedure of Hengen et al. (4), and analyzed by reverse-phase HPLC using a methanolic potassium citrate buffer as the mobile phase. In 1982, Carter (5) described an HPLC method for the quantification of niacin metabolites in human urine. Interfering substances were removed from the samples by anion exchange column chromatography prior to HPLC analysis. N^1MN was analyzed on a reverse-phase column with a mobile phase containing 10 mM potassium phosphate, 5 mM sodium 1-octanesulfonate as an ion pairing agent, and 10% acetonitrile by volume at pH 7.0. The same column was used for 2-PYR analysis with a mobile phase containing 10 mM potassium phosphate, 2% acetonitrile, and no sodium 1-octanesulfonate. Because the ion exchange clean-up step resulted in a 3- to 25-fold dilution of the samples, Carter (5) suggested that for very dilute urines or samples from deficient subjects, the purified samples may require lyophilization prior to HPLC analysis.

This paper describes a modification of Carter's HPLC procedure which eliminates the need for extraction or ion exchange clean-up of samples prior to N^1MN analysis by HPLC. Human and rat urine samples can be analyzed directly, with minimal dilution and without lyophilization.

MATERIALS AND METHODS

Samples

Random urine samples or 24-hour collections from normal subjects admitted to the Human Nutrition Unit of this Center for metabolic studies, and 24-hour urine collections from adult male Wistar rats fed either a corn-based control diet or a corn-based niacin-deficient diet were used.

Sample Preparation

One-tenth mL of a concentrated buffer solution [0.5 M K_2HPO_4 and 0.25 M sodium 1-octanesulfonate (Eastman Kodak Company, Rochester, NY), pH 7.0] was added to 0.25 - 0.75 mL urine and adjusted to pH 7.0 with dilute H_3PO_4 or KOH as necessary. Distilled water was added as required to provide a final volume of 1.0 mL. The sample was then vortexed and filtered through a 0.2 micron nylon filter (Rainin Instrument Company, Woburn, MA) into a vial for HPLC analysis.

Standard Solutions

The solutions used to construct the standard curve were prepared by mixing graded amounts of N^1MN stock solution (0.1 mg/ml; Sigma, St. Louis, MO) with 0.1 mL of the concentrated buffer solution (as above) and diluting each standard solution to 5 mL with water. The prepared standard solutions ranged from 4 to

24 µg/mL. The standard solutions were mixed and filtered through 0.2 micron filters into HPLC vials. The volume of each standard solution injected on the column was 150 µL.

High Performance Liquid Chromatography Analysis

A Hewlett-Packard 1084B liquid chromatograph with a variable wavelength ultraviolet detector (Hewlett-Packard, Santa Clara, CA) was fitted with a 25 cm x 4.6 mm Altex Ultrasphere ODS (C18) reverse-phase analytical column (Rainin Instrument Company, Woburn, MA) and a 3 cm Brownlee Spheri-5 RP-18 guard column (Rainin Instrument Company, Woburn, MA). The particle size of both columns was five microns. The mobile phase contained 10 mM K_2HPO_4 and 10 mM sodium 1-octanesulfonate in 8% acetonitrile (Burdick and Jackson, Muskegon, MI) at a final pH of 7.0. The buffer was filtered through a 0.2 micron nylon filter and degassed before it was introduced into the HPLC system. Conditions were isocratic with a 1.25 mL/minute flow rate, a 30°C column and mobile phase temperature, and a wavelength setting of 264 nm in the sample cell. The reference cell wavelength was 430 nm. Injection volumes varied from 25 to 150 µL. Urinary N^1MN was quantitated by comparing its peak area with that of N^1MN standards analyzed under the same conditions.

RESULTS

Identification

N^1MN in urine samples was identified by comparison of its retention time with that of an N^1MN standard. Both were about

12.1 minutes, with a small day-to-day variation due to slight differences in mobile phase composition. The retention time for 20 samples run in an 8-hour interval was 12.09 minutes (C.V.=0.2%). For 25 samples run 5 per day on 5 separate days over a 3-week interval, it was 12.16 minutes (C.V.=5.5%). N^1MN standard added to urine samples co-eluted with the urinary N^1MN. Scans of the ultraviolet spectrum between 190 and 350 nm showed the same wavelength of maximum absorbance (264 nm) for N^1MN both in standards and urine samples. Scanning both slopes of the urinary N^1MN peak produced the same wavelength of maximum absorbance (264 nm), providing additional evidence that no other substances were co-eluting with N^1MN. In addition, N^1MN peaks from samples of pooled normal rat urine were collected and reanalyzed using a mobile phase containing no counter-ion and only 2% acetonitrile. In each case the chromatogram corresponded to that of N^1MN standards run under the same conditions.

Standard Curve

A standard curve was constructed by plotting absorbance, expressed in units of area, versus μg N^1MN injected onto the column for five standard points ranging in concentration from 4 to 24 μg/mL. The volume injected was 150 μL. The plot was linear over this range and fit the equation y=84981x - 8995, where y is integration units and x is μg N^1MN injected (r=0.9998). When multiple 150 μL injections of standard were made, the coefficient of variation at each level was $\leq$ 1.00% (n=5

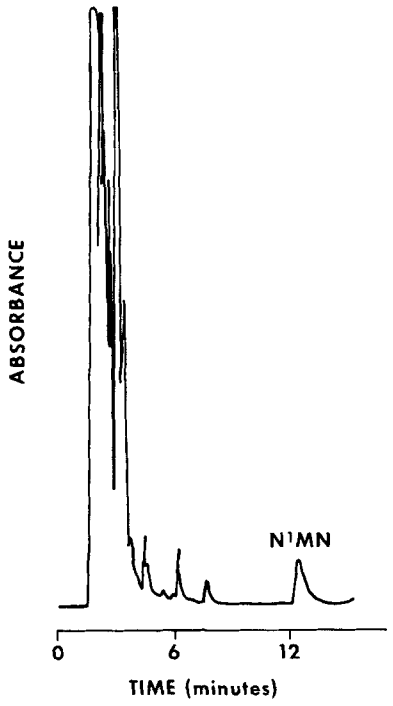

FIGURE 1. Chromatogram of a random sample of normal human urine. A 0.5 mL aliquot of urine was prepared as described in the text. Injection volume of the prepared sample was 25 µL. Attenuation setting of the ultraviolet detector = 2^8.

at each level). For multiple 25 µL injections of a urine sample containing 99 µg/mL, the coefficient of variation was 1.10% (n=10).

Recovery Studies

Recovery studies were performed by determining N^1MN concentrations in urine samples with and without the addition of an equal volume of 0.1 mg/mL N^1MN stock standard to 0.5 mL of each urine sample during the sample preparation step. For three

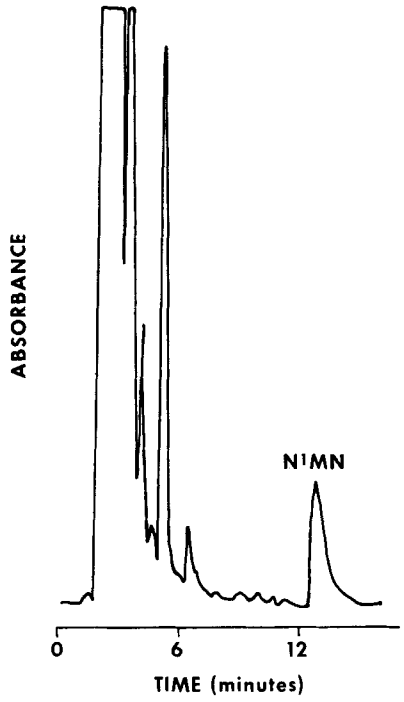

FIGURE 2. Chromatogram of a 24-hour collection of normal rat urine. A 0.5 mL aliquot of urine was prepared as described in the text. Injection volume of the prepared sample was 50 μL. Attenuation setting of the ultraviolet detector = 2^8.

normal rat urine samples, the recoveries of added N^1MN were 105.2%, 100.3%, and 99.7%; for a normal human urine sample, the recovery was 105.2%. Chromatograms of human and rat urine samples without added N^1MN standard are shown in Figures 1 and 2, respectively.

DISCUSSION

The application of HPLC techniques to the determination of urinary niacin metabolites represents an advance in nutrition

status assessement. HPLC methods are simpler, faster, and more sensitive than conventional colorimetric and fluorometric methods (1,5). However, HPLC methods have required a preliminary sample clean-up step by either extraction or ion exchange column chromatography. Direct assay of the urine sample saves time, eliminates the possibility of losses due to poor recovery, and avoids excessive sample dilution. The latter is especially important when working with small urine volumes and/or deficient states. By modifying the mobile phase composition of Carter's analysis procedure, the N^1MN retention time can be increased enough to isolate it from the substances present in urine which would otherwise interfere with HPLC analysis. This is accomplished by doubling the counter-ion concentration from 5mM to 10 mM and reducing the acetonitrile content from 10% to 8%. Using these modifications, no interference is observed in human or rat urine chromatograms, while reproducibility and recovery are maintained. The method has been used successfully to analyze N^1MN in the urine of niacin-deficient rats and in dilute 24-hour collections of human urine. The system described in this paper incorporates a guard column and has been used to conduct over 1000 determinations during a three-month period without significant reduction in performance or efficiency. Attempts to eliminate the ion exchange column clean-up step from Carter's 2-PYR analysis procedure by mobile phase modification were not successful. Changing the acetonitrile concentration did not sufficiently isolate 2-PYR

from the neighboring UV-absorbing substances present in both normal and deficient human or rat urine to allow its quantitation in unpurified samples. An entirely different mobile phase or HPLC column may be required for the analysis of 2-PYR in these samples.

Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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