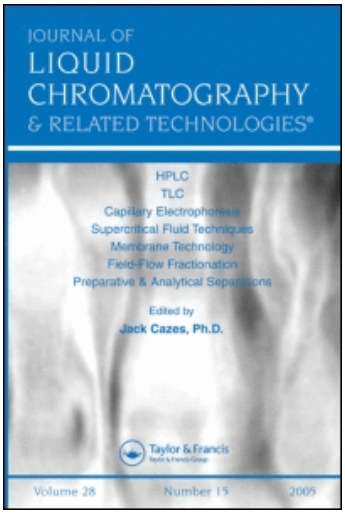

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DETERMINATION OF N-METHYLNICOTINAMIDE IN URINE WITH CAPILLARY ZONE ELECTROPHORESIS

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ABSTRACT

In the present study a method is described for the quantitation of N-methylnicotinamide (NMN) in rat and human urine with capillary zone electrophoresis (CZE). Because NMN is considered as a possible biomarker for nitrite exposure, a quantitative method of analysis is required for further evaluation studies. The CZE method for NMN analysis turned out to be very simple and practicable. No pretreatment of the human urine sample was needed before analysis and large series of samples can be handled. Although only a few human urine samples have been analyzed at present, the CZE method looks very promising.

In addition, rat urines from a toxicity experiment have been analyzed. At this point it is questionable whether a dose-response relationship exists in the low concentration exposure range because the statistical significance disappeared after correction for the produced urine volume or creatinin content.

INTRODUCTION

In a toxicity experiment in which rats have been exposed to nitrite and nitrate, it was found with 1H-nuclear magnetic resonance (NMR) that the concentration of N-methylnicotinamide (NMN) was increased in urines of rats exposed to nitrite [1]. To prove the hypothesis that NMN was a metabolite of tryptophan, experiments have been performed with labelled tryptophan which confirmed that NMN indeed originated from tryptophan [2]. Because NMN was raised only in the nitrite group, the use of this compound as a biomarker for nitrite exposure in human biomonitoring experiments was further investigated.

Because NMR is a relatively insensitive technique, the quantitation of NMN in rat urines with NMR was not satisfactory. Especially in urines from control animals the quantitation of low concentrations of NMN could only be performed in a semi-quantitative manner. Therefore, more sensitive detection methods are required for a better quantitation of NMN. Besides high performance liquid chromatography (HPLC), capillary zone electrophoresis (CZE), which is a very suitable technique for the separation and detection of charged small molecules, can be used for the quantitation of NMN.

In the present report we describe the detection and quantitation of NMN in both rat and human urine with CZE.

MATERIALS AND METHODS

MATERIALS

Citric acid, sodium citrate, sodium hydroxide, ammonium sulfate, ethanol and trichloroacetic acid were obtained from Merck-Schuchardt (Darmstadt, Germany). Cytosine, 1-methylnicotinamide iodine salt, nicotinic acid, tryptophan, kynurenine, 3-hydroxyanthranilic acid and 3-[cyclohexylamino]-1-propaansulfonic acid (CAPS) were obtained from Sigma (St. Louis, U.S.A.). A

kit for measuring creatinine in urine was obtained from Hoffmann - La Roche Diagnostica (Basel, Switzerland). Separation buffers were made by mixing a solution of 20 mM citric acid in water (from a Milli-Q system) and a solution of 20 mM sodium citrate to obtain the desired pH. The rinsing solution which was used for the capillary column was 1 M sodium hydroxide. A CAPS buffer (50 mM, pH 10) and a cytosine solution (500 mg/l CAPS) were used for testing the system with regard to the plate height and reproducibility. Other standard solutions were made by dissolving the compound of interest in 20 mM citrate buffer with a pH of 5. 3-Hydroxy anthranilic acid did not dissolve in the buffer. The suspension was filtered and used as a standard solution. The concentration of this standard is therefore unknown.

EQUIPMENT

The Capillary Zone Electrophoresis (CZE) system consisted of a Prince injection system with power supply, an Applied Biosystems 759A absorbance detector suitable for CE , and a GynkoSoft Chromatography datasystem V4.10 (Separations, H.I. Ambacht, The Netherlands). Chromatograms were printed on a Laserjet IIP printer (Hewlett Packard, Amstelveen, The Netherlands). SGE deactivated silica with an I.D. of 75 μm was used as column (Bester, Amstelveen, The Netherlands). The effective length of the column was about 0.8 m. From a part of the column which was situated in the light path of the detector the polyimide coating was removed with a cigarette lighter.

Creatinine was measured with a Cobas Bio centrifugal analyzer (Hoffmann-La Roche Diagnostica, Basel, Switzerland).

SAMPLES

SPF male rats (Wistar Riv:TOX strain) were about 3.5 weeks old at the start of the experiment with a body weight range of 55-70 grams. The animals were housed individually in a one wire cage. During the two weeks of the experiment, the animals were placed in a cage for metabolic studies for 24

hours every 5 days. The urine was collected, and the volume of the urine was determined. In this experiment, 12 animals had free access to a grounded semi-purified diet (SSP-TOX flour). The animals (four per group) were exposed to 35.25 mmol KCl, 35.25 mmol KNO_2 or 35.25 mmol KNO_3 per liter tapwater. The total study will be reported in detail elsewhere [2]. The 24-hours urine of the rats exposed for two weeks to KCl, to KNO_2 and to KNO_3 were used for the determination of NMN with CZE.

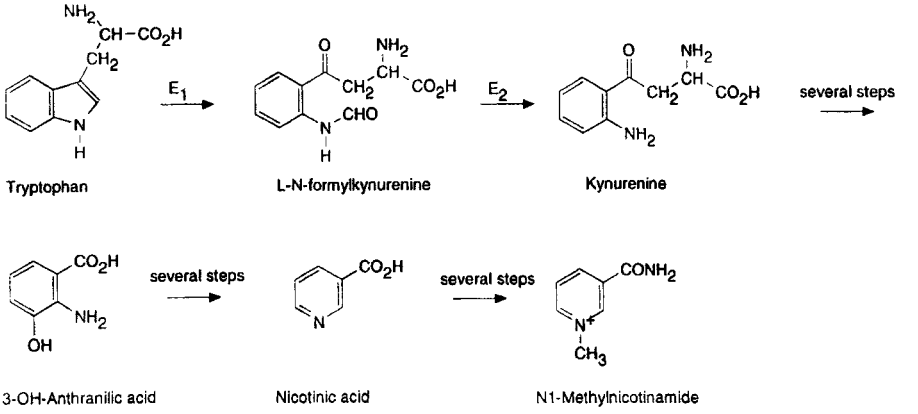
The human urine was a void from a healthy female volunteer.

SAMPLE PRETREATMENT

The advantage of CZE is the relatively simple sample pretreatment. The human urine was only filtered through a HV13 0.45 μm filter (Millipore, Etten-Leur, The Netherlands). The urine from rat origin contained more proteins which had to be removed. The following methods for removing the proteins were tested: the addition of 120 mg ammoniumsulfate to 200 μl urine, the addition of ethanol to a total of 50% in the urine and the addition of trichloroacetic acid to a total of 10% in the urine. After centrifugation the supernatant was filtered through the filter mentioned before.

CZE METHOD

Before each injection, the column was rinsed with 1 M sodium hydroxide for 1 minute with a pressure of 1000 mbar. The column was equilibrated with the buffer (20 mM citric acid/citrate buffer, pH 3) during 1 minute with a pressure of 2000 mbar. 30 kV was applied during 0.3 minute before introducing the sample. Sample introduction was done by applying a pressure of 40 mbar during 0.1 minute. The analysis was performed in the constant voltage mode of 30 kV applied at a ramp speed of 6 kV/s. The column was kept at a temperature of 30 degrees and no pressure was applied during the analysis. Compounds were detected by measuring the absorbance at 264 nm (at this wavelength NMN has an absorbance maximum). The signal of the detector was collected and integrated with the GynkoSoft software package.



E_1 = Tryptophan pyrrolase

E_2 = Kynurenine formylase

Figure 1: Simplified metabolic pathway of tryptophan

RESULTS AND DISCUSSION

SEPARATION OF TRYPTOPHAN METABOLITES

NMN is a metabolite in the tryptophan dioxygenase metabolisme. A simplified representation of the metabolic pathway is shown in figure 1. The first step is a ring opening catalyzed by the enzyme tryptophan pyrrolase. This enzyme can be influenced by several physiological parameters, such as corticosteroids. Since there is some evidence that nitrite can act on the adrenal gland [3], which can result in the release of corticosteroids, a possible indirect effect of nitrite on the tryptophan metabolism can be expected.

The separation of the main compounds, being NMN, kynurenine, tryptophan, nicotinic acid and 3-hydroxyanthranilic acid was tested with 20 mM citrate buffers with different pH values, being 3.0, 4.0 and 5.0. The other variables were kept the same as with the analysis of NMN. At pH 5.0, kynure-

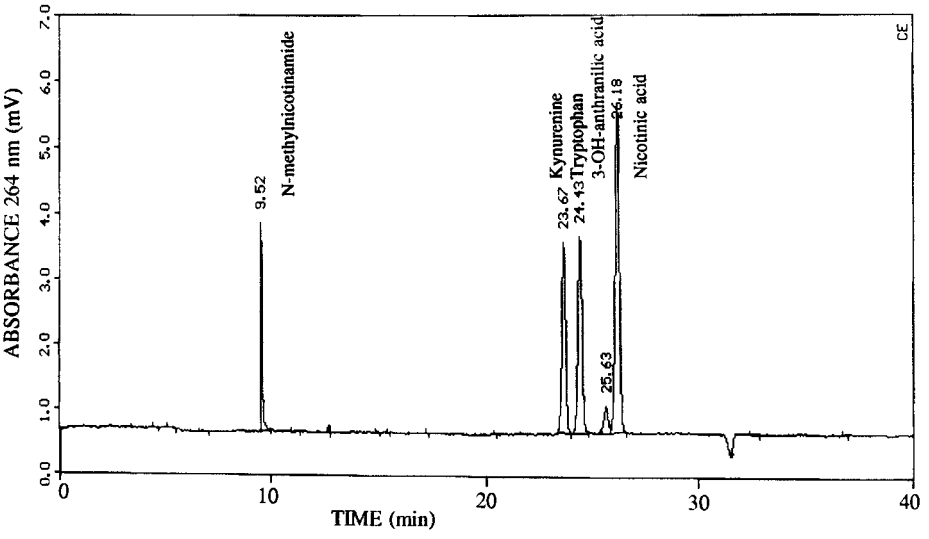


Figure 2: Separation of different tryptophan metabolites with a 20 mM citrate buffer, pH 3.

nine and tryptophan were not separated. The total run time was 30 minutes. At pH 4.0, only three peaks were visible after 40 minutes. The optimum separation of all compounds was achieved with the buffer with a pH of 3.0. All the compounds were separated within 30 minutes. An example of a representative chromatogram is shown in figure 2.

REPRODUCIBILITY

With the above described optimum separation, nine injections were made to determine the reproducibility of the technique. Both the reproducibility of the migration time and the peak height and the peak area were considered. The variation in the migration time is within 2%, but the system is only stable after six injections. Although the column is thermostatted, the initial instability is probably due to the warming up of the whole system. Since with CZE high

plate numbers can be achieved, the migration time must be constant for adequate peak identification and quantitation based on peak height. An autosampler can overcome the problem of initial instability.

The variation in the peak area is within 7.5% and in the peak height within 5%. The use of an internal standard can further decrease the variation in the analyses.

DETERMINATION OF NMN IN HUMAN URINE

Filtered human urine was analyzed upon the presence and concentration of NMN. The resulting chromatogram can be seen in figure 3. A small, very sharp peak appeared at the migration time of a standard of NMN. Since this peak was so small (the corresponding plate number was 11 million) and the chromatogram was very clean even without prepurification of the urine, we had some doubt upon the origin of this peak. Therefore, NMN standard addition to the urine was done which indeed increases the peak area. The correspondending plate number dropped to 4.5 million plates. No other peak appeared, so it was concluded that the peak at a migration time of 9.5 minutes was indeed NMN. To confirm this conclusion, the pH of the buffer was changed from 3 to 5. The NMN moved both in the standard and in the urine from 9.5 minutes to 8.3 minutes. Also the peak area remained the same, although the plate number for NMN in the urine dropped to 50,000. Attempts to determine an UV-spectrum by varying the absorbance wavelength was only successfull with the standard solution. The NMN peak in urine had a too low intensity for a reliable UV-spectrum.

DETERMINATION OF NMN IN RAT URINE

Rat urine contains more proteins than human urine. To remove these proteins, three methods were used. It appeared that with ammonium sulfate the peak moved 3 minutes backwards and the peak area dropped to 35% of the original standard. Also precipitation with trichloroacetic acid was not succes-

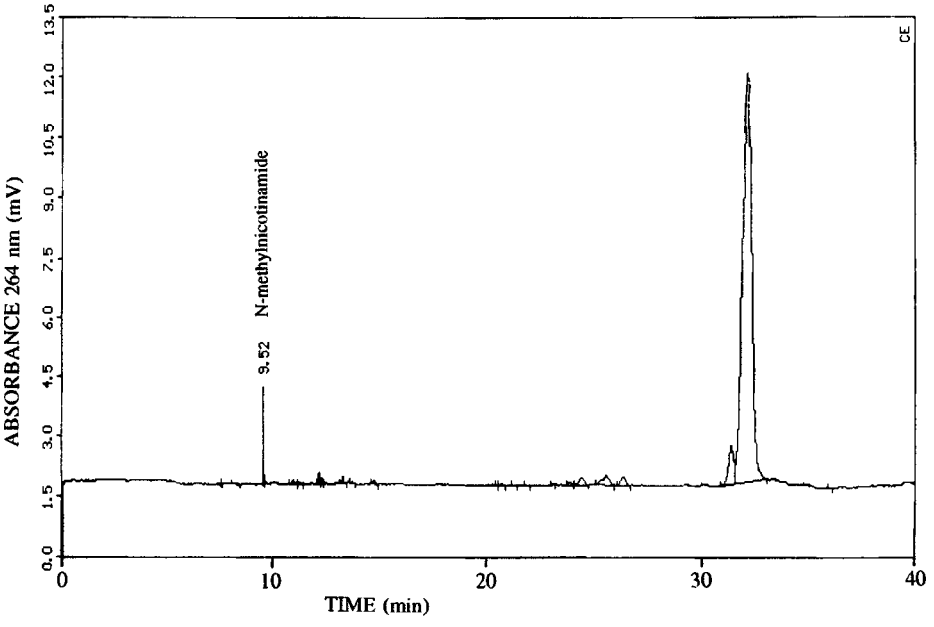


Figure 3: CZE-chromatogram of normal human urine.

full. The peak doubled and became very broad. The addition of 50% ethanol was the best method because the migration time remained the same. Therefore, this procedure was used for removing the proteins from rat urine.

All the rat urines were measured twice. The mean values of the urinary NMN concentration of the animals of the three groups which have been exposed to KCl, KNO_2 or KNO_3 have been listed in table 1. A statistical difference is observed between the nitrite and the control (chloride) group. The nitrate group shows similar NMN concentrations as the nitrite group. If the concentration of NMN is corrected, however, for the dilution factor the statistical difference disappears. As is shown in Table 1 in the third and fourth column, upon correction both for the total volume of the produced urine within 24 hours and for the creatinine content of the urine, still a difference is

Table 1:

Summary of the NMN determinations (mean ± standard deviation) of the three groups. NMN values have been expressed as mg/l urine (uncorrected), in mg/24 hr urine and in g/mol creatinine.

Exposure group	NMN (mg/l)	NMN (mg) per 24 hr	NMN (g) per mol creat.
KCl	13 ± 2	0.13 ± 0.02	2.8 ± 0.6
KNO_2	29 ± 8 **	0.18 ± 0.08	3.9 ± 2.0
KNO_3	13 ± 4	0.10 ± 0.02	2.1 ± 0.5

** $p < 0.01$.

observed between the nitrite and the two other groups but the statistical significance has disappeared.

NMN AS BIOMARKER FOR NITRATE/NITRITE EXPOSURE

The human population is exposed to very low amounts of nitrite in comparison to the higher amounts of nitrate. The intake of nitrate, however, results in an internal nitrite exposure caused by the blood-salivary cycle in the human body [4]. In addition, it must be taken into account that humans have a relatively high endogenous production of nitrate [5] which can make it difficult to distuiguish between variations in the endogenous nitrate levels and low exogenous exposure levels of nitrate.

From the present study it can be concluded that NMN is promising as a biomarker for nitrite exposure, although additional experiments are required.

Because of the relatively high concentrations of the biomarker NMN in urine, the control levels can be measured without any problem with probably a number of analytical methods. In the present study CZE was evaluated as

method of analysis which turned out to be very suitable. No pretreatment of the urine sample was needed before analysis and as a result large series of samples can be handled. Although only a few urine samples have been analyzed at present, the CZE method looks very promising. To develop this technique into a routine-like method of analysis for NMN more experience must be obtained especially in the quantitation of the very small peaks that are obtained with this technique.

The use of NMN as a biomarker for nitrite exposure, a clear dose-response relationship between nitrite and the biomarker NMN should exist. This has at present not yet adequately been assessed. In addition, the sensitivity must be determined for small changes in nitrite exposure. Also the disappearence of the statistical significance after correction of the produced urine volume must be further investigated.

At present experiments with human volunteers are undertaken under controlled conditions which probably will give an answer to the still existing questions.

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