

Concentrations of Nicotinamide in Plasma by RP-HPLC With Fluorescence Detection

Zhipeng Pan¹, Da Li², Xiaokun Yao¹, Shisheng Zhou¹ and Yongzhi Lun^{1,a}

¹Liaoning Provincial University Key Laboratory of Biophysics, College of Medicine, Dalian University, Dalian, Liaoning 116622, P.R. China

²Department of Obstetrics and Gynecology, Shengjing Hospital of China Medical University, Shenyang, Liaoning 110004, P.R. China

Abstract. The purpose of this study is to establish a new method for detecting nicotinamide concentration in plasma. In the experiment, the high performance liquid chromatography (HPLC) method was used, with a fluorescence detector. The nicotinamide in the plasma was first converted to N1- methylnicotinamide, then reacted with acetophenone under certain conditions to produce fluorescent derivatives for testing. The method is a kind of highly sensitive detection, of which the lower limit is 10 ng/mL, the recovery rate is between 92.75% and 105.13%, and the relative standard deviation (RSD) is between 3.76% and 4.43%. The results showed that this measurement method is accurate, sensitive and rapid. It meets the requirements of the experiment, and applies to the detection of nicotinamide concentration in plasma.

Nicotinamide (NM), namely Nike amide, vitamin B3 and vitamin PP, is a water-soluble vitamin (Figure 1) [1]. Nicotinamide is an alkylated derivative of nicotinic acid, which is also the precursor of coenzyme I (NAD) and coenzyme II (NADP) in the body, thus the pharmacokinetic observation of nicotinamide is an important tool for monitoring metabolism levels in the body [2]. Our research shows that long-term nicotinamide overload can cause insulin resistance and oxidative stress, and eventually lead to diabetes [3, 4]. Currently, the detection of nicotinamide in plasma samples is a complex method, which requires high instrument conditions and liquid chromatography - mass spectrometry [5-7]. This method is also highly time-consuming, mainly due to the very low levels of nicotinamide in the plasma. In this study, concentrations of nicotinamide were measured using the ordinary high-performance liquid chromatography method with cleverly designed, simple processing and saving supplies, and satisfactory results were achieved [8-14].

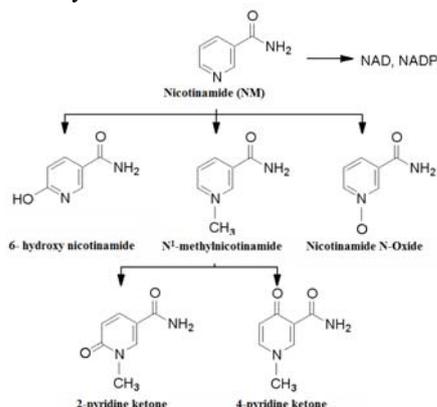


Figure 1. Vitamin PP metabolism in human

^a Corresponding author: Yongzhi Lun:lunyz@163.com

1 Methods

1.1 Instruments: High-performance liquid chromatography (Shimadzu LC-9A pump, Rheodyne 7725i injector, 20 μ L sample loop, Shimadzu Co.), Waters470 fluorescence detector (Waters, USA), N2000 chromatography workstation (Zhejiang University Zhida Information Engineering Co., Ltd.).

1.1.1 Reagents: Ethyl acetate (AR), acetophenone (AR), formic acid (AR), NaCl (Beijing Jingke Hongda), 0.5M HCl; 0.5M NaH₂PO₄, 0.4M KOH, 20% trichloro acetic acid (TCA), Nicotinamide (NM), and N1-methylnicotinamide (NMN) (Japan Takeda Co.). N1-ethylnicotinamide (NEN) was synthesized according to the methods described by Hirayama et al. [15, 16], with a purity of 99.99%, and this was used as an internal standard.

1.2 Chromatographic conditions: The column in the experiments: ODS-C18 column (Thermo, Bellefonte, PA), 250 mm*4.6 mm (id), particle size 5 μ m; mobile phase: 10 mM heptane sulfonate, 50 mM triethylamine solution (pH 3.2): acetonitrile (78:22 v/v); excitation wavelength 366 nm, emission wavelength 418 nm; flow rate 1.0 mL/min; injection volume 20 μ L.

1.3 Sample preparation: 200 μ L blood was input into the EP tube with 100 μ L 20% TCA and then mixed by vortex, and 12000 rpm of centrifugation for 10 min; next, 200 μ L supernatant was removed and placed in a new EP tube. 25 μ L 0.5M HCl was added with 100 mg NaCl into the supernatant, then shocked by vortex until the water phase was fully saturated by the NaCl, which was extracted three times by 1 mL of water-saturated ethyl acetate extract, then the upper phase of ethyl acetate was

discarded and the residual ethyl acetate was blow-dried with N₂. 25 μL 0.5M NaH₂PO₄ and 25 μL 0.4M KOH were continuously added, and these were shocked and mixed well, followed by the addition of 1 mL anhydrous ethyl acetate, which was shocked and mixed. Centrifugation was performed at 3000 rpm for 3 min, then the 800 μL upper phase was taken in another EP tube, followed by N₂ drying^[17]. 250 μL iodomethane was added and the two were mixed together, then saved in dark for 24 h before being dried with N₂. Then 200 μL double distilled water was added, and extracted three times with ethyl acetate saturated with water after the shocking; the residue in the ethyl acetate was dried by N₂, at which point all of the nicotinamide in the plasma was converted into the N1-methylnicotinamide. 200 μL internal standard was added (50 ng/mL), then 200 μL of 100 mM acetophenone was added^[18], and placed in the ice bath for 10 min after shocking. 400 μL of 6M NaOH was added and mixed well, then placed in the ice bath for 60 min. Then 200 μL of formic acid was added, and placed in the ice bath for 60 min. Finally, the solution was placed in the boiling water bath for 3 min to generate the fluorescent derivatives, then mixed well after cooling for machine testing.

1.3.1 Determination: using the internal standard method and analyzed by HPLC with fluorescence detection, and the N2000 data acquisition system collected the data and calculated the concentration of nicotinamide in the plasma. The nicotinamide concentration was calculated according to the following formula [Equation (1)] based on N1-methylnicotinamide determination:

$$m = 9.375 m_0 \times M / M_0 \quad (1)$$

Where m is the concentration of nicotinamide in the plasma, unit: ng/ml; m₀ is the mass of the N1-methylnicotinamide, unit: nanograms (ng); M₀ is the molecular weight of the N1-methylnicotinamide, molecular weight: 172.61; and M is the molecular weight of the nicotinamide, molecular weight: 122.12.

2 Results

2.1 Chromatogram of nicotinamide in plasma:

Figure.2 is a blank of the chromatogram of the nicotinamide in the plasma, and Figure.3 is a marked chromatogram of nicotinamide in the plasma. Peak 1 of the chromatogram is nicotinamide, and peak 2 is the internal standard in the figure.

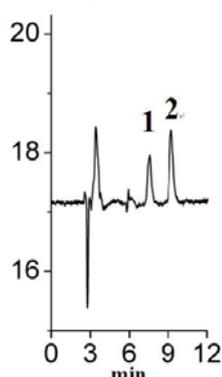


Figure.2 Chromatogram of nicotinamide in plasma

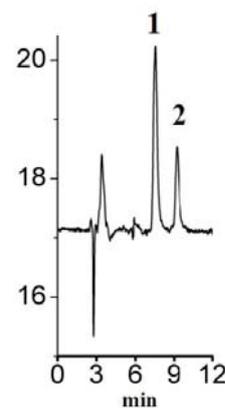


Figure.3 Marked chromatogram of nicotinamide in plasma

2.2 The minimum detection limit: the minimum detection limit for the RP-HPLC fluorescence detection method in the experimental conditions and chromatographic conditions, according to the peak response value of 3 times of the noise, the sample volume is calculated as the minimum detectable amount^[19, 20]. The detection lower limit of this method is 10 ng/mL.

2.3 The recovery rate and precision of the experiment:

Three plasma samples were treated using the same method, once per day for 6 days. The results are as shown in Table 1.

Table 1. Precision experiments among batches (n = 6)

Sample*	Number	Measured value (ng/mL)				
X±SD	RSD(%)					
Sample1	158.05, 168.67, 175.58, 148.47, 154.76, 149.43					
	159.16±18.86	4.43				
Sample2	161.40, 140.98, 154.97, 156.69, 168.46, 170.53					
	158.84±10.72	4.38				
Sample3	141.65, 138.79, 159.85, 144.19, 133.77, 150.23					
	144.75±9.21	3.76				

*Samples 1-3 are all plasma of normal humans.

Precision and recovery rate experiment among batches: taking 3 parts of normal human plasma, which is divided into three parts after mixing, the standard of nicotinamide was added by the concentration gradient, according to the same procedure used to deal with the samples, which were then detected. Under the same conditions, each sample was continuously injected six times, and according to the corresponding peak area to calculate the RSD, the experimental results are as shown in Table 2.

Table 2. Recoveries and precision (n = 6)

Sample	background value	marked value	measured value
recovery value	recovery rate	RSD	
Sample4	153.67	100.00	246.42
	92.75	4.72	
Sample4	153.67	200.00	363.93
	210.26	105.13	3.94
Sample4	153.67	500.00	635.57
	481.90	96.38	3.27

3 Discussions

3.1 The high performance liquid chromatography with fluorescence detector was used in this paper to measure the concentration of nicotinamide in the plasma. Its

operation is simple and reproducible, with a high recovery rate, and the experimental results were satisfactory compared with those of other liquid chromatography-mass spectrometry methods, showing that it provides an experimental method for the study of pharmacokinetics and dynamics of nicotinamide.

3.2 In the sample processing, it is necessary to be careful when shaking with ethyl, otherwise the ethyl may overflow, possibly causing the loss of nicotinamide, thus causing the results to decrease in accuracy. When N1-methylnicotinamide was converted into nicotinamide, the saturated ethyl acetate in the acidic conditions can be extracted to the original N1-methylnicotinamide of plasma, but this cannot be done under neutral conditions, thus it is important to pay particular attention to the pH value. The final amount of N1-methylnicotinamide was solely converted from the nicotinamide, thus the final amount of N1-methylnicotinamide is equal to the nicotinamide.

3.3 Due to the fact that the concentration levels of nicotinamide in the body differ among people, the nicotinamide content of samples 1, 2 and 3 in this experiment are different. Nicotinamide is easily oxidized in air to form oxidized nicotinamide, thus it is necessary to pay particular attention to sample storage; the samples should be stored in a -20°C refrigerator, and processed and detected promptly after treatment.

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