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Validation of an HPLC method for the determination of urinary and plasma levels of N^1-methylnicotinamide, an endogenous marker of renal cationic transport and plasma flow

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Abstract

N^1-Methylnicotinamide (NMN) is an endogenous cationic metabolite of nicotinamide (niacine, vitamine PP) whose renal clearance reflects both the capacity of the renal tubular transport system to secrete organic cations and renal plasma flow. NMN is present in human plasma and urine at the 1–117-ng ml^{-1} and 0.5–25-μg ml^{-1} concentration range, respectively, and its level depends notably on pathophysiological (age, renal or hepatic diseases) conditions. We report the optimization and validation of an HPLC method for the measurement of endogenous NMN in biological fluids after derivatization into a fluorescent compound. Plasma is first deproteinized with TCA 20% and the urine diluted 1:10 with HCl 10^{-4} M prior to the derivatization procedure, which includes a condensation reaction of NMN with acetophenone in NaOH at 0°C, followed by dehydration in formic acid and subsequent formation of the fluorescent 1,6-naphthyridine derivatives after heating samples in a boiling water bath. The synthetic homologous derivative N^1-ethylnicotinamide (NEN) reacts similarly and is added as internal standard into the biological fluid. The reaction mixture is subjected to reverse phase high performance liquid chromatography on a Nucleosil 100-C18 column using a mobile phase (acetonitrile 22%, triethylamine 0.5%, 0.01 M sodium heptanesulfonate adjusted to pH 3.2), delivered isocratically at a flow rate of 1 ml min^{-1}. NMN and NEN are detected at 7.8 and 10 min by spectrofluorimetry with excitation and emission wavelengths set at 366 and 418 nm, respectively. The addition–calibration method is used with plasma and urine pools. Calibration curves (using the internal standard method) are linear ($r^2 > 0.997$) at concentrations up to 109 ng ml^{-1} and 15.7 μg ml^{-1} in plasma and urine, respectively. Both intra- and inter-assay precision of plasma control samples at 10, 50 and 90 ng ml^{-1} were lower than 3.3% and concentrations not deviating more than 2.7% from their nominal values. In urine intra- and inter-assay CVs of control samples at 1, 5 and 9 μg ml^{-1} are lower than 8.3%, with concentrations not deviating more than −9.0 to +11.8%

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from their nominal values. This analytical method has therefore the required sensitivity and selectivity to measure NMN in plasma and urine, enabling the non-invasive determination of the tubular secretory capacity of the kidney and the renal plasma flow.

Keywords: N^1-Methylnicotinamide; Derivatisation; Fluorescence detection; Renal plasma flow; Renal cationic transport; Clearance

1. Introduction

N^1-Methylnicotinamide (NMN) (Fig. 1) is an endogenous cationic metabolite of nicotinamide (Vitamin PP, niacin). It is filtered at the glomerulus and highly extracted (secreted into the tubules) during its passage through the kidney, without being reabsorbed to a significant extent, and its renal handling reflects therefore both the capacity of the renal tubular transport system to secrete organic cations and the renal plasma flow (RPF). Clearance of endogenous NMN could substitute to *p*-aminohippuric acid (PAH) clearance for the determination of RPF [1], with the advantages of not being an exogenous product and not having to be infused, eliminating therefore not only technical and financial burden but also adverse effects, though admittedly rare, of PAH administration. Furthermore, though considered the gold standard, PAH has the drawback of being cleared in part (10–15%) by extra-renal mechanisms [2].

To the best of our knowledge, NMN has not been evaluated as a marker of RPF yet in humans. The use of NMN for renal plasma flow determination and clinical tubular function studies has presumably been hampered by the difficulty of routinely analyzing NMN in complex biological fluids. NMN has a very low UV extinction coefficient precluding its direct quantitation at the low nanomolar concentrations found in human plasma samples.

In a different context, the measurement of NMN urinary excretion has been used as a surrogate of niacin nutritional status [3]. Since it is present in urine in the μg ml^{-1} range, NMN could be directly analyzed in this milieu after ion-exchange chromatography and UV detection [4,5]. However, UV detection may not provide the necessary selectivity in complex urine matrices found in treated patients. NMN has therefore been commonly analyzed in urine after derivatization procedures without [6,7] or with [8] subsequent separation of reaction mixtures by high performance liquid chromatography (HPLC). Such an approach has also been applied for measuring subnanomolar levels of endogenous NMN in plasma. The derivatization of NMN requires a preliminary off-line step including a condensation of N^1-alkylnicotinamides with acetophenone in NaOH at 0°C. This is followed by dehydration of the condensed products in formic acid, and a final heating in boiling water, yielding the fluorescent 1,6-naphthyridine derivatives (Fig. 1) [9] subsequently quantitated by reverse phase HPLC with spectrofluorimetric detection.

This approach has been notably evaluated by Somogyi et al. [10] who proposed an optimization of the derivatization method for its application to endogenous NMN in plasma, where sensitivity is a major issue. This important contribution did not made clear whether the very early NMN peak elution could always be resolved from the relatively high baseline noise at the beginning of the

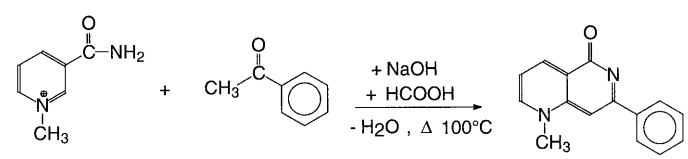


Fig. 1. Derivatization reaction of NMN with acetophenone into a fluorescent dervative.

relatively short HPLC run. Moreover, the reported direct derivatization of whole plasma samples [10] resulted in our hands in erratic reaction mixtures, producing unstable analytical samples (with clouding and even precipitation in vials over time) not suitable for subsequent HPLC analysis.

We report an adaptation and optimization of the method of Somogyi et al. [10] leading to improved chromatographic profiles, notably for plasma samples, with satisfactory sensitivity and enhanced selectivity, enabling the baseline separation and quantitation of NMN from its synthetic homologous N^1-ethylnicotinamide (NEN)-added as internal standard — and from minor nearby interfering peaks. The detailed analytical method validation has been based on the recommendations published as a conference report of the Washington Conference on Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies [11].

The method has been applied to the determination of endogenous NMN in plasma and urine of subjects included in a study assessing the adaptation of renal function during high altitude hypoxia and the development of acute mountain sickness (AMS).

2. Experimental

2.1. Chemicals and reagents

1-Methylnicotinamide chloride salt (NMN) and trichloroacetic acid (TCA) were purchased from Sigma (Buchs, Switzerland). Formic acid 98–100% and hydrochloric acid 25% were from E. Merck (Darmstadt, Germany). Sodium hydroxide pellets, acetophenone, sodium 1-heptanesulfonate monohydrate, triethylamine and *ortho*-phosphoric acid 85% were purchased from Fluka (Buchs, Switzerland). HPLC grade acetonitrile was from Romil (Cambridge, UK). All chemicals were of analytical grade and used as received. Ethanol absolute puriss. (Fluka, Switzerland) was used as aldehyde- and ketone-free ethanol. Ultrapure water was obtained from a Milli-Q UF-Plus apparatus (Millipore).

2.2. Synthesis of the internal standard N^1-ethylnicotinamide

N^1-Ethylnicotinamide iodide salt (NEN) was synthesized according to the method outlined by Hirayama et al. [8] from nicotinamide (Fluka, Switzerland) and ethyliodide (Buchs, Switzerland). Briefly, 1.0 g of nicotinamide was dissolved in 5 ml absolute ethanol (EtOH) by gentle heating under reflux at 50–60°C. After solubilization, a 1.5-ml volume of ethyliodide was added in one portion, and the resulting mixture was stirred at 50°C under reflux for 5 h. After cooling, a solid amorphous material was obtained, which was collected by filtration. The crude material was recrystallized twice in 10 ml MeOH and 110 mg of colorless crystals of pure N^1-ethylnicotinamide were obtained. This material was used for the preparation of a stock solution of internal standard used throughout all quantitative analysis.

2.3. Chromatographic system

The chromatographic system consisted of an L-6000A isocratic pump connected to an L-7200 autosampler (Merck–Hitachi, Japan), a LC 240 fluorescence detector (Perkin–Elmer, UK) with excitation and emission wavelengths set at 366 and 418 nm, respectively. The fixed scale factor on the spectrofluorimetric detector was set at 2.0 and 0.25, for the analysis of plasma and urine samples, respectively.

Chromatographic separations were performed using a ChromCart® Nucleosil 100-5 μm C18 (250×4 mm I.D.) column (Macherey–Nagel, Germany) equipped with a guard column CC 8/4 filled with the same packing material. The mobile phase consisted of 0.01 M sodium heptanesulfonate, 0.5% triethylamine and 22% acetonitrile in water adjusted to pH 3.2 with *ortho*-phosphoric acid 85% before the final addition of acetonitrile, and was delivered isocratically at a flow rate of 1 ml min^{-1}.

2.4. Stock solutions and calibration standards

Concentrated stock solutions of NMN and NEN were prepared in 10^{-4} M HCl at a concen-

tration of 1 mg ml^{-1}. The stock NMN solution was diluted with 10^{-4} M HCl to achieve working solutions at concentrations of 50 μg ml^{-1} and 500 ng ml^{-1}, which were used for the preparation of calibration samples in urine and plasma, respectively. The 1-mg ml^{-1} NEN (I.S.) solution was diluted with 10^{-4} M HCl down to 1 μg ml^{-1} and 50 ng ml^{-1} for the preparation of the I.S. solutions used for analysis of urine and plasma samples, respectively.

Plasma calibration standards were prepared by adding an appropriate volume of the 500-ng ml^{-1} NMN working solution, corresponding to the addition of 0-, 20-, 40-, 60-, 80- and 100-ng ml^{-1} NMN above the 'endogenous' NMN plasma levels, into a plasma pool collected from outdated frozen single plasma unit bags, or from Vaquez disease's patients, at the occasion of their regular phlebotomy. (Endogenous 'basal' NMN levels of the constituted plasma pool has been first determined by standard addition). Calibration samples and the similarly prepared control samples at 10, 50 and 90 ng ml^{-1} (added to basal NMN level) were stored as 200-μl aliquots in polypropylene Eppendorf tubes at −20°C until use and thawed the day of the analysis.

For the analytical method validation, urine calibration standards were prepared accordingly by adding an appropriate volume of the 50-μg ml^{-1} NMN working solution into a urine from one healthy donor (having an NMN endogenous level of 2.2 μg ml^{-1}, quantitated by standard addition), corresponding to the 0–10-μg ml^{-1} NMN range of added concentrations, into which, after (1:10) dilution, the response signal of fluorescence detector was found linear. They were stored together with control samples at 1, 5, and 9 μg ml^{-1} (added) as 1500-μl aliquots in polypropylene Eppendorf tubes at −20°C and thawed the day of the analysis.

For the analysis of urine samples collected in the clinical study (see below), the urine pools used for each urine calibration were prepared ex tempore on each day of operation with aliquots (600 μl) taken from each thawed urine sample ($n = 20$, from the same subject) to be analyzed within the same series. This procedure enables to obtain urine calibration standards with endogenous NMN levels averaging those found in the subsequent series of analysis. Serial volumes of the 50-μg ml^{-1} NMN working solution were therefore added to the urine pool constituted the day of the analysis for the preparation of calibration standards.

2.5. Sample preparation

The procedure applied to derivatize NMN in biological sample is an adaptation of the method proposed by Somogyi et al. [10] and was optimized specifically for plasma by including a protein precipitation step with TCA 20% prior to the derivatization reaction.

2.5.1. Urine

Urines were diluted 1:10 prior to analysis (i.e. 300 μl + 2700 μl HCl 10^{-4} M). To an aliquot (200 μl) of diluted urine sample, 200 μl of the internal standard solution (1 μg ml^{-1}) and 200 μl of 100 mM acetophenone in aldehyde- and ketone-free ethanol were added. The tubes were briefly (10 s) vortex-mixed and placed in an ice-bath for 10 min. A volume of 400 μl of 6 M NaOH was added; the tubes were briefly mixed again and incubated on ice for 10 min. After the addition of 200 μl of formic acid, vortex-mixing and a further incubation on ice for 15 min, the samples were placed in a boiling water bath for 3 min. After cooling, the samples were vortex-mixed and introduced into HPLC vials. A volume of 10 μl of the solution was injected onto the HPLC column.

2.5.2. Plasma

To an aliquot (200 μl) of plasma sample, 200 μl of the internal standard solution (50 ng ml^{-1}) and 100 μl of 20% TCA were added, the samples were vortex-mixed and centrifuged at 12 000 × g (Hettich Mikroliter 2043 benchtop centrifuge) for 10 min at room temperature. The resulting clear supernatants were treated similarly to urine samples for the derivatization procedure, except for a longer duration of the incubation time after NaOH addition (experimentally found optimal when prolonged up to 60 min). After cooling, the resulting solution was placed into HPLC vials and

an aliquot of 50 μl was injected onto the HPLC column.

2.6. Calibration curves

In the absence of NMN-free biological matrix, quantitation of NMN was performed using the addition–calibration method. Matrix-matched samples were used to establish calibration curves and their parameters (slope and offset) were used for quantitative analysis of all subsequent samples. All measurements were made in duplicate, with the duplication process starting at the sample processing procedure, to detect variability associated with the NMN derivatization step.

Since there was some concerns initially that the heterogeneity of the biological matrix composition may preclude the application of the addition-calibration approach, the slopes of standard addition were measured with plasma ($n = 4$) and urine ($n = 18$) pools from different subjects (i.e. with presumably different matrix composition). Since the influence of the heterogeneous biological matrix — notably urine — could be eliminated with the derivatization treatment (see below), the use of the addition-calibration method for the quantitative determination of all subsequent samples was indeed possible. Calibration curves were obtained by unweighted least-squares linear regression analysis of the peak-area ratio (NMN/NEN) versus the amount of NMN added in each standard sample. On each operation day, the parameters (slope and offset) of the standard addition calibration curves — established with a previously prepared pool — were used for the quantitative determination of NMN in all subsequent samples (absolute values in μg ml^{-1} respectively ng ml^{-1}), using the spreadsheet EXCEL 7.0 for WINDOWS 95 (Microsoft®).

The homogeneity of variances was examined by plotting the residuals (difference between experimental and calculated values, using the established linear regression) vs the concentration levels, using the spreadsheet EXCEL 7.0 for WINDOWS 95 (Microsoft®). The hypothesis of homoscedasticity (homogeneity of variances) has been statistically verified according to the Levene test [12] using the Robvar procedure from STATA 6.0 for Windows.

2.7. Analytical method validation

The detailed analytical method validation was based on the recommendations published as a conference report of the Washington Conference on Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies [11]. The Washington Conference provides however guiding principles for the validation of analytical method for the assay of *drugs and their metabolites* — i.e. xenobiotics — in biological fluids. Since *N*-methylnicotinamide is an *endogenous* compound naturally present in the biological matrices, some adjustments of these recommendations were necessary, but all efforts were done to follow them at best.

Each level of the calibration curve was measured with two sets of calibration standard samples: one set at the beginning and a second set at the end of the HPLC run.

In the absence of NMN-free biological samples, the recovery (i.e. difference between the measured and nominal levels of NMN added to samples containing endogenous NMN) reflects the accuracy of the method. The three control standards spiked with low, medium and high levels of NMN (1, 5, 9 μg ml^{-1} and 10, 50, 90 ng ml^{-1} for urine and plasma, respectively) were analyzed 12 times within the same run and on 6 separate days.

The control samples were used for the determination of the precision and accuracy of the method. The precision was calculated as the coefficient of variation (C.V.%) within a single run (intra-assay) and between different runs (inter-assays). Accuracy was determined as the percentage of deviation between nominal and measured concentrations obtained from the calibration curves.

Throughout the routine analysis of the biological samples collected in the metabolic study (see below), control samples at three relevant concentration levels (10, 50 and 90 ng ml^{-1}, and 1, 5 and 9 μg ml^{-1}, added onto plasma and urine pools, respectively) were assayed every four samples.

2.8. Lower limits of quantitation (LLOQs)

The assessment of LLOQs were determined by analyzing serial dilution of urine and plasma samples —whose NMN levels have been first determined — providing measurements with a precision and accuracy within the recommended $\pm 20\%$ from their nominal values.

2.9. Limits of detection

The limit of detection (LOD) of NMN in plasma and urine was determined by analyzing serial dilutions of plasma and urine samples whose NMN levels were first determined by the standard addition method.

2.10. Selectivity

The selectivity of the assay was determined by subjecting aqueous solutions to the same derivatization reaction, with or without the presence of NMN and NEN.

2.11. Stability studies

The stability of NMN in biological samples was assessed by subjecting control and calibration standards to various storage conditions: at room temperature for 8, 24 and 48 h; kept frozen at -20°C; after three successive thawing–freezing cycles.

The stability of the fluorescent 1,6-naphthyridine derivatives had never been studied. Since relatively large series of samples are to be analyzed in a row, the stability of the processed samples (i.e. after derivatization) was evaluated during their storage at $+4$°C and at room temperature into HPLC vials in the autosampler rack for the maximum duration encountered in our series of analysis (typically 17 h).

2.12. Application of the method to the NMN clearance determination

According to a protocol previously approved by the Ethics Committee of the Hospital, 18 healthy volunteers were included in a metabolic study assessing the modification of the renal function during high altitude hypoxia and the development of AMS. The alteration in fluid homeostasis at high altitudes could suggest a pathophysiological role of the kidney. This question was addressed by investigating the renal function of volunteers before (low altitude, 560 m) and after exposure to high altitude hypoxia (at 4559 m). Subjects were on a standardized metabolic diet and activity starting 5 days prior to the study days. They were submitted to segmental tubular (fractional excretion of trace lithium [13], and electrolytes) and global renal function tests (sinistrin, a polyfructosane related to inulin [14], PAH [2] and NMN clearances), as well as body composition and hormonal profile evaluation.

The proposed analytical method was used for the determination of the endogenous NMN levels in plasma and urine enabling the evaluation of the individual N^1-methylnicotinamide clearance over time. The results of this metabolic study will be reported in detail elsewhere (Biollaz et al., in preparation) and their discussion are beyond the scope of the present report. Only data from the baseline assessment performed at the University Hospital at 560 m are reported and briefly discussed here.

At predetermined times, blood samples were drawn into 4-ml Monovettes tubes (Sarstedt, Nürnberg, Germany) containing EDTA-K and immediately centrifuged for 10 min at 4°C. Similarly, at scheduled times, urine was collected and its volume measured. Plasma and urine samples were stored at -25°C prior to analysis.

3. Results and discussion

3.1. Samples preparation

The sample treatment proposed by Somogyi et al. [10] was found satisfactory and could be directly applied to diluted urine samples. Urine samples had to be diluted (1:10) with HCl 10^{-4} M prior to derivatization because the native NMN concentrations yielded levels of naphthyridine derivative whose fluorescence signals exceeded the detector response limits. All standard

solutions for urine (calibration and control) were diluted similarly (1:10) before the derivatization procedure.

For plasma, the reported direct derivatization of whole plasma samples [10] resulted in our hands in erratic reaction mixtures producing unstable analytical samples with clouding and even precipitation occurring in vials over time, and was therefore not suitable for HPLC determination of samples left at room temperature in the autosampler instrument.

The prior removal of plasma proteins by precipitation with TCA 20%, centrifugation and subsequent derivatization reaction of the clear supernatants made it possible to achieve reproducible analytical results. The preliminary protein precipitation step appears therefore adequate since NMN is not bound to plasma proteins [15]. Such a procedure was in fact proposed in an earlier fluorimetric NMN assay [7].

Compared to urine, derivatization of NMN in plasma samples needed a longer incubation time (60 min) after NaOH addition as recommended by Somogyi et al. [10]. This was confirmed during the setting-up and validation of our method.

3.2. Chromatograms

The typical chromatographic profiles of a derivatized urine pool, either 'blank' (containing 3.3-μg ml^{-1} endogenous NMN) (upper trace) and spiked with a 6-μg ml^{-1} NMN standard solution (lower trace) are shown in Fig. 2a. Similarly, the chromatograms of a derivatized plasma pool sample (from outdated transfusion bag) containing 7.4-ng ml^{-1} NMN (upper trace), and spiked with a 40-ng ml^{-1} NMN standard solution (lower trace) are shown in Fig. 2b. The I.S. (NEN) solution (50 ng ml^{-1} and 1 μg ml^{-1}, in plasma and urine) was added in both 'blank' and spiked samples. Examples of chromatograms of derivatized urine (A) and plasma (B) samples (containing 12.5 μg ml^{-1} and 16 ng ml^{-1}, respectively) from the healthy volunteers enrolled in the metabolic study are shown in Fig. 3. The fluorescent naphthyridine derivatives of NMN and NEN have a retention time of 7.8 and 10 min, respectively, with good baseline separation, and are well resolved from minor fluorescent components eluted early, as shown in Fig. 2a and b, and Fig. 3. Compared to the rather noisy baseline profile of chromatograms reported in Somogyi's publication [10], the chromatograms of derivatized samples were significantly improved, especially for plasma. This was presumably made possible by using Nucleosil reverse phase column, with enhanced performance over older packing material, and surely by the preliminary protein precipitation step performed in plasma, with clear supernatants much more suitable for the subsequent derivatization reaction.

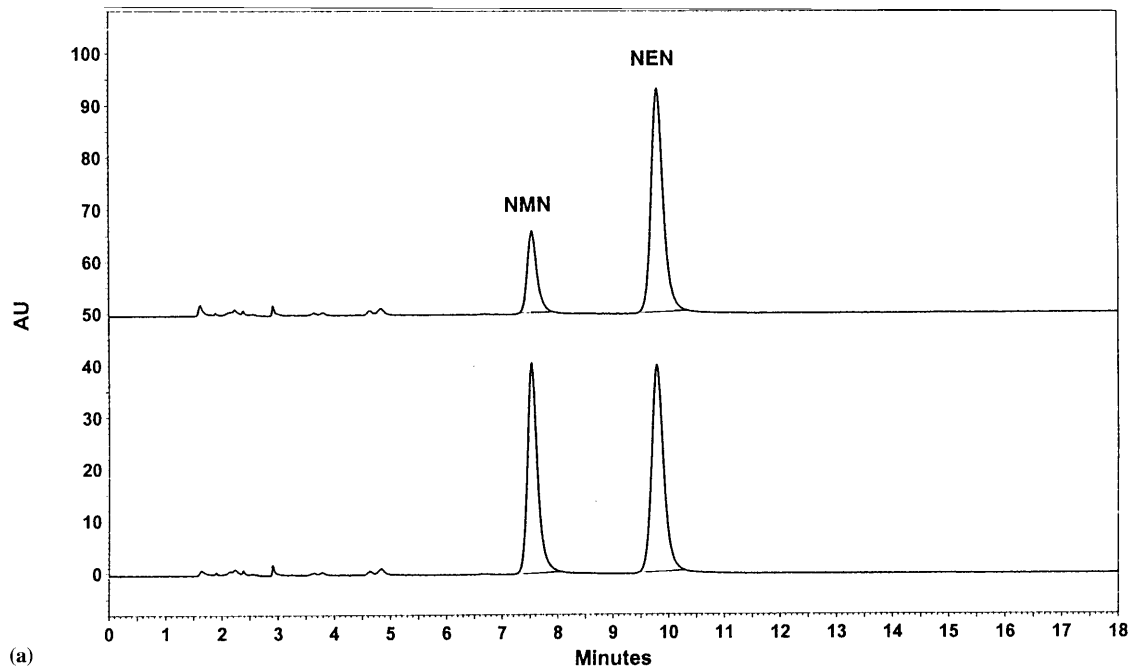
3.3. Calibration curves

Calibration curves established with the addition-calibration method were linear (regression coefficient values r^2 always > 0.997) up to 15.7 μg ml^{-1} in urine and to 109 ng ml^{-1} in plasma (i.e. up to the highest NMN levels observed in our experiments, using different urine and plasma pools onto which NMN has been added for the calibrations).

The linearity obtained with *unweighted* calibration curves of NMN in urine and plasma were satisfactory and a weighting was not considered. Indeed, visual inspection of the plot of residuals (difference between experimental and calculated values using the established linear regression) vs. concentration levels in the calibrations curves for plasma ($n = 6$) and urine ($n = 18$) samples indicates no trend in variability values throughout the delineated range of concentrations, both in plasma or urine. The homogeneity of variances has been statistically verified according to the Levene's test [12] yielding Pr values > 0.535 and 0.637, for plasma and urine calibration, respectively, indicating that the hypothesis of homoscedasticity (homogeneity of variances) is verified, and that the unweighted model is indeed adequate.

Typical standard addition–calibration curves in urine are described by $y = 0.093x + 0.3242$, in which y is the peak area ratio of NMN to IS and x is the concentration of NMN added (Table 1).

The initial concern that the non-homogenous urine matrix composition may preclude the appli-



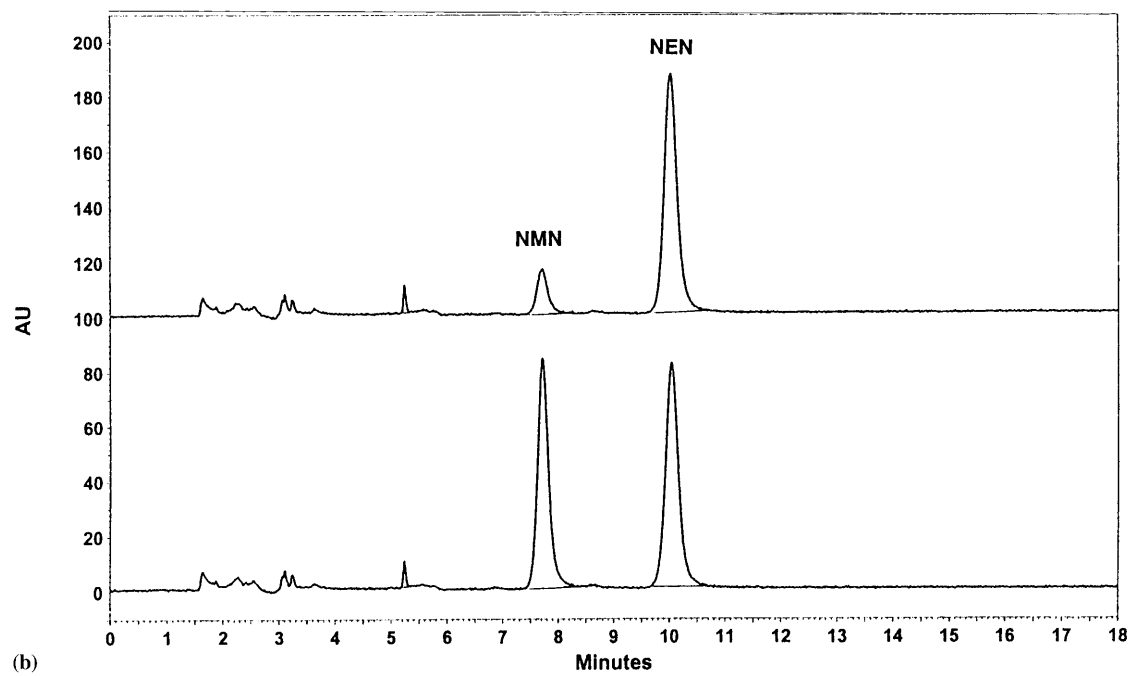


Fig. 2. (a) Chromatographic profile of a urine pool sample (obtained from healthy donors 'blank' (upper trace) and spiked with a 6 μg ml^{-1} NMN standard solution (lower trace). NEN, internal standard. (b) Chromatographic profile of a plasma pool sample (from outdated transfusion bag) 'blank' (upper trace) and spiked with a 40 ng ml^{-1} NMN standard solution (lower trace). NEN, internal standard).

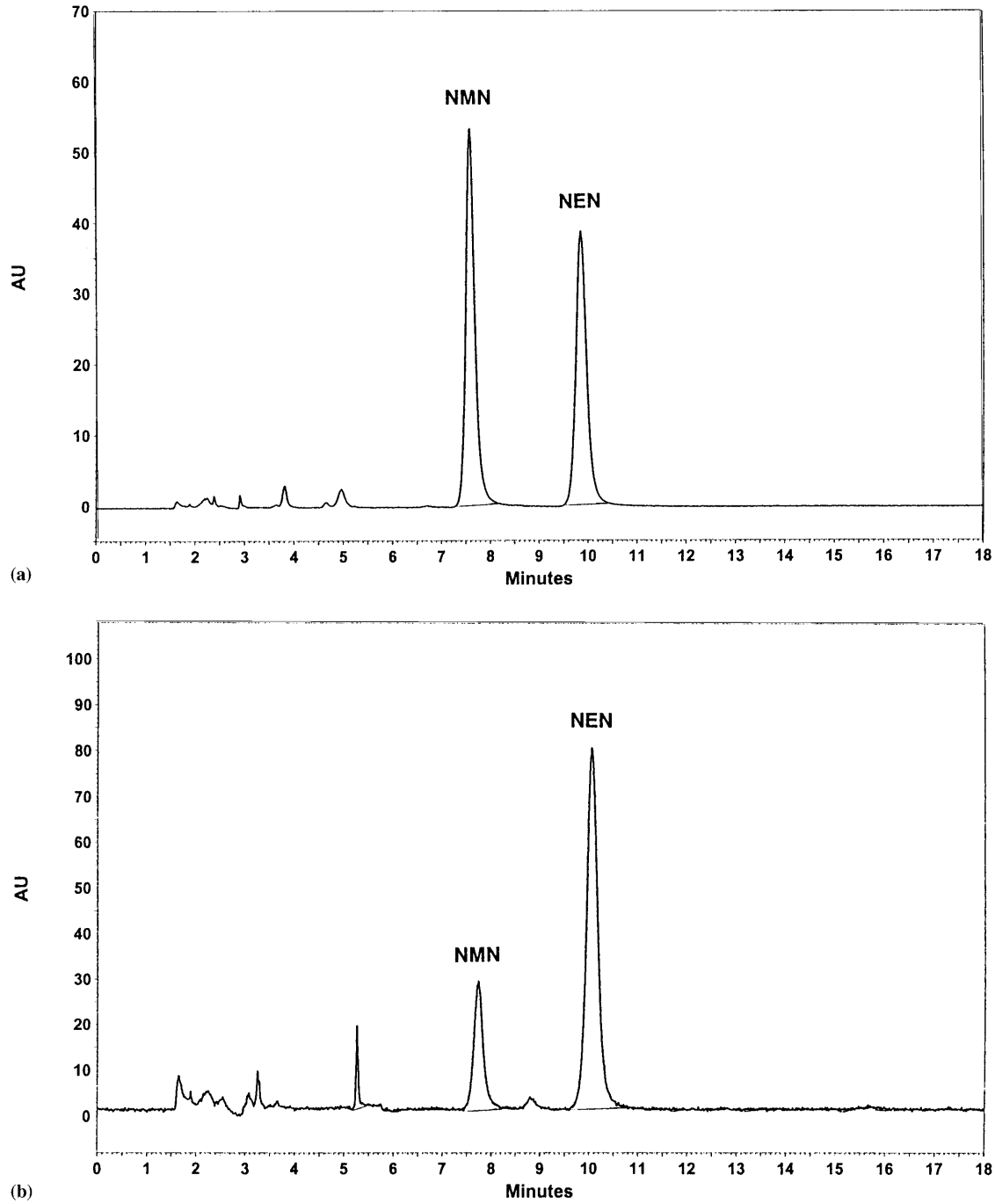


Fig. 3. Typical chromatograms of urine (A) and plasma (B) samples (12.5 μg ml^{-1} and 16 ng ml^{-1}, respectively obtained from healthy voluteers.

cation of the addition-calibration approach was not confirmed. The slopes of the addition-calibration performed with urine pool constituted from 18 different donors were reproducible ($0.09 \pm 2.8\%$), as shown in Table 1. The heterogeneity of the urine matrix does not affect, at least after dilution, the yield and rate of the derivatization reaction. Consequently, it was found a posterori that a unique pool could have been conveniently used to prepare calibration and control standards for all subsequent measurements in urine.

In plasma, the protein precipitation step also decreases, if not eliminates, any matrix effect likely to perturb the derivatization reaction. This treatment renders the plasma matrix quite uniform as assessed by the small CV(%) achieved with the addition-calibration curves slopes obtained with four different plasma matrices ($0.019 \pm 2\%$). A plasma pool containing an average NMN concentration of 8.8 ng ml^{-1} was used for the preparation of all calibration and control samples. The slopes of calibrations established with this same plasma pool ($n = 6$) were stable, with CVs never exceeding 0.8%.

3.4. Validation of the method: precision, accuracy and recovery

3.4.1. Plasma

The NMN concentrations added to the plasma control samples at 10, 50 and 90 ng ml^{-1} were selected to encompass the reported ranges of NMN concentrations of 6–40, 7–58, 13–101, 14–117 ng ml^{-1} found in plasma from young and elderly subjects, and from patients with renal or liver diseases, respectively [10]. The precision and accuracy of the plasma control samples achieved during the validation of the method are given in Table 2.

The mean intra-assay ($n = 12$) and inter-assay ($n = 6$) precision (CV%) of the NMN-fortified plasma samples were lower than 3.3% with concentration values not deviating more than -0.6 to $+2.7\%$ from their nominal values.

3.4.2. Urine

The urine NMN concentrations were comparatively high and urine samples had to be diluted at least 1:10 to bring the fluorescence signal of the naphthyridine derivative within the linearity range of the detector. The concentrations added to the

Table 1
Parameters of addition-calibration curves of NMN in 18 urine pools[a]

Patient #	Slope	Offset	R^2	Concentration pool (μg ml^{-1})
1	0.0987	0.2789	0.9992	2.83
2	0.0930	0.3242	0.9992	3.49
3	0.0964	0.2182	0.9992	2.26
4	0.0926	0.1594	0.9990	1.72
5	0.0925	0.4469	0.9976	4.83
6	0.0919	0.5152	0.9997	5.61
7	0.0931	0.4123	0.9981	4.43
8	0.0936	0.5303	0.9995	5.67
9	0.0885	0.3785	0.9986	4.28
10	0.0922	0.3237	0.9998	3.51
11	0.0921	0.3396	0.9989	3.69
12	0.0901	0.3902	0.9994	4.33
13	0.0918	0.2994	0.9997	3.26
14	0.0929	0.2664	0.9989	2.87
15	0.0975	0.2880	0.9983	2.95
16	0.0957	0.1726	0.9997	1.80
17	0.0901	0.1968	0.9995	2.18
18	0.0916	0.2480	0.9997	2.71

[a] Mean 0.09 ± 0.003, CV% 2.8.

Table 2
Precision and accuracy of the measurements of NMN in urine and plasma

Urine				Plasma			
Nominal concentration ($\mu g\ ml^{-1}$)	Concentration found ($\mu g\ ml^{-1}$)	Precision (C.V.%)	Accuracy* (deviation, %)	Nominal concentration ($ng\ ml^{-1}$)	Concentration found ($ng\ ml^{-1}$)	Precision (C.V.%)	Accuracy* (deviation, %)
Intra-assay (n = 12)				*(n = 12)*			
1.0	1.0 ± 0.04	3.9	4.9	10.0	10.3 ± 0.13	1.3	2.7
5.0	4.5 ± 0.11	2.4	−9.0	50.0	51.1 ± 0.36	0.7	2.2
9.0	8.6 ± 0.14	1.6	−4.1	90.0	91.2 ± 0.57	0.6	1.3
Inter-assay (n = 18)				*n = 6)*			
1.0	1.1 ± 0.09	8.3	11.8	10.0	10.2 ± 0.34	3.3	2.0
5.0	5.1 ± 0.27	5.3	1.1	50.0	50.5 ± 1.02	2.0	0.9
9.0	9.2 ± 0.52	5.6	2.2	90.0	89.5 ± 0.95	1.1	−0.6

* = 100* (found value − nominal value)/nominal value [%]

urine control samples were chosen accordingly at 1, 5 and 9 μg ml^{-1}. The precision and accuracy of the urine control samples achieved during the validation of the method (intra-assay) and semi-routine analysis (inter-assay) of urine samples of the 18 volunteers are given in Table 2.

Slightly lower precision and accuracy were obtained with urine samples compared to those achieved with plasma. When performing the assay on a semi-routine basis, improved experience with the urine sample derivatization procedure was attained and the mean intra-assay and inter-assay CV% for the 1-, 5- and 9-μg ml^{-1} urine controls were lower than 8.3%. Similarly, the intra-assay ($n = 12$) and inter-assay ($n = 18$) deviation from nominal values achieved for the urine control samples were also satisfactory, with values within the −9.0 to + 11.8% range.

3.5. Limit of detection

The limit of detection (LOD) of NMN in plasma and urine was experimentally found to be 8 ng ml^{-1} in urine and at least 0.25 ng ml^{-1} in plasma.

3.6. Lower limits of quantitation (LLOQs)

The lower limit of quantitation (LLOQs) of NMN was experimentally found to be 1.3 ng ml^{-1} and 0.15 μg ml^{-1} in plasma and urine, respectively. In fact, at concentration slightly below this level in plasma (i.e. 1.28 ng ml^{-1}) the precision was still acceptable (CV = 14%) but the deviation of + 22% from the theoretical level exceeds the ± 20% recommended values.

These LLOQ values should be selected in principle for the *lowest* concentration levels of the calibration curves, according to the Washington guidelines [11]. With the addition-calibration approach however, the lowest level of the calibration is constituted by a pool onto which *no* exogenous NMN is added (intercept of the curve at the y-axis when $x = 0$). The lowest levels of the calibration curve does contain endogenous NMN concentrations, which may *vary depending on the plasma or urine pools* and which indeed does *not* correspond to the LLOQ. This explains the difference between the lowest concentration level of the calibration curves and LLOQs values.

3.7. Selectivity

The derivatization procedure (see Fig. 1) applied to biological samples appears rather specific for alkylnicotinate species and makes it unlikely that other endogenous compounds — stable enough in both strongly alkaline and acidic medium — would interfere with the assay, especially when the resulting reaction mixture is subjected to a final reverse-phase chromatography. In our hands, no signal peak was observed at the retention time of NMN or NEN, when performing the assay with NMN- and NEN-free samples, confirming the observations of Somogyi et al. who also tested for interference a large number of drugs and potential metabolites (including NAD+ and NADP+) [10].

3.8. Stability of samples at room temperature

The variations over time of the NMN levels in plasma and urine samples (containing 14.6 ng ml^{-1} and 2.4 μg ml^{-1}, respectively) left at room temperature for 48 h, were 3.1 and 4.2% in plasma and urine respectively, indicating that no significant decomposition of NMN can be expected at room temperature. However, an apparent increase of the measured concentrations was noticeable, even when plasma and urine samples were contained in tight propylene tubes. This increase arises presumably from evaporation, indicating that the time during which samples are let at room temperature should be kept to a minimum to reduce the likelihood of spuriously elevated concentrations.

3.9. Stability of derivatize samples into HPLC vials (i.e. ready for HPLC analysis) at room temperature and at + 4°C

The stability of plasma and urine extracts (containing the fluorescent derivatize NMN) was checked with calibration samples placed in HPLC vials left at room temperature (rt) for 24 h and at + 4°C for 48 h. The slope of the established

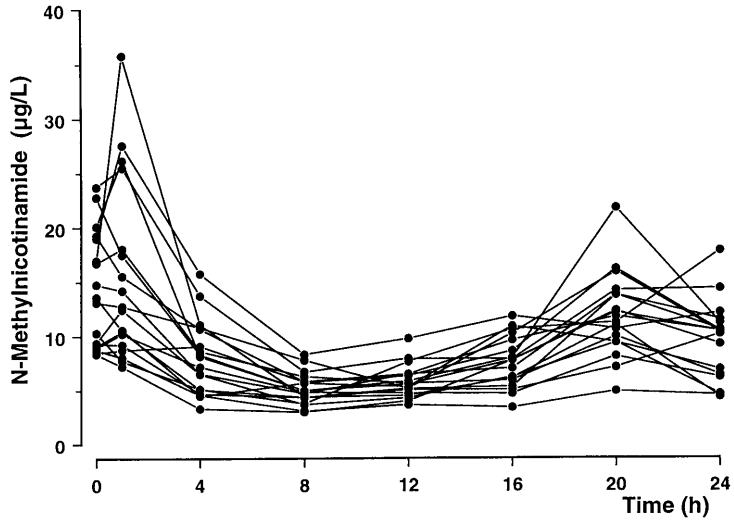


Fig. 4. Twenty four-hour profiles of the NMN concentrations in plasma from 18 healthy subjects.

calibration curves were 0.0224, 0.0219 and 0.0222, when analyzed immediately, after 24 h at rt, and after storage for 48 h at +4°C, respectively. This indicates that the processed samples are stable over time, which may be of importance when confirmatory analysis are required.

3.10. Application of the method

This method was applied to the analysis of plasma and urine samples from 18 male healthy volunteers participating to the study mentioned above. During the 24-h-baseline assessment, the mean NMN concentrations of all plasma samples ($n = 144$) was 9.7 ± 5.5 ng ml^{-1} (median 8.5 ng ml^{-1}, range 3.0–35.8 ng ml^{-1}). These values are in good agreements with the 6–40-ng ml^{-1} range of concentrations reported by Somogyi et al. in plasma from young subjects. Fig. 4 shows the plasma NMN concentrations profiles of the 18 subjects over 24 h. Interestingly, an apparent nyctemeral variation of NMN levels can be observed, with NMN levels consistently higher in the morning and lower in the evening. A similar variation in NMN levels over time was also reported by Somogyi et al. in a young healthy subject [10]. Circadian variations of metabolic variables are well known [16].

The urine NMN concentrations in the same volunteers were 4.97 ± 2.26 µg ml^{-1} (median 4.73 µg ml^{-1}, range 0.83–12.79 µg ml^{-1}) ($n = 144$).

4. Conclusion

An optimization and validation of the assay of NMN in biological fluids is presented, using a derivatization reaction first proposed by Huff and Perlzweig [6] and further improved by Clark et al. [7]. Efforts have been made not only for improving the method but also for enabling a fully automatable HPLC analysis of derivatize samples, especially in plasma, where stability problems had to be solved. Indeed analysis of large number of samples over a prolonged period of time (overnight), makes it necessary that an automatable method meets the desired precision and accuracy requirements. Plasma protein precipitation prior to derivatization enables better chromatographic profiles and improved sensitivity (LOD $\approx$ 0.25-ng ml^{-1} NMN in plasma).

The applicability of the method was demonstrated by analyzing endogenous NMN levels in plasma and urine samples from healthy subjects taking part in a study assessing the effect of high altitude hypoxia on renal function.

The use of NMN in investigating the renal function, in particular as a marker of RPF in humans, has been presumably hampered by the analytical limitations of quantitating low nanomolar NMN concentrations in complex biological fluids. This assay represents therefore a useful tool for evaluating the cationic transport system and for assessing RPF in the clinical setting, without the need to administer exogenous marker, such as PAH.

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