Simultaneous Quantitation of Nicotinamide Riboside and Nicotinamide in Dietary Supplements via HPTLC–UV with Confirmation by Online HPTLC–ESI–MS

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The identification and quantitation of nicotinamide riboside (NAR) and its main related compound (nicotinamide) were achieved using high-performance thin-layer chromatography (HPTLC)–ultraviolet (UV) densitometry with confirmation by online electrospray ionization (ESI)–mass spectrometry (MS). As the stationary phase, HPTLC Si 60 F254 glass plates were employed; the mobile phase was ethanol–1 M ammonium acetate–formic acid (7:1:0.1, v/v/v). No derivatization was applied, and UV densitometry was performed in the absorbance mode (270 nm). The method was validated by specificity, linearity, accuracy, precision, and robustness.

**Keywords:** Nicotinamide riboside, nicotinamide, dosage forms, HPTLC–UV, online HPTLC–ESI–MS

Introduction

The main building blocks of nicotinamide riboside (NAR), a pyridine-nucleoside form of vitamin B3, are nicotinamide (NA) and ribose. NAR can be found in milk, yeast, and beer but also in bacteria and mammals. NAR enriched foodstuffs are not well established. Presumably, products that contain yeast represent great natural sources for the compound [1, 2]; also, dairy products like, whey fractions, have been noted to incorporate NAR [3, 4]. The amount found in foodstuff is extremely low, and most likely, it does not surpass the low micromolar limit.

NAR is a known precursor for oxidized nicotinamide adenine dinucleotide (NAD⁺). NAD⁺ is a flexible receiver of hydride equivalents in order to create the reduced NAD (NADH), chemistry also common with the phosphorylated derivatives, nicotinamide adenine dinucleotide phosphate (NADP⁺), and reduced nicotinamide adenine dinucleotide phosphate (NADPH). NAD⁺ and its derivatives work as coenzymes for dehydrogenases and oxidoreductases and perform fundamental roles in basic energy metabolism, e.g., glycolysis, the cycle of citric acid, and the mitochondrial electron transport. NAD⁺ is also an essential substrate for signaling enzymes, e.g., poly[adenosine diphosphate (ADP)-ribosyl] polymerases, sirtuins, and ADP-ribosyltransferases, called ‘NAD⁺ consumers’ [5, 6]. Consequently, NAD⁺ is an essential and abundant metabolite in every mammalian cell, engaged in various cellular mechanisms, for instance, metabolism and cell signaling both fundamental for survival.

The low amount of NAR in foodstuff (some quantitative studies exist) and the relative difficulty of obtaining high amounts of pure substance have restricted the research concerning the impact of NAR on cells and tissues [7]. Nevertheless, recently new and refined synthesis strategies for producing NAR have been developed [8], so that higher amounts are accessible for cell-based investigations [9] and for animal feeding tests [9, 10].

Since July 2013, NAR became accessible in dietary supplement form under the brand name NIAGEN® (Chromadex Incorporated, Irvine, California, USA). A lot of different other products that contain the compound have been commercially available since then [11].

In the recent specialty literature, quantification of NAR and NA has been performed by means of liquid chromatography (LC)–mass spectrometry (MS) [12, 13], LC–MS/MS [4, 14].

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ultra-high performance liquid chromatography (UHPLC)–MS [15, 16], UHPLC–MS/MS [7], nuclear magnetic resonance (NMR) spectrometry [17], and fluorometric/enzyme-coupled assay [18]. Also, various methods of analyzing NA such as in admixture with nicotinic acid in biological fluids and pharmaceutical dosage forms are well documented as follows: colorimetry [19], fluorometry [20], luminescence [21], surface-enhanced Raman detection [22], differential pulse polarography and cyclic voltammetry [23], normal phase (NP) or reversed-phase (RP) thin-layer chromatography (TLC) [24, 25], RP-high-performance thin-layer chromatography (HPTLC) [26], and HPLC–spectrophotometry [27–29]. TLC with postcolumn ultraviolet (UV) irradiation and fluorometric detection [30], micellar LC–UV absorbance detection [31], RP–LC [32], and LC–UV–MS in the multiple reaction mode [33]. Often, these analytical methods are time-consuming, and some of them are with low selectivity, requiring difficult procedures for sample preparation.

This study describes, for the first time, a fast, specific, and cheap HPTLC–densitometric method to identify and quantify not only NAR but also its main related compound (NA) from capsules of dietary supplements.

Experimental

Chemicals and Solvents. The reference compound for NAR was chloride salt (99% purity) obtained from MedKoo Biosciences Inc. (Morrisville, North Carolina, USA), and the NA reference (99.5% purity) was purchased from Sigma (St. Louis, Missouri, USA). Three different capsules of dietary supplements with NAR were purchased from an online market. The ammonium acetate and solvents (LiChrosolv®) – ethanol, formic acid, methanol, tolune, ethyl acetate, chloroform, cyclohexane, n-hexane, acetone, and water – were all purchased from Merck (Burlington, Massachusetts, USA). The HPTLC Si 60 F<sub>254</sub> 20 cm × 10 cm glass plates were also obtained from Merck.

Equipment. For sample preparation, an Eppendorf 5804 centrifuge (Eppendorf, Hamburg, Germany) and a Bandelin Sonorex DL102H ultrasound bath (Bandelin Electronic GmbH & Co. KG, Berlin, Germany) were used. A CAMAG (Muttenz, Switzerland) Linomat 5 was used for sample application on the plates, and a TLC Scanner 3 (also from CAMAG) was employed for the densitometric data acquisition. Both instruments were controlled through a CAMAG visionCATS v2.5 software package. The plates were developed in twin-tough glass chambers (20 cm × 10 cm or 10 cm × 10 cm). The plates were documented using a Nikon Coolpix S8000 digital camera (Nikon Instruments Europe B.V.) [34]. The online MS confirmation was accomplished by means of a CAMAG TLC–MS Interface 2 coupled with an Acquity QDa mass detector and a HPLC Binary Pump 1525 controlled using an Empower 3 v1.7 software package, all from Waters (Milford, Massachusetts, USA).

Preparation of Reference and Sample Solutions. For quality purposes, 2.5 mg of each reference compound, NAR and NA, were dissolved in 5 mL of methanol ≥99.9% purity (GC LiChrosolv® grade (500 μg/mL). In order to obtain the calibration curve, the following amounts of reference compound were dissolved in 25 mL of methanol: 2 mg, 3 mg, 4 mg, 5 mg, 6 mg, and 7 mg to have the following concentrations per band (2 μL): 160 ng, 240 ng, 320 ng, 400 ng, 480 ng, and 560 ng, respectively (80 μg/mL, 120 μg/mL, 160 μg/mL, 240 μg/mL, and 280 μg/mL, respectively).

The content of 10 capsules from each type of dietary supplement was thoroughly mixed. Fifteen milligrams of each capsule content were added to a 25-mL volumetric flask and ultrasonicated for 15 min with 20 mL methanol. After 15 min, the volume in each flask was completed to the 25-mL mark, shaken for another 5 min, and filtered through 0.2-μm hydrophobic syringe filters (Merck–Millipore [Ireland] Millflex–FG Non-Sterile Hydrophobic Fluoropore [PTFE] Membrane 0.2 μm × 25 mm). The samples must be freshly prepared and immediately analyzed.

Application of Reference and Sample Solutions. Two microliters of reference solutions and 2 μL of sample solution(s) were each applied as 8 mm (10 cm × 10 cm plate) or 6 mm (20 cm × 10 cm plate) bands, at 11.4 mm or 8.4 mm apart and 8 mm from the lower edge.

Chromatography. The developing solvent that was employed was a mixture of ethanol–1 M ammonium acetate and formic acid (7:1:0.1, v/v/v), 5 mL (10 mL for 20 cm × 10 cm chamber, respectively) developing solvent in front trough, and 10 mL (20 mL for 20 cm × 10 cm chamber, respectively) in rear trough. The developing distance was 40 mm from the lower edge of the plate (32 mm from the application position). Prior to elution, the chamber was saturated with the mobile phase for 20 min. After the elution, the plate was dried for 5 min using a hair dryer at room temperature.

Densitometric and Mass Spectrometry Investigations. The densitometric investigation was performed using a CAMAG TLC Scanner 3 in the absorbance mode. The wavelength used for the scan was 270 nm, which is the absorbance maximum for both NAR and NA, with a deuterium lamp as a radiation source. The settings for the TLC Scanner were as follows: absorption measurement mode, 20 mm/s scanning speed, data resolution of 100 μm per step, and a slit dimension of 5 mm × 0.2 mm. All the data acquired by the scanner were processed via the visionCATS software.

The mass spectrometry investigation was accomplished using a CAMAG TLC–MS Interface 2 and a Waters Acquity QDa detector coupled with a 1525 binary pump. The spectra were acquired via the Empower 3 software. The settings for the detector were positive electrospray ionization (ESI+) mode, capillary voltage 0.8 V, cone voltage 10 V, probe temperature 450 °C, and full scan between 100 to 500 m/z. The mobile phase used for the online MS detection of NAR and NA was methanol–0.1 M ammonium acetate (95:5, v/v). The flow rate of the mobile phase was 0.2 mL/min. The nebulizing and drying gas used was nitrogen.

Method Validation. The proposed method was validated by specificity, linearity range, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and robustness, according to the International Conference on Harmonization (ICH) guidelines [35, 36].

The specificity of the method was analyzed through chromatography of working standard (NAR) and the related compound (NA) and NAR samples extracted from capsules.

The linearity of the HPTLC method was checked by analyzing 10 standard solutions of NAR and NA of the following concentrations: 160 ng, 240 ng, 320 ng, 400 ng, 480 ng, 560 ng, 640 ng, 720 ng, 800 ng, and 880 ng per band. The solutions (2 μL) were all applied on the same plate. Lastly, the plate was developed using the abovementioned mobile phase and scanned. The bands were applied in duplicate.

The accuracy of the HPTLC method was confirmed by measuring the recovery. An established amount of NAR reference compound was added to the capsule of known NAR content.
For the intra-day precision, three portions of each reference compound were individually prepared according to the previously described method. Onto three 10 cm × 10 cm plates, 2 µL of each reference solution were applied in triplicate. The plates were chromatographed subsequently using fresh portions of developing solvent in the same chamber. The plates were documented according to the method. The same was done for the inter-day precision, with only a plate per day for another two days. The results from plate to plate and across each plate were evaluated.

A specific calibration curve was studied using NAR and NA in the linearity range of 160 ng, 240 ng, 320 ng, 400 ng, 480 ng, and 560 ng per band. LOD and LOQ were calculated from the calibration curve expressed through the standard deviation method.

The robustness was investigated by checking the stability of the analytes in solution, on the plate, and during chromatography. Reference (NAR and NA) and sample solutions were prepared according to the “Experimental” section (with the statement that, only for robustness determination, solutions from capsules were obtained by dissolving 5 mg from each capsule content in 5 mL methanol). Two microliters of these solutions were applied onto a 20 cm × 10 cm plate. The samples and the plate with the applied samples (wrapped in aluminum foil) were set aside. After 3 h, similar solutions were prepared. Two microliters of the set-aside samples were applied next to the first samples, followed by 2 µL of the new solutions on the set-aside plate.

Results and Discussion

Analysis of Dosage Forms

Specificity. In order to choose the optimal mobile phase which allows separating NAR and its related compound (NA) from commercial products, different compositions and ratios of ammonium acetate, toluene, ethyl acetate, chloroform, cyclohexane, methanol, ethanol, n-hexane, acetone, and formic acid were examined. Of all the mobile phases used in the experiment, applying the mixture of ethanol, 1 M ammonium acetate, and formic acid in volume compositions of 7:1:0.1 as the mobile phase, we established the method specificity, which resulted in compact bands, sharp, and symmetric peaks of NAR and NA. The mixture used as the mobile phase resulted in optimum NAR (RF = 0.34) migration and a resolution (RS) of 2.304 (to separate two components to at least 98%, a resolution of RS = 1.0 is necessary) from its related compound, NA (RF = 0.79), without interference from other components from the formulation matrix (Figure 1). The absorption maxima of NAR and NA are equal to 270 nm and 225 nm. After the separation of the two compounds, the bands were eluted from the plate with a mobile phase comprised of methanol–0.1 M ammonium acetate (95:5, v/v) directly into the MS detector. The mass spectra were obtained for both NAR ([M – Cl + H]+, m/z 255) and NA ([M + H]+, m/z 123) using the CAMAG TLC MS Interface 2 directly from the plate (Figure 2).

Linearity Range. As reported above, we first tried the linearity with 10 different concentrations; the statistical data indicate that the linear relationship which exists between the peaks’ area [AU], and the concentrations of NAR and NA references (ng/band) was linear in the range of 160 ng to 560 ng, for both compounds (Table 1; Figure 3, a & b). All concentrations were applied in duplicate. Starting from the label claim, the levels of NAR and NA were set into the linearity range (Table 2).

Accuracy. The accuracy was confirmed by measuring the recovery, when a known amount of NAR reference compound was added to the capsules of known NAR content. Quantitative recoveries of 94.661–98.122% (mean 96.391%) were obtained. All samples were applied in duplicate. Low coefficient of variation values (CV < 5%) are indicative for the accuracy of the method (Table 3).
Table 1 Method validation data for the quantitative determination of nicotinamide riboside and related compound (nicotinamide)

| Method characteristics | NAR (%) | NA (%) |
|------------------------|---------|--------|
| Specificity            | Specific| Specific|
| Range (ng per band)    | 160–560 |        |
| Linearity (ng per band)| \(y = 1.52 \times 10^{-4} + 6.095 \times 10^{-4}\) | \(y = 1.8 \times 10^{-4} + 6.095 \times 10^{-4}\) |
| LOD (ng per band)      | 41.673  | 41.556 |
| LOQ (ng per band)      | 126.282 | 125.93 |

NAR: nicotinamide riboside; NA: nicotinamide; \(R^2\): coefficient of determination; CV: coefficient of variation; F value: the ratio of the mean regression sum of squares divided by the mean error sum of squares; \(p\): probability value (measures the level of statistical significance); LOD: limit of detection; LOQ: limit of quantification; LOD and LOQ were calculated from the slope \((\sigma)\), using the following equations: LOD = 3.3 \(\times\) S; LOQ = 10 \(\times\) S. The calibration curve was obtained by a plot between the amount of analyte versus the average response (peak area).

**Precision.** The precision of the method was investigated as the intra- and inter-day repeatability of the process. The results of the precision testing were expressed as the relative standard deviation (%RSD) of the response factors (a relationship between the peak area and concentration of NAR and NA and their \(R^2\) as well). All samples were applied in triplicate. Because %RSD was less than 5%, the method was precise. The intra-day %RSD for NAR and NA \(R^P\) was 1.264 and 0.972, respectively. The intra-day %RSD for NAR and NA response was 0.99919 and 3.59061, respectively. The inter-day %RSD for NAR and NA \(R^P\) was 0.633 and 0.262, respectively. The inter-day %RSD for NAR and NA response was 4.81743 and 1.65707, respectively (Tables 4 and 5).

**LOD and LOQ.** LOD and LOQ were 41.673 ng per band and 126.282 ng per band for NAR and LOQ for NA and 125.93 ng per band for NA, respectively (Table 1).

**Robustness.** The robustness of the method was tested on 3 main stages of the assay: the stability of analytes in solution and on the plate and the stability of analytes during chromatography.

Concerning the stability of analytes in solution and on the plate, no notable degradation takes place during the 3-h window, as observed from the chromatogram. If we read the plate with the TLC Scanner, we can actually detect a small amount of degradation in some of the capsules both in solution and on the plate (C2 and C3). Only one product (C2) seems to be already degraded (Table 6; Figure 4).

A mixture of 1 mg/mL of NAR and NA was prepared. Two microliters were applied as spot at the lower left corner of a 10 cm \(\times\) 10 cm plate (10 mm from each edge). The plate was developed and dried according to the method (see the “Chromatography” subsection). The plate was then turned 90 degrees to the left and developed a second time according to the same method with a fresh portion of developing solvent. The samples are stable during chromatography because all the spots are positioned on the diagonal, which connects the application position with the intersection of the 2 solvent fronts (Figure 5).

A thorough search in the literature, we did not manage to find any reference regarding a HPTLC–densitometric method for the separation and quantitation of NAR and its main related compound NA. Since 2013, when NAR has become available on the market as a supplement, many dietary formulations that contain it have emerged. It was only normal to develop a method that could not only identify and quantify NAR but also NA from those specific formulations. The results of this study indicate that the validated HPTLC–densitometric method can be applied successfully for the determination of the specified compounds in selected dietary supplements.
Conclusion

The proposed HPTLC–densitometric method for the simultaneous quantification of nicotinamide riboside and nicotinamide indicates that the silica gel 60 F254 20 cm × 10 cm glass plates are suitable as a stationary phase and the mixture of ethanol, 1 M ammonium acetate, and formic acid (7:1:0.1, v/v/v) as the mobile phase. The abovementioned chromatographic conditions resulted in optimum migration of nicotinamide riboside and complete resolution of its related compound (nicotinamide) from commercial products in capsule dosage forms.

Conflict of Interests

The authors declare that they have no conflict of interests or financial benefits from specifying the names of the companies or the trademarks.

Table 4 Intra-day precision data for NAR and related compound (NA)

| Sample     | Intra-day precision |         |         |
|------------|---------------------|---------|---------|
|            | \( R_f \) | Average | Deviation | %RSD |
| NA         | 0.793  | 0.799   | 0.008    | 0.972 | 0.01364 |
|            | 0.797  | 0.808   | 0.328    | 0.326 | 0.334 |
| NAR        | 0.326  | 0.329   | 0.004    | 1.264 | 0.0122 |
|            | 0.334  | 0.01197 | 0.01211 | 0.00012 | 0.999919 |

NA: nicotinamide; NAR: nicotinamide riboside; %RSD: relative standard deviation.

Table 5 Inter-day precision data for NAR and related compound (NA)

| Sample     | Inter-day precision |         |         |
|------------|---------------------|---------|---------|
|            | \( R_f \) | Average | Deviation | %RSD |
| NA         | 0.793  | 0.795   | 0.002    | 0.262 | 0.01344 |
|            | 0.797  | 0.796   | 0.328    | 0.331 | 0.327 |
| NAR        | 0.331  | 0.329   | 0.002    | 0.633 | 0.01335 |
|            | 0.327  | 0.01255 | 0.01268 | 0.00061 | 4.81743 |

NA: nicotinamide; NAR: nicotinamide riboside; %RSD: relative standard deviation.

Table 6 Results of analytes' stability in solution and on the plate

| Track | Sample | Assigned compound | \( R_f \) | Area (%) |
|-------|--------|-------------------|---------|---------|
| 1.    | NAR plate 3 h | NAR | 0.331 | 100 |
| 2.    | NA plate 3 h  | NA | 0.775 | 100 |
| 3.    | C1 plate 3 h  | NAR | 0.331 | 100 |
| 4.    | C2 plate 3 h  | NAR | 0.331 | 100 |
| 5.    | C3 plate 3 h  | NAR | 0.334 | 95.558 |
| 6.    | C3 plate 3 h  | NA | 0.768 | 4.441 |
| 7.    | NA solution 3 h | NA | 0.778 | 100 |
| 8.    | C1 solution 3 h | NAR | 0.334 | 100 |
| 9.    | C2 solution 3 h | NAR | 0.334 | 91.089 |
| 10.   | C3 solution 3 h | NA | 0.768 | 8.91 |
| 11.   | NAR fresh | NAR | 0.334 | 100 |
| 12.   | NA fresh | NA | 0.784 | 100 |
| 13.   | C1 fresh | NAR | 0.337 | 100 |
| 14.   | C2 fresh | NA | 0.778 | 7.271 |
| 15.   | C3 fresh | NAR | 0.337 | 100 |

NAR: nicotinamide riboside; NA: nicotinamide; C1: capsule 1; C2: capsule 2; C3: capsule 3.

Figure 4. HPTLC chromatogram of the stability of analytes in solution and on the plate. The samples on the plate represent the following: tracks 1, 2, 3, 4, and 5 – samples on the plate for 3 h prior to chromatography; tracks 6, 7, 8, 9, and 10 – samples prepared 3 h prior to chromatography (in solution); and tracks 11, 12, 13, 14, and 15 – fresh samples applied immediately prior to chromatography. The image was captured under UV light (254 nm).

Figure 5. HPTLC chromatogram of the stability of analytes during chromatography. The image was captured under UV light (254 nm).
Authors' Contribution

All the authors contributed equally to the manuscript.

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