Short Communication

A new and highly sensitive LC-MS/MS method for simultaneous quantification of NMN and related pyridine compounds in different mouse tissues.

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Abstract

Nicotinamide (NAM) nicotinic acid (Na), nicotinamide mononucleotide (NMN), nicotinamide riboside (NR) and nicotinamide adenine dinucleotide (NAD) represent key metabolites through which a variety of cellular processes are regulated. NAD biosynthesis and its consumption are enzymatically controlled pathways belonging to a finely-tuned network in charge of maintaining the robustness of physiological systems in response to a variety of nutritional and environmental stimuli. Here a quantitative simultaneous determination of NMN, NAD and their pyridine precursors, in several murine tissues, has been developed by UHPLC-MS/MS method. All the analytical steps, from sample preparation and compounds extraction, RP-LC separation and ESI-MS monitoring and identification, were optimized and validated in order to achieve low LOQ, high sensitivity and good robustness. The compounds under interest have been identified and quantified in the tissues analyzed in a range of concentration of the order of magnitude of picomoles/mg of tissue.

1. Introduction

Nicotinamide adenine dinucleotide (NAD) has central role in cellular metabolism and is both a coenzyme for hydride-transfer enzymes and a substrate for NAD-consuming enzymes such as ADP-ribose transferases, poly(ADP-ribose) polymerases, cADP-ribose synthases and sirtuins. These enzymes regulate diverse cellular processes including cell survival [1], transcription [4,5], apoptosis [2,3], and calcium signalling [6]. The recently discovered role of NAD biosynthetic pathways in axon survival [10] is very important due to the involvement of axon degeneration in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and Huntington's disease.

Four building blocks and four corresponding pathways may be used for biosynthesis of NAD. Quinolinic acid (QA) in the *de novo* pathway and nicotinamide (NAM), nicotinic acid (NA) and nicotinamide riboside (NR) in the salvage pathways [7,8].

In mammals, both the *de novo* and salvage pathways are available but salvage pathways are favoured because *de novo* synthesis is limited and requires exogenous salvage of nicotinamide precursors to meet metabolic needs [7].

The biosynthetic pathways from all four NAD precursors converge at the level of dinucleotide formation, catalysed by the enzyme NMNAT, which is able to use both NMN and NAMN with comparable efficiency [9]. Hence, measuring simultaneously NMN and related pyridine compounds levels inside the cells with a rapid and highly sensitive method is very important to better understand their role in cellular metabolism and disease.

Different HPLC-UV methods already exist to measure NAD metabolite levels with a LOQ ranging from millimolar to micromolar [11-15]. Other methods, based on the use of electrospray ionization (ESI) and selected reaction monitoring (SRM), are powerful and sensitive [16,17], even if one is based on hydrophilic interaction liquid chromatography (HILIC) and the only example in RP-HPLC misses monitoring of NR and NaR.

Recently it has emerged that fungi and vertebrates encode eukaryotic NR kinases (Nrk isozymes) to salvage NR, a third vitamin precursor of NAD [18, 19]. Furthermore, NAR although poorly imported can also be utilized by Nrk1 [20] to form nicotinic acid mononucleotide (NaMN). Current data suggest that NR may be the only vitamin precursor that supports neuronal NAD synthesis [8], leading to an increased interest toward detection and quantification of this nucleoside in natural sources.

We developed a rapid and more sensitive UHPLC-MS/MS method for simultaneous quantification of NMN and related pyridine compounds in different tissues derived from *Mus musculus*, a species often used as a model for NAD-biosynthesis-related physiology and disorders, such as peripheral neuropathies.

2. Experimental

2.1 Materials

All reagents and solvents were HPLC or LC/MS grade. NaMN, NMN, NaAD, NAD, Na, NaM, Benzamide, 3-Methoxybenzamide, ammonium acetate, HPLC-grade methanol and SUPELCO Discovery® C18 HPLC Column (250 mm x 4.6mm ID, 5 μm) were purchased from Sigma–Aldrich Corp. (St. Louis, MO,USA). NR and NaR were produced both enzymatically and chemically as described [8, 21]. Ultra-pure water was prepared using a Milli-Q water system equipped with a 0.22-μm point-of-use membrane filter cartridge (Millipore–Waters, Milford, MA, USA). Minisart® RC4 Syringe Filters 0,2 μm were purchased from Sartorius Stedim Biotech GmbH (Goettingen, Germany).

2.2 Sample preparation and extraction of NMN and related compounds from mouse tissues.

Procedures using live animals were authorised under Project Licence 80/2254 that was approved both by the Babraham Research Campus Animal Welfare, Experimentation and Ethics Committee (AWEEC) and the UK Home Office. Tissues from wild-type (C57BL/6) mouse were obtained from a breeding colony purchased from Harlan Laboratories (UK). Collected tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C until the time of processing. Frozen mouse tissues were dipped into liquid nitrogen and ground by mortar and pestle to a fine powder. Thereafter, weighed tissue aliquots were extracted according to Gonzalez et al. [22]. Briefly, this consisted on the addition of 10 µl of boiling buffered ethanol (75% ethanol in 10 mM Hepes, pH 7.1) for each mg of fresh tissue and 30 µl of Benzamide 25 µM (Internal Standard). The homogenate was incubated for 3 min at 80°C, cooled at room temperature and sonicated 3 times at 50 watts with 1-min intervals on ice. After centrifugation, supernatant was dried by Speedvac and stored at -80°C until use. Prior to LC-MS analysis, samples were resuspended in 300 µl of Ammonium Acetate 5 mM pH 3, filtered with Minisart® RC4 Syringe Filters 0,2 µm and divided in two equal parts (100 µl each). One of them was spiked with 10 µl of standard solution mix and 10 µl of 3-Methoxybenzamide 25µM (process standard); the other one was spiked with 10 µl of Ammonium Acetate 5 mM pH 3 and 10 µl of 3-Methoxybenzamide 25 µM, to evaluate the matrix effect for each sample.

2.3 UHPLC-MS/MS conditions

Separation was carried out using an Agilent 1290 Infinity UHPLC system coupled to an Agilent 6420 Triple Quadrupole mass spectrometer equipped with an Agilent electrospray ionization source. Agilent MassHunter workstation B.06.00 software was used for data acquisition and analysis.

The UHPLC conditions are summarized in Table 1 and the 6420 Triple Quadrupole parameters are shown in Table 2. Analysis was carried out with positive electrospray ionization in MRM mode. Transitions and conditions for each compound were optimized using the MassHunter Optimizer software with flow injection of the diluted stock solutions of each pure analyte (Table 3). Time segment was applied to ensure each compound detected with high sensitivity without interfering transitions. Effluent was directed to waste from 0 to 5 minutes.

2.4 Preparation of stock solutions and calibration curves

Stock solutions of NaMN, NMN, NaAD, NAD, Na, NaM, NR, NaR, Benzamide and 3-Methoxybenzamide were each prepared at a concentration of 10 mM in ultrapure water and stored at -80°C until use. Calibration curve standards were prepared taking into consideration the endogenous baseline level of each nucleotide. The calibration range of each analyte is reported in Table 3.

2.5 Method validation

The method performance requirements were established according to Commission Decision 2002/657/EC [23]. More in details the linearity was evaluated determining the corresponding correlation coefficient (R^2) that was ≥0.997 for each analyte; precision expressed as RSD was < 15%; accuracy expressed as mean recovery was assessed to be in the range of 70-87%, uncertainty was less than 50%. The LOQ was calculated for each analyte and reported in table 3. All listed parameters comply the Commission Decision 2002/657/EC requirements.

3. Results and discussion

In order to achieve simple and accurate quantification of physiological components in biological samples, in developing a method LC-MS/MS some crucial steps have to be investigated, and strategies to overcome these problems have to be planned. Between them the most important are: (i) high recovery percentage of analytes, (ii) high sensitivity because of the general small amount of available samples, (iii) high selectivity and specificity, (iv) evaluation of the matrix effect. To obtain these goals both analytical and preparative steps have to be optimized.

3.1 Optimization of LC-MS/MS analysis

Different columns and mobile phases were evaluated to optimize the chromatographic conditions. The use of HILIC (Luna-NH_2) at pH 9.9, reported in previous studies [17], was avoided for the risk of silica release at pH>8. Furthermore RP-LC was preferred because

of its high robustness, flexibility and applicability in pharmaceutical industry. After several trials a SUPELCO Discovery® C18 was selected for the analysis given the surprising separating performances with the 250 mm long column in terms of reproducibility, peak symmetry and separation of all analytes.

The aim to achieve a chromatographic separation was prominent for the presence of interfering transitions between the target analytes even in absence of matrix. More in details the couples Na/Nam, NaAD/NAD, NaMN/NMN, NaR/NR differ of 1 amu (atomic mass unit) for the change from the carboxylic to amidic function in the structural formula, so the isotopic peak of the lightest component of each couple (Nam, NAD, NMN, NR) interferes with the heaviest component transition in case of coelution. Moreover these interferences remain even working in tandem mass mode, because in the fragmentation process each analyte loses the discriminant part of the molecule leading to the same daughter ion as the most abundant fragment for each couple of analytes. This problem is especially important for the couple NaR/NR where the physiological concentration levels of the two analytes are comparable. To choose a different transition or change polarity are solutions that led to an unacceptable loss in sensitivity.

The other two crucial steps to be explored in order to assure accurate analysis were the matrix effect (ME) and the recovery percentage calculated for each sample. The ME can be assessed using different approaches, generally the use of stable isotopic labelled internal standards (SIL-ISs) is recommended [24-25], even if analog internal standard are admitted when SIL-ISs are not commercially available and the only chance to obtain them is in house synthesis with expensive and time consuming procedures [17]. We quantified the ME in each sample splitting the aqueous extracts in two identical aliquots just before injection, one is spiked with a standard mixture adjusted at the same range as the expected NMN and relative compounds in the sample, instead the other aliquot is added with the same volume of buffer. MeBe was chosen as analogue IS and it was added to both aliquots just before injection. By comparing the results of the two aliquots, we obtained the standard mixture response in presence of matrix. At the same time the standard mixture in net solvent was injected replacing the volume of matrix extract with net solvent. The difference in response provides an accurate quantification of ME for each target analyte in each sample. In addition a second IS was used and added before the extraction process. In order to mime the target compounds behavior Benzamide was chosen for its structural similarity. The ratio between Benzamide and MeBe gave the recovery percentage calculated for each sample. Both ISs were chosen in order to have a

retention time out from the ME window in order not to be affected by it. The final concentration of NMN and relative compounds was calculated taking into account the ME and recovery percentage quantified for each sample analyzed.

4. Application of the method

The method described in this study was sensitive enough to detect endogenous levels of nicotinic acid, nicotinamide, NMN and related pyridine compounds and would be suitable for many lines of investigation.

NAD metabolism regulates diverse biological processes, including ageing, circadian rhythm and axon survival (10). Quantifying NAD precursors NMN, NR, NaR, Na and nicotinamide in animal tissues is becoming more and more relevant in drug discovery and several tissues were investigated in this study as reported in Figure 1 (HPLC/MS-MS chromatogram of lung) and Table 4: the brain, the kidney, the lung, the spleen, the hearth, the small intestine, the thymus and skeletal muscle of mice.

5. Conclusions

An accurate and sensitive method was developed for the determination of NMN and related pyridine compounds in several mouse tissues. These analytes are highly relevant because of their recently recognized role in neurodegeneration, besides in human nutrition. In recent studies, accumulation of high levels of NMN after nerve injury has been demonstrated to promote axon degeneration [26]. It is of great significance to quantify NMN and NAD related metabolites in different tissues of animal models of neurodegeneration: NAD levels in tissues are in fact subjected to fluctuations due to different expression of key enzymes in NAD biosynthesis, such as NAMPT (nicotinamide phosphoribosyltransferase) and NMNAT (nicotinamide mononucleotide adenylyltransferase), and enzymes that use it as substrate, like sirtuins. Defects in NAD biosynthesis and thereby reduced sirtuins activity are considered crucial underlying processes during aging [27].

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Table 1. UHPLC Parameters

UHPLC column	SUPELCO Discovery® C18 HPLC Column (25 cm × 4.6 mm, 5 µm particle size) equipped with Discovery® C18 Supelguard™ Guard Cartridge at 11°C.		
Temperature	11°C		
Mobile phase	A: 5 mM ammonium acetate pH 3 in water B: Methanol		
Gradient program	Time (min)	%B	Flow (mL/min)
	5	0	0,6
	12	35	0,6
	15	100	0,6
	15,2	100	0,8
	18	100	0,8
	18,2	100	0,6
	23	0	0,6
Injection volume	20 µL		

Table 2. Triple Quadrupole Parameters

Ionization mode	Positive ESI
Scan type	MRM
Gas temperature	350 °C
Gas Flow	12 L/min
Nebulizer pressure	45 psi
Capillary voltage	4000 V
Maximum number of concurrent MRMs	3
Minimum dwell time	200 ms
Maximum dwell time	300 ms
Resolution	Unit/Enh

Table 3. Optimized MRM parameters

Analyte	Precursor ion	Product ion	Dwell (ms)	Frag. (V)	CE (V)	CAV (V)	RT (min)	Calibration range (μM)	LOQ (pmol)
NaMN	336	124	300	86	8	7	6.09	0.05-0.5	0.007
NMN	335	123	200	94	8	7	6.13	0.02-2	0.020
NR	255	123	200	83	4	7	6.82	0.005-0.5	0.003
NaR	256	124	200	72	4	7	7.20	0.0025-0.25	0.005
Na	124	78	200	112	24	7	7.87	0.01-0.1	0.227
Nam	123	80	200	112	20	7	10.64	1.25-20	0.450
NaAD	665.2	136.06	200	140	60	7	11.62	0.1-1	0.020
NAD	663.9	136.06	200	155	50	7	11.96	1.5-150	0.201
Benzamide	152	109	200	110	12	7	17.58		-
Met-Benz	122	77	200	85	28	7	17.99		-

Table 4. Concentration (pmoles/mg of fresh tissue) of each metabolite in different mouse tissues

	Brain	Lung	Kidney	Spleen	Small Intestine	Heart	Thymus	Skeletal Muscle
NAD	88,506	32,621	246,366	45,457	65,948	179,665	29,932	118,204
NaAD	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0,845	0,023	<LOQ
NMN	0,677	0,455	3,502	0,527	1,476	1,891	0,559	0,176
NaMN	<LOQ	0,335	0,151	0,091	0,088	0,304	0,048	0,007
NR	0,061	0,014	0,322	0,047	0,113	0,486	0,037	0,023
NaR	0,002	0,011	0,009	0,006	0,062	0,021	0,006	<LOQ
Na	0,031	<LOQ	<LOQ	0,077	0,127	<LOQ	0,019	<LOQ
Nam	32,129	14,119	28,332	48,056	42,402	15,537	22,197	9,164