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Note

Direct urinary assay method for N^1-methylnicotinamide by soap chromatography

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Various analytical methods have been reported for the measurement of urinary excretion of N^1-methylnicotinamide (1-MNA). One of the earlier methods used the formation of a fluorescent condensation product between 1-MNA and acetone in strong alkali and the removal of interfering fluorescent impurities by charcoal [1]. This method has been improved by various workers, with the removal of the impurities by ion-exchange [2, 3] or descending-paper chromatography [4].

Recently, an improved method for the determination of 1-MNA in urine, based on the removal of interfering substances with a single strong cation-exchange chromatographic step, followed by quantitative high-performance liquid chromatographic (HPLC) assay of the isolated 1-MNA was reported [5]. While this method was shorter than former methods, it also required cleanup before the HPLC assay.

We report here a rapid quantitative method for 1-MNA in rat and human urine by direct injection onto a reversed-phase HPLC column operating in the soap chromatography mode.

This system employs a reversed-phase packing in combination with a hydrophilic eluent containing methanol as an organic modifier and a small concentration of a detergent which forms an ion-pair with an ionized form of a solute. To keep the solutes in the preferred ionic forms, the pH of the solution is controlled. This is a rather simple, rapid, and specific method for the direct assay of 1-MNA in urine, requires no pretreatment of the sample, and has a high degree of precision and accuracy.

EXPERIMENTAL

Materials

N^1-Methylnicotinamide chloride was obtained from Sigma (St. Louis,

Mo., U.S.A.). Sodium dodecyl sulfate (SDS) was purchased from Bio-Rad Labs. (Richmond, Calif., U.S.A.). Glass-distilled methanol (Burdick & Jackson, Muskegon, Mich, U.S.A.) and deionized water were used.

Preparation of urine samples

Sprague-Dawley rats were placed in metabolism cages (two rats per cage) and urine was collected for 24 h. To remove debris, collected urine was centrifuged on a GLC-1 (Sorvall, Norwalk, Conn., U.S.A.) at 3000 rpm for 15 min, decanted, and the supernatant stored at -70° if not used immediately. An aliquot of the above sample (or of fresh normal human urine) was passed through a 0.45-μm Nalge membrane filter (Fisher Scientific Co., Silver Spring, Md., U.S.A.) before direct HPLC analysis.

Recovery of N^1-methylnicotinamide

A stock solution of 1-MNA chloride (10 μg/μl) was prepared in deionized water. Aliquots of urine (1 ml) were spiked with 60, 150, and 300 μg of 1-MNA, and 1-MNA recoveries were determined by direct injection of a few microliters of spiked urine onto an HPLC column.

Apparatus

All analyses were performed at ambient temperature on a Waters Assoc. (Milford, Mass., U.S.A.) Model ALC 202 liquid chromatograph with U6K septumless injector and a Schoeffel (Westwood, N.J., U.S.A.) Model SF-770 variable wavelength (200—700 nm) ultraviolet (UV) detector. Chromatograms were recorded on a 10 mV full-scale Omniscribe recorder (Houston Instruments, Austin, Tex., U.S.A.) operated at 0.1 in./min chart speed.

A 25 cm × 4.6 mm I.D., Partisil-10 ODS analytical column (Whatman, Clifton, N.J., U.S.A.) was used. A 4 cm × 2.1 mm I.D. precolumn, packed with Waters Assoc. pellicular Bondapak C_{18}/Corasil (37—50 μm), was used to ensure the stability of the analytical column. The column was eluted with a mixture of 0.005 *M* SDS, 47.5% methanol, and 0.01% H_2SO_4, at a flow-rate of 1.5 ml/min. The eluent was de-gassed by ultrasonification for 10 min before use. Peak areas and retention times were determined with a Hewlett-Packard (Avondale, Pa., U.S.A.) 3352-A laboratory data system linked through a Hewlett-Packard 1865 A/D converter to the UV detector output of the liquid chromatograph. The output from the data system was registered on a Texas Instruments' (Houston, Tex., U.S.A.) silent 700 thermal line printer.

RESULTS AND DISCUSSION

The recent introduction of soap chromatography [6, 7] has enlarged the scope of HPLC application to biochemical analysis. The method has a particular advantage when applied to urine analysis, since the bulk of the excreted compounds are polar, show UV absorption and will not be retained. We have developed a rapid HPLC method to measure 1-MNA in urine without prior cleanup.

The result of a direct injection of 3 μl of rat urine onto the column is shown in Fig. 1. N^1-Methylnicotinamide is separated from the interfering compounds

under the conditions shown. Several methanol concentrations in the mobile phase were investigated; namely, 40, 45, 47.5 and 50%. All provided adequate resolution of 1-MNA from interfering compounds in urine, and the retention time of 1-MNA was increased by lowering the methanol concentration. Lower SDS (0.002 *M*) concentrations also gave longer retention of 1-MNA. The average retention time for ten identical injections (1—3 μl) was 16.4 ± 0.15% (± S.D.) under the conditions in Fig. 1.

A standard calibration curve covering the range 0.1—0.9 μg of the 1-MNA standard showed excellent linearity (correlation coefficient 0.999). Linearity of response of 1-MNA in urine for urine injection volumes covering the range 1—6 μl was also excellent (correlation coefficient 0.999). The method was used to determine the 1-MNA content of normal human urine, which was found to be 56 μg/ml. Recovery experiments were carried out on 1 ml of control urine samples spiked with 60, 150 or 300 μg of 1-MNA. The overall recovery and its coefficient of variation (C.V.), as shown in Table I, was reasonable, indicating that the method is precise and accurate. The concentration of 1-MNA in rat urine by this method was found to be 117 μg/ml, whereas the method reported in ref. 5 gave 112 μg/ml. This indicates good agreement between the two methods. However, it should be noted that the present method utilizes

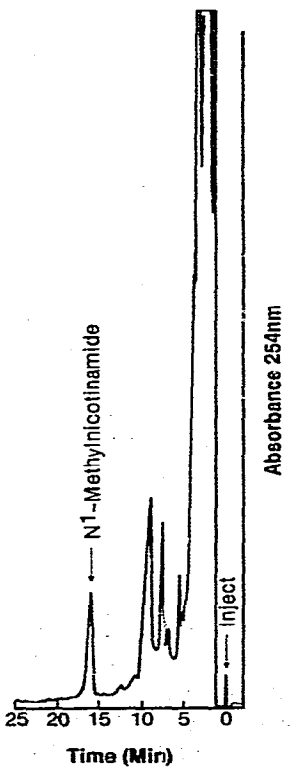

Fig. 1. Soap chromatography of N^1-methylnicotinamide in urine. Conditions: column, Partisil-10 ODS; detection, 254 nm, 0.04 a.u.f.s.; eluent, water—methanol—SDS (52.2:47.4: 0.01, v/v/w) with 0.01% added sulfuric acid.

TABLE I

RECOVERY OF 1-MNA ADDED TO URINE SAMPLES

Amount of 1-MNA added (μg)	Recovery (%)
60	91.9
	105.8
	104.1
150	95.5
	100.5
	102.7
312	99.0
	97.7
	103.4
Mean	100.1
C.V.*(%)	4.5

*C.V. (%) = (S.D./mean) × 100.

direct injection of urine onto the HPLC column and the analysis is effected in less than 20 min. In contrast, the method in ref. 5 required pre-cleanup before HPLC analysis, resulting in several hours for the overall assay.

The identity and purity of the 1-MNA peak was established by the following techniques. (A) Alteration of the SDS concentration (0.002 *M vs.* 0.005 *M*) and methanol content (40% *vs.* 47.5%) of the mobile phase gave identical quantitative results for urinary 1-MNA content. (B) A UV spectrum run on collected urinary 1-MNA peaks was identical to that of authentic 1-MNA. The peak effluents were combined and taken to dryness on a rotary evaporator and reconstituted with 3 ml of deionized water for UV spectral analyses. The reference cell and standard compound solutions were prepared from the mobile phase (0.005 *M* SDS, 47.5% methanol, 0.01% H_2SO_4) and treated as above. Both the standard and collected urinary 1-MNA peaks showed a similar UV spectrum with a maximum at 265 nm. (C) The HPLC peak height ratios of urinary and standard 1-MNA were determined and compared at two wavelengths, 254 nm (a general wavelength) and 265 nm (absorption maximum for 1-MNA). The HPLC peak height ratio for 254:265 of urinary 1-MNA (0.65) was similar to that of standard 1-MNA (0.66), and was also similar to the 254:265 absorption ratio from the UV spectrum (0.66).

These techniques, especially the matching of ratios of both standard and sample compound, indicates that the component of interest (1-MNA) in urine is completely resolved from contaminants, thus the peak height ratio method in combination with the other two could be used to obtain an accurate quantitative analysis. The soap chromatographic method described here is hence suitable for assaying 1-MNA in urine, since this compound is well retained and readily separated from other UV-absorbing components. Furthermore, the need for tedious processes for cleanup previously required is avoided by direct injection of a few microliters of urine samples onto the HPLC column. Repeated

injections of urine cause no notable deterioration in column performance. The method can be used for a reliable quantitative assay of 1-MNA for studies in human nutrition, and NAD tryptophan metabolism associated with certain diseases including cancer.

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