Journal of Chromatography B, 878 (2010) 895–902

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

# Simultaneous determination of nicotinic acid and its four metabolites in rat plasma using high performance liquid chromatography with tandem mass spectrometric detection (LC/MS/MS)

Malgorzata Szafarz[a,*], Magdalena Lomnicka[b], Magdalena Sternak[b], Stefan Chlopicki[b], Joanna Szymura-Oleksiak[a]

[a] Department of Pharmacokinetics and Physical Pharmacy, Jagiellonian University Medical College, Medyczna 9, 30-688 Krakow, Poland
[b] Department of Experimental Pharmacology, Chair of Pharmacology, Jagiellonian University Medical College, Grzegorzecka 16, 31-531 Krakow, Poland

ARTICLE INFO

*Article history:*
Received 30 October 2009
Accepted 9 February 2010
Available online 17 February 2010

*Keywords:*
Nicotinic acid
Metabolites
N-methylnicotinamide
LC/MS/MS
Rat
Plasma

ABSTRACT

A sensitive and specific liquid chromatography electrospray ionization–tandem mass spectrometry method for the simultaneous quantitation of nicotinic acid (NicA) and its metabolites nicotinamide (NA), 1-methylnicotinamide (MNA), 1-methyl-2-pyridone-5-carboxamide (M2PY) and 1-methyl-4-pyridone-5-carboxamide (M4PY) in rat plasma has been developed and validated. As an internal standard, 6-chloronicotinamide was used. The samples (100 μL) were subjected to deproteinization with acetonitrile (200 μL) and then, after centrifugation, 150 μL of the supernatant was transferred into conical vial and evaporated. Dry residue was reconstituted in 100 μL of the ACN/water (10:90, v/v) mixture. Chromatography was performed on a Waters Spherisorb® 5 μm CNRP 4.6 × 150 mm analytical column with gradient elution using a mobile phase containing acetonitrile and water with 0.1% of formic acid. The full separation of all compounds was achieved within 15 min of analysis. Detection was performed by an Applied Biosystems MDS Sciex API 2000 triple quadrupole mass spectrometer set at unit resolution. The mass spectrometer was operated in the selected reactions monitoring mode (SRM), monitoring the transition of the protonated molecular ions *m/z* 153–110 for M2PY, 153–136 for M4PY, 124–80 for NicA, 123–80 for NA and 137–94 for MNA. The mass spectrometric conditions were optimized for each compound by continuously infusing the standard solution at the rate of 5 μL/min using a Harvard infusion pump. Electrospray ionization (ESI) was used for ion production. The instrument was coupled to an Agilent 1100 LC system. The precision and accuracy for both intra- and inter-day determination of all analytes ranged from 1.3% to 13.3% and from 94.43% to 110.88%. No significant matrix effect (ME) was observed. Stability of compounds was established in a battery of stability studies, i.e. bench-top, autosampler and long-term storage stability as well as freeze/thaw cycles. The method proved to be suitable for various applications. In particular using this method we detected increased concentration of MNA and its metabolites in rat plasma after treatment with exogenous MNA (100 mg/kg), as well as increased concentration of endogenous NA and MNA in rat plasma in the early phase of hypertriglyceridemia development in rats fed high-fructose diet.



## 1. Introduction

Nicotinic acid (NicA) possesses the ability to decrease LDL cholesterol level and to increase HDL cholesterol level which led to its clinical use in the treatment of dyslipidemia and prevention of atherosclerosis. Nicotinamide (NA, Vitamin B3 or Pellagra Preventive vitamin), a metabolite of NicA, has neuro- and vasculo-protective properties as well as anti-inflammatory activity. Nicotinamide deficiency causes pellagra with symptoms including dermatitis, diarrhea, depression and dementia. 1-methylnicotinamide (MNA) has been considered, upto now, to be an inactive metabolite of NicA and NA, but recent studies have proved its anti-inflammatory and anti-thrombotic activity [1–3].

Previously reported methods quantifying nicotinic acid and its metabolites usually do not include MNA since it was considered an inactive compound [4,5]. A LC/MS method for the simultaneous determination of NA and MNA together with its major metabolites 1-methyl-2-pyridone-5-carboxamide (M2PY) and 1-methyl-4-pyridone-5-carboxamide (M4PY) has been developed recently, but it had poor reproducibility and lacked the fragments that would allow detection in tandem MS mode [6]. As to the best of our knowledge, there is only one method in literature available,

* Corresponding author. Tel.: +48 126205720; fax: +48 126570262.
*E-mail addresses:* mszafarz@cm-uj.krakow.pl, mszafarz@poczta.fm (M. Szafarz).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.02.009

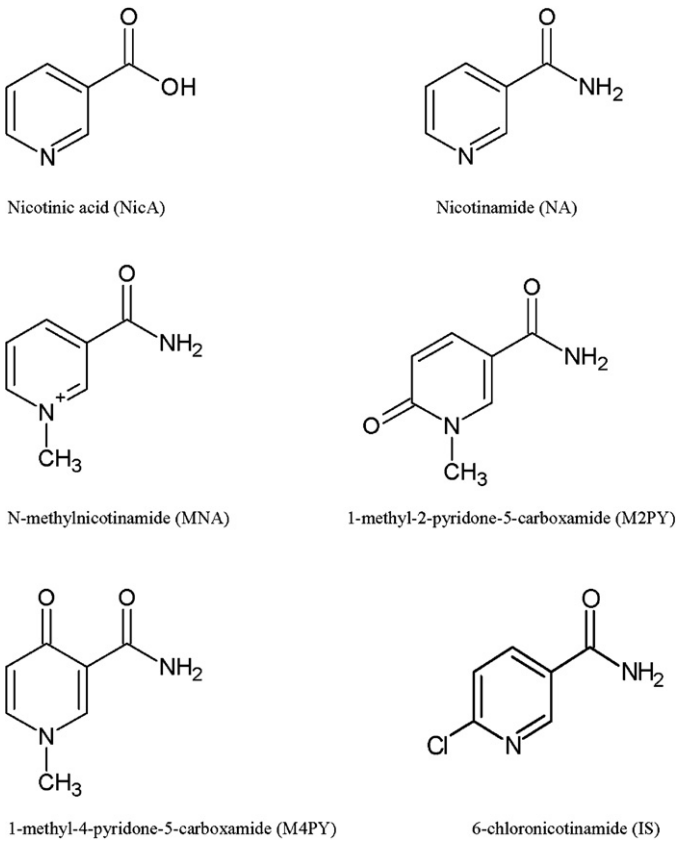


**Fig. 1.** Structures of nicotinic acid and its metabolites.

which quantifies simultaneously NicA and its major metabolites including NA, MNA, M2PY and M4PY. Separation of the target compounds was performed within 60 s with tandem mass spectrometric detection. Authors used stable isotopes of the corresponding analytes as internal standards. This approach is very useful for the correction of signal deviation, however, isotopically labeled internal standards are usually not easily available due to difficulties associated with synthesis and/or costs. The described method was used for two different matrices (urine and plasma) therefore the extraction procedure applied was quite complicated and time consuming [7]. Because of difficulties with obtaining isotopically labeled standards and laborious sample preparations, which may not be needed in case of plasma, application of this method in routine plasma analysis might be limited.

The purpose of this study was to develop and validate a simple, fast and inexpensive method for simultaneous determination and quantification of NicA and its four metabolites NA, MNA, M2PY and M4PY in rat plasma using high performance liquid chromatography with tandem mass spectrometric detection (LC/MS/MS).

## 2. Experimental

### 2.1. Chemicals and reagents

Nicotinic acid, nicotinamide, 1-methylnicotinamide, 1-methyl-2-pyridone-5-carboxamide and 1-methyl-4-pyridone-5-carboxamide were synthesized by Dr. Adamus from Technical University in Lodz, Poland. Chloronicotinamide used as an internal standard was obtained from Sigma (St. Louis, MO, USA). The chemical structures of NicA and metabolites are shown in Fig. 1. HPLC grade acetonitrile, water and formic acid were purchased from Merck (Darmstadt, Germany).

**Table 1**
Optimized parameters for SRM analysis of NicA, NA, MNA, M2PY and M4PY.

| Compound | NicA | NA | MNA | M2PY | M4PY |
|---|---|---|---|---|---|
| Declustering potential (DP) | 45 | 30 | 30 | 25 | 8 |
| Focusing potential (FP) | 350 | 350 | 400 | 400 | 200 |
| Entrance potential (EP) | 10 | 10 | 10 | 10 | 8 |
| Collision cell entrance potential (CEP) | 20 | 6 | 12 | 8 | 14 |
| Collision cell exit potential (CXP) | 4 | 4 | 3 | 2 | 3 |
| Collision energy (CE) | 30 | 30 | 30 | 30 | 20 |

Control plasma, containing heparin as anticoagulant, was obtained from adult male Wistar rats Krf:(WI)WV (Charles River Labolatory, Germany). Rats were injected intraperitoneally with thiopental (70 mg/kg) and blood samples were collected to heparinized tubes by decapitation. The plasma samples were separated by centrifugation (1000 × *g*, 10 min) and stored at −30 °C until used. The study was approved by the Institutional Animal Care and Ethics Committee of the Jagiellonian University.

### 2.2. Instrumentation and operating conditions

#### 2.2.1. Liquid chromatographic conditions

An Agilent 1100 system (Agilent Technologies, Waldbronn, Germany) consisting of a degasser, a binary pump, a column oven and an autosampler was used for solvent and sample delivery. Chromatographic separation was achieved on a Waters Spherisorb® 5 μm CNRP 4.6 × 150 mm analytical column. Autosampler temperature was set at 10 °C and the column was at ambient temperature. Mobile phase consisted of acetonitrile (ACN) and water ($H_2O$) with an addition of 0.1% of formic acid. Each LC/MS/MS run took 15 min. Initial mobile phase composition was 45% A (ACN), with a linear gradient to 10% of mobile phase A in the first 6 min. Then the HPLC operation was continued at isocratic mode (10/90, ACN/$H_2O$, v/v) for the next 3 min. The second linear gradient was the fast change within 0.1 min back to the 45% of mobile phase A and for the remaining 6.9 min isocratic mode (45/55, ACN/$H_2O$, v/v) was applied. The flow rate was maintained at 800 μL/min. The effluent from the HPLC before being directed into the ESI probe was split in the proportion of 1–5 (one part to the mass spectrometer and five parts to waste). A sample volume of 20 μL was injected into LC-ESI/MS/MS system.

#### 2.2.2. Mass spectrometric conditions

Mass spectrometric detection was performed on an Applied Biosystems MDS Sciex (Concord, Ontario, Canada) API 2000 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface. ESI ionization was performed in the positive ion mode. The tandem mass spectrometer was operated in the selected reaction monitoring mode (SRM) and Q1 and Q3 quadrupoles were set at unit mass resolution. The mass spectrometric conditions were optimized for each compound by continuously infusing standard solutions at the rate of 5 μL/min using a Harvard infusion pump. The ion source temperature was maintained at 450 °C. The ionspray voltage was set at 3500 V. The curtain gas (CUR) was set at 10 and the collision gas (CAD) at 6. The collision energy (CE) and the most favorable parameters of ion path for all analyzed compounds are presented in Table 1. The quantification was performed via peak area ratio. The Applied Biosystems Analyst version 1.4.2 software was used to control the LC-ESI/MS/MS system and to collect and treat the data.

### 2.3. Preparation of stock solutions

The stock solutions of M2PY, M4PY, NicA, NA and MNA were prepared at 1.0 mg/mL in water. Then they were further diluted with water to make working solutions at concentrations of 20, 10, 5, 2.5, 1, 0.5 and 0.1 μg/mL. The stock solution of the 6-

chloronicotinamide (IS) was prepared at 1.0 mg/mL in the mixture of acetonitrile and water (50/50, v/v). Internal standard working solution was prepared by diluting the stock solution of internal standard with water to give a concentration of 2.5 μg/mL. All stock and working solutions were stored at 4 °C.

### 2.4. Calibration curves

Calibration curves were prepared by spiking pooled blank plasma with an appropriate amount of working solution to produce the calibration curve points equivalent to 2000, 1000, 500, 250, 100, 50 and 10 ng/mL of each compound. Samples were made in five replicates and each of them also contained 10 μL of the IS working solution. The results (peak area of analyte/peak area of IS) versus concentration were fitted to the linear equation. The quality of fit was evaluated by comparing back-calculated concentrations to the nominal ones.

### 2.5. Quality control samples

The quality control samples used during the validation and in the following studies were prepared in the same way as the calibration curves. Namely, they were made by spiking pooled blank plasma with an appropriate analyte, IS and produced at different concentrations along the calibration range (LLOQ at 10 ng/mL, low at 40 ng/mL, medium at 400 ng/mL and high at 1800 ng/mL).

The calibration standards and QCs were stored under the same conditions and extracted without differences in thaw times.

### 2.6. Sample preparation

Samples were prepared using deproteinization with acetonitrile. A 100 μL aliquot of rat plasma was pipetted out into an eppendorf vial. The working IS solution (10 μL) and 200 μL of acetonitrile were added and then shaken for 90 s on the vortex shaker. Afterwards, samples were stored in the refrigerator (+4 °C) for 20 min and centrifuged at 16,600 × *g* for 10 min. The supernatant (150 μL) was transferred to the new eppendorf vials and evaporated to dryness at 37 °C under the stream of nitrogen gas in the TurboVap evaporator (Zymark, Hopkinton, MA, USA). The residue was reconstituted with 100 μL of the mixture of acetonitrile and water (10/90, v/v) and then vortex-mixed. An aliquot of 20 μL was injected onto the LC-ESI/MS/MS system for analysis.

### 2.7. Method validation

The bioanalytical method was validated according to the FDA guidelines.

#### 2.7.1. Assay selectivity and "cross-talk" effect issues

The absence of "cross-talk" between channels used for monitoring all five compounds and an IS was confirmed by injecting separately samples (pure solutions) containing an IS at the concentration used in the assay and monitoring the response in the MS/MS channels used for detecting the analyzed substances, and by injecting samples of the compounds of interest at the highest concentration on the standard line and monitoring the response in the IS channel. In the similar way the possibility of "cross-talk" between the analytes was monitored considering both masses and the retention times.

#### 2.7.2. Assessment of matrix effect

An assessment of matrix effect (ME) was done by comparing the peak areas of analytes in extracted samples of blank plasma spiked with the test compounds with the corresponding peak areas obtained by injection of standard solutions at appropriate concentration. Since the compounds of interest have an endogenous nature samples of blank plasma were earlier analyzed to obtain the basic levels, which were subtracted in all following calculations. Three different concentrations were evaluated (40, 400, and 1800 ng/mL) by analyzing five samples at each level. For evaluation of the relative matrix effect five different sources of rat plasma were used. The relative ME was expressed as RSD (%) and also as a comparison of precision (CV) of standard line slopes constructed in different lots versus repeated analysis in a single lot.

#### 2.7.3. Precision and accuracy

Precision of the assay was determined from the QC samples by replicate analysis of four concentration levels of compounds of interest (10, 40, 400 and 1800 ng/mL). Accuracy (percent of recovery) was evaluated, using a formula for calculating the recovery of endogenous substances in their biological matrices, as [(mean found concentration − basal concentration)/theoretical concentration] × 100. Precision was expressed as percent relative standard deviations (RSD, CV%).

Within-day and between-day precision and accuracy of the assay were assessed by performing replicate ($n = 5$) analyses at different QC levels (10, 40, 400 and 1800 ng/mL of each analyte) in plasma on the same day and further QC samples were run on 5 different days. The concentration of each sample was determined using the calibration curve prepared and analyzed on the same batch.

The criteria for acceptability of the data included accuracy within ±15% of the nominal values except for LLOQ where it should not deviate by more than 20% and a precision not exceeding 15% of the coefficient of variation (CV) except for LLOQ, where it should not exceed ±20% of CV.

The lower limit of quantification (LLOQ) based on QC samples was defined as the lowest added analyte concentration to the biological sample that can be measured with a RSD ≤ 20% and an accuracy of 100 ± 20% on a day-to-day basis and that can be discriminated significantly from the basal concentration of the analyte in rat plasma. The rLLOQ (relative lower limit of quantification) was calculated as a percentage fraction of the analyte, which upon addition to the biological sample that contains this analyte in basal concentration can be measured with accepted accuracy and precision. The value of rLLOQ was calculated by formula (F1), i.e. by dividing the value of the lower limit of quantification $C_{LLOQ}$ by the experimentally measured basal value of $C_0$ and by multiplying the result by 100.

$$\text{rLLOQ} = (C_{LLOQ} : C_0) \times 100 \quad \text{(F1)}$$

The smaller the rLLOQ value the greater the discriminatory power of the analytical method for the analyte.

#### 2.7.4. Extraction recovery

Extraction recovery was evaluated at three replicates of each QC samples (10, 40, 400 and 1800 ng/mL) in plasma. The recovery value of the IS was determined at a single concentration of 250 ng/mL. The recoveries were determined by comparing peak area obtained from plasma samples with the analytes spiked before extraction to those spiked with the appropriate standard concentration after the extraction, which represented the 100% recovery value. In both cases (analyte added after or post-extraction) the peak area corresponding to the basal concentration of the analyte was deducted before the percent value of extraction recovery was calculated.

#### 2.7.5. Stability

The stability of analytes was determined by injecting replicate preparations of processed samples for up to 48 h (in the autosampler batch at 10 °C) after the initial injection. The peak areas of the

analyte and IS obtained at initial cycle were used as the reference to determine the stability at subsequent points (12 and 48 h). Stability in the matrix during 2 h (bench-top storage) was determined at ambient temperature (25 ± 3 °C) at concentrations of QC samples. Freezer stability of analyzed compounds in rat plasma was assessed by analyzing the QC samples stored at −30 and −80 °C for 60 days. The stability was also assessed after three freeze/thaw cycles using the concentrations of QC samples. The samples were stored at −30 °C between freeze/thaw cycles then they were thawed by allowing them to stand at room temperature for approximately 30 min. The samples were then returned to the freezer for 24 h. Samples were considered to be stable when 85–115% of the initial concentrations were found.

## 3. Results and discussion

### 3.1. Selection of the analytical conditions

Electrospray ionization operated in the positive ion mode was used for the LC-ESI/MS/MS analysis to provide optimum sensitivity and selectivity. Because MNA is already positively charged due to the quaternary nitrogen in the pyridine ring the mass of the dominant ion in Q1 spectra was equal to that of the MNA. For the other analyzed compounds protonated forms of each analyte $[M+H]^+$ were the dominant ions in the Q1 spectra, and were used as the precursor ions to obtain Q3 product ion spectra. The resulting SRM transitions (precursor ion $m/z \rightarrow$ product ion $m/z$) for each analyte were as follows: 153 → 110 for M2PY, 153 → 136 for M4PY, 124 → 80 for NicA, 123 → 80 for NA, 137 → 94 for MNA and 157 → 157 for IS.

Different chromatographic conditions were investigated to optimize sensitivity, speed and peak shape. Various HPLC columns were tested and the Waters Spherisorb® 5 μm CNRP 4.6 × 150 mm analytical column gave good retention and baseline separation of the five analytes within a 15 min run time. To achieve symmetrical peak shapes, a short chromatographic analysis time, and to minimize the matrix effect, a mobile phase consisting of acetonitrile, water and formic acid was used with the gradient conditions described in Section 2.2.1. The presence of a low amount of formic acid in the mobile phase improved the sensitivity by promoting ionization of the analytes. Baseline separation of NicA and NA was needed to prevent peak area contribution from one to the other due to isotope effect, since the precursor ions for those analytes are 1 amu apart (124 versus 123 $m/z$) and both product-ion settings are at 80 $m/z$. Similar situation was with the separation of M2PY and M4PY. These two isomers have some product ions in common and therefore have isotopic interference to each other. According to Li et al. [7] the interference can be avoided by selecting the transition of $m/z$ 153 → 110 instead of 153 → 108 for M2PY as the product ion $m/z$ 110 is unique to M2PY as presented in Fig. 2. Because of the high flow rate as well as the considerable content of water in the mobile phase the LC column effluent was split via a T-piece directly in front of the ESI-probe to feed the ESI source with a reduced flow. This flow was adjusted by selecting PEEK tubings of appropriate internal diameter and length for the waste line. A reduction of the flow directed towards the ESI interface increased the instrumental sensitivity probably because of the reduction of droplet size and consequent increase of the surface area, which is favorable for ion desorption into the gas phase [8]. The representative extracted ion chromatograms (XIC) of a quality control sample at the concentration of 400 ng/mL presenting the separation of the analytes, extracted from supplemented plasma is shown in Fig. 3.

Since the analytes of interest have very different physicochemical properties, plasma protein precipitation, followed by evaporation of the supernatant and reconstitution of the dried extract proved to be the most effective. Acetonitrile was chosen as a precipitating agent. A reconstitution solvent for the dried extracts was selected that optimized recovery of all five analytes while at the same time sufficiently reduced the presence of endogenous material. The mixture of acetonitrile/water (10/90, v/v) was the best to accomplish the given task.

### 3.2. Matrix effect

Coeluting, undetected matrix components may reduce or enhance the ion intensity of the analytes and affect the reproducibility and accuracy of the assay. The majority of matrix effects occur in the solvent front of the chromatographic run therefore if the analytes can be retained to some degree chromatographically then matrix effect can be minimized [9]. In this method such parameters of gradient elution were selected that the first analyte was retained on the analytical column for at least 2 min. Gradi-

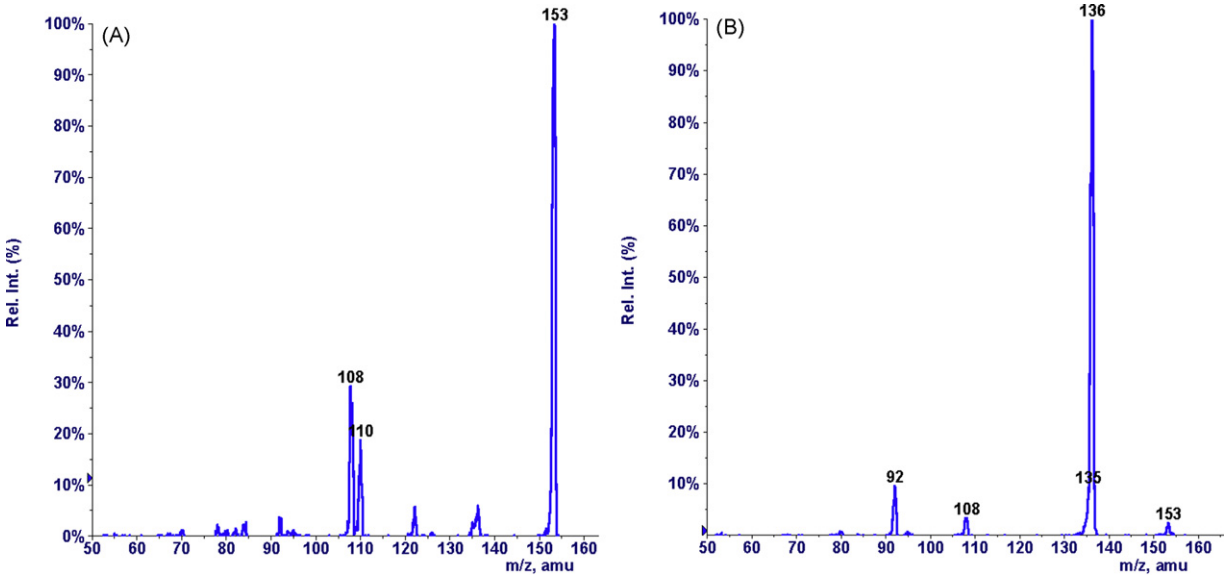


**Fig. 2.** Product-ion spectra of M2PY (A) and M4PY (B).

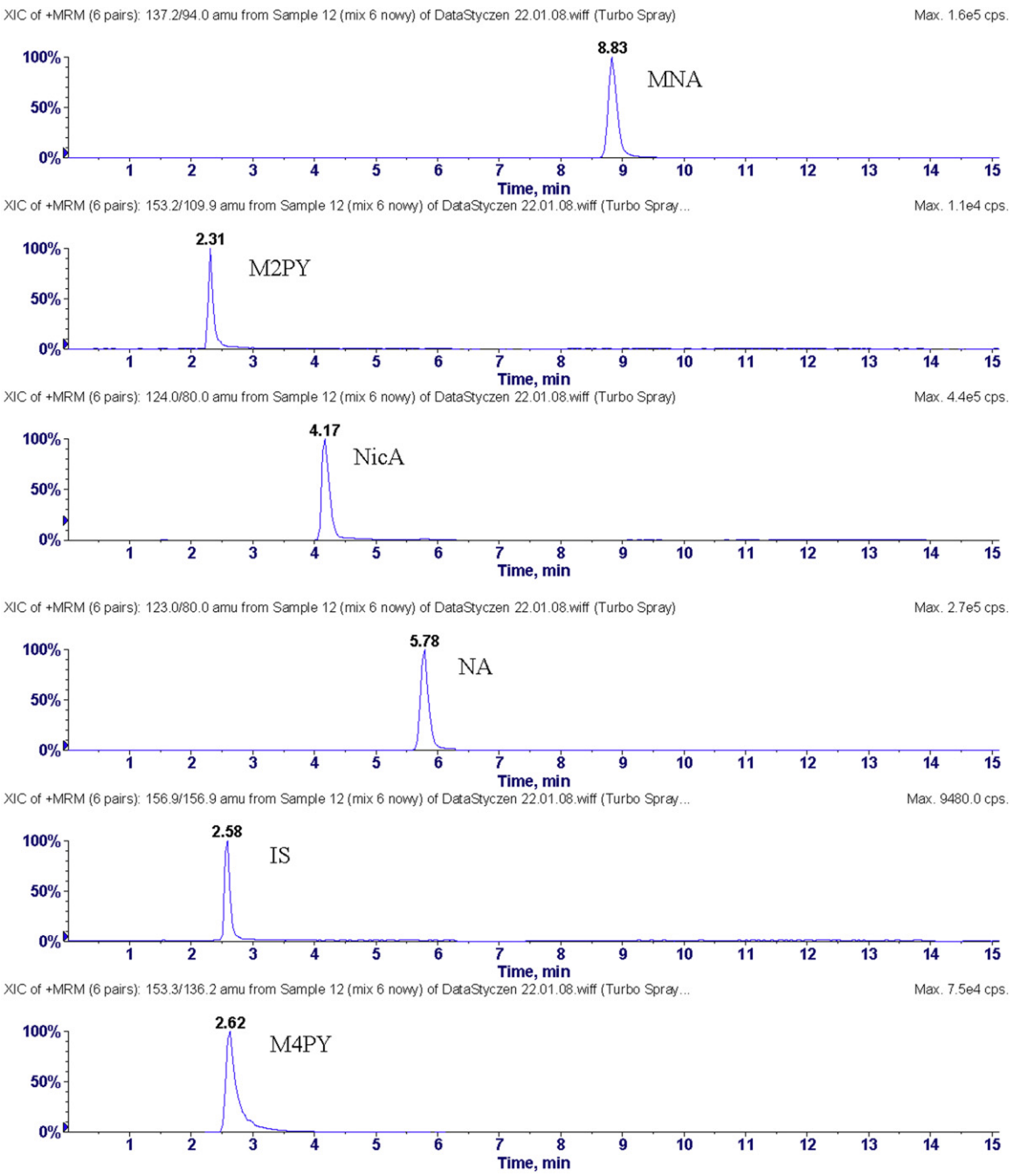


**Fig. 3.** Representative extracted ion chromatogram (XIC) of a QC sample at the concentration of 400 ng/mL.

ent elution can also help to wash the column after injection and prevent late-eluting compounds from the previous injection interfering. Both the absolute and the relative ME were assessed. The relative matrix effect (lot-to-lot) was expressed as RSD (%) and also evaluated using the standard lines slope concept as described by Matuszewski [10].

The results showed a good repeatability from the samples coming from the pooled plasma as well as those coming from different animals and CV values were below 15%. Furthermore the variability of standard line slopes in different lots of rat plasma did not exceed 5%. Therefore the method can be considered practically free from the relative matrix effect and calibration curve quantification approach could be applied. The analysis of absolute matrix effect did not show significant changes in ionization.

### 3.3. Method validation

Due to the varying basal concentrations of NicA, NA, MNA, M2PY and M4PY in rat plasma coming from different animals all calibration and quality control samples used for this method validation were prepared from the same pooled plasma to assure the same basal levels of analytes in question. Furthermore the animals were chosen with as low as possible concentrations of all analytes. The calibration curve fit was attained by subtracting the mean blank plasma response of the analyte from the calibration standards response, to generate a weighted by $1/x$ linear regression forced through zero. The resulting linear range was from 10 to 2000 ng/mL for each analyte. For each point of calibration standards, the concentrations were back calculated from the equation of the linear

**Table 2**
Back-calculated concentrations of calibrators for NicA, NA, MNA, M2PY and M4PY.

| Analyte | Nominal concentration (ng/mL) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 10 | 50 | 100 | 250 | 500 | 1000 | 2000 |
| NicA | | | | | | | |
| Mean ($n=5$) | 10.18 ± 1.08 | 52.23 ± 1.30 | 94.50 ± 11.18 | 249.01 ± 16.91 | 536.18 ± 31.57 | 1048.74 ± 88.17 | 2050.80 ± 98.70 |
| % CV | 10.67 | 2.49 | 11.83 | 6.79 | 5.89 | 8.41 | 4.81 |
| NA | | | | | | | |
| Mean ($n=5$) | 10.39 ± 1.03 | 51.64 ± 5.52 | 105.44 ± 4.67 | 244.70 ± 29.15 | 498.40 ± 34.53 | 1004.04 ± 110.70 | 2000.61 ± 150.81 |
| % CV | 9.92 | 10.69 | 4.43 | 11.89 | 6.93 | 11.02 | 7.54 |
| MNA | | | | | | | |
| Mean ($n=5$) | 8.58 ± 0.85 | 49.11 ± 4.28 | 96.05 ± 7.97 | 239.62 ± 8.35 | 515.67 ± 35.49 | 930.16 ± 44.00 | 2017.38 ± 90.8 |
| % CV | 9.88 | 8.72 | 8.3 | 3.48 | 6.88 | 4.73 | 4.5 |
| M2PY | | | | | | | |
| Mean ($n=5$) | 9.73 ± 0.68 | 50.16 ± 3.06 | 102.83 ± 6.77 | 244.86 ± 19.93 | 475.31 ± 32.56 | 1012.67 ± 59.58 | 1924.28 ± 177.8 |
| % CV | 7.04 | 6.1 | 6.58 | 8.14 | 6.85 | 5.88 | 9.24 |
| M4PY | | | | | | | |
| Mean ($n=5$) | 9.41 ± 1.17 | 48.52 ± 4.87 | 102.41 ± 12.6 | 248.02 ± 14.55 | 499.21 ± 40.3 | 1023.78 ± 34.57 | 1991.04 ± 142.1 |
| % CV | 12.38 | 10.04 | 12.34 | 5.87 | 8.07 | 3.38 | 7.146 |

regression curves and the relative standard deviations were computed. The results are presented in Table 2.

Since the components of interest are endogenous the LLOD was not established [7]. The LLOQ for analyzed plasma was 10 ng/mL and precision at this concentration did not exceed 14%. The rLLOQ calculated as proposed by Tsikas [11] was 40.34% for NicA, 33.9% for NA, 40.57% for MNA, 11.76% for M2PY and 28.1% for M4PY. The value of LLOQ depends on the basal levels of analyzed compounds in particular animal or group of animals. For different basal concentrations probable range of LLOQ can be calculated based on the rLLOQ.

To assess the accuracy and precision of the method, QC samples at four concentrations (LLOQ, low, medium and high) were processed on the same day and also on five separate days of analysis. As seen in Tables 3 and 4 values of precision ranged from 1.3% to 13.3% and those for accuracy from 94.43% to 110.88%.

The absolute recoveries ranged from 75.33% to 108.91% for all analytes across the concentrations range. The absolute recovery of internal standard at 250 ng/mL was 73.2 ± 4.1%.

To evaluate post-preparative stability the analytes concentrations in freshly prepared QC samples were determined and later compared to the measured ones for the same QC samples re-analyzed after 24 and 48 h in the autosampler at 10 °C. The original calibration results were used to determine the analytes' concentrations in the re-analyzed samples. As shown in Table 5 for the period of 48 h only the concentration of MNA stayed within the range of ±8% of the nominal one, similar results were obtained for 24 h. The second study performed for the period of 12 h shows that all the analytes were stable for that period of time, therefore no greater batches than 48 samples analyzed at one run are recommended. Short-term stability was assessed for samples kept at room temperature for 2 h. The predicted concentrations for analyzed compounds at QC samples in some cases deviated within almost 30% of the nominal concentrations, hence the sample preparation time should be as short as possible and preferably performed on ice. The results of QC samples following three repeated freeze/thaw cycles also have shown that analyzed compounds were unstable under that condition. To avoid adverse changes in concentrations fresh samples which are to be analyzed later can be packed in the volume required for analysis in separate vials, this way they will be thawed only once. All analyzed compounds were stable in plasma at −80 °C for 60 days.

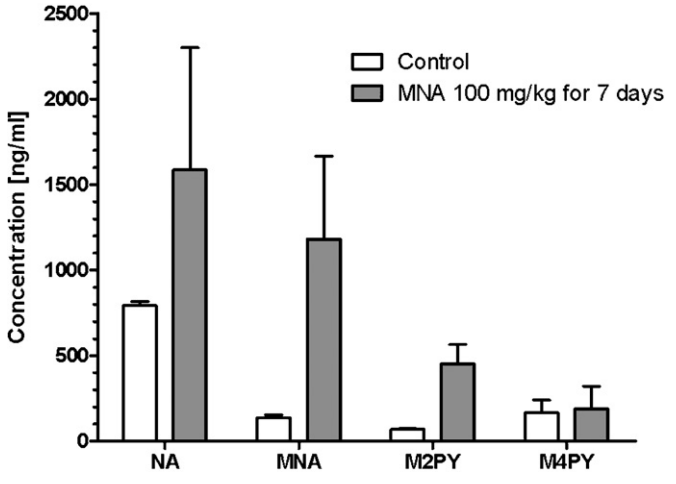


**Fig. 4.** NA, MNA, M2PY and M4PY concentrations in plasma of rats given MNA 100 mg/kg in drinking water for 7 days ($n=4$) as compared to control rats.

## 4. Application of the method

This method was successfully applied to measure basal concentration of MNA and its metabolites in rat plasma, and to

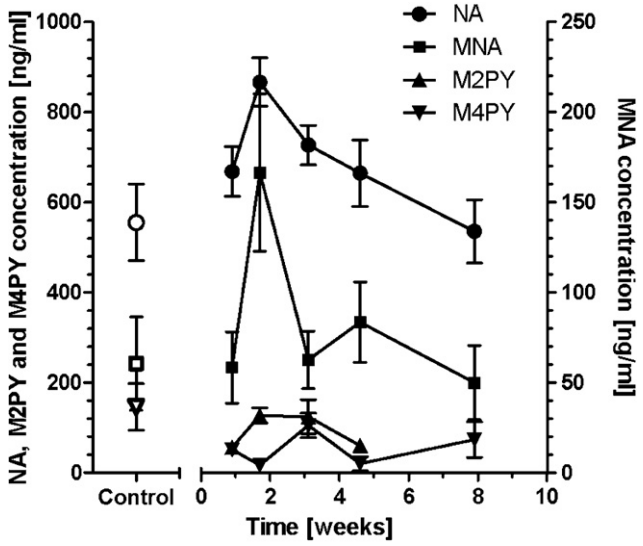


**Fig. 5.** NA, MNA, M2PY and M4PY concentrations in plasma of rats with hypertriglicerydemia induced by high-fructose diet. To induce hypertriglicerydemia rats were given AIN93G diet containing 60% of fructose for 1, 2, 3, 4 or 8 weeks as described previously [12]. Control group obtained standard AIN93G diet for 8 weeks ($n=4$–5)

**Table 3**
Intra-day accuracy (% of nominal concentration) and precision (%RSD) of NicA, NA, MNA, M2PY and M4PY in rat plasma.

| QC i.d. | QC nominal concentration (ng/mL) | Analyte NicA | | Analyte NA | | Analyte MNA | | Analyte M2PY | | Analyte M4PY | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean% accuracy | %RSD | Mean% accuracy | %RSD | Mean% accuracy | %RSD | Mean% accuracy | %RSD | Mean% accuracy | %RSD |
| LLOQ | 10 | 108.35 | 9.79 | 95.88 | 13.3 | 97.55 | 10.18 | 101.07 | 11.99 | 110.88 | 9.31 |
| Low | 40 | 94.43 | 7.53 | 99.08 | 9.7 | 93.21 | 3.41 | 105.85 | 8.4 | 103.52 | 6.42 |
| Medium | 400 | 100.1 | 4.37 | 100.63 | 3.35 | 105.52 | 2.29 | 98.17 | 6.5 | 98.73 | 3.67 |
| High | 1800 | 95.6 | 4.57 | 100.00 | 7.21 | 109.82 | 3.13 | 100.08 | 1.3 | 99.77 | 5.92 |

**Table 4**
Inter-day accuracy (% of nominal concentration) and precision (%RSD) of NicA, NA, MNA, M2PY and M4PY in rat plasma.

| QC i.d. | QC nominal concentration (ng/mL) | Analyte NicA | | Analyte NA | | Analyte MNA | | Analyte M2PY | | Analyte M4PY | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean% accuracy | %RSD | Mean% accuracy | %RSD | Mean% accuracy | %RSD | Mean% accuracy | %RSD | Mean% accuracy | %RSD |
| LLOQ | 10 | 103.98 | 8.13 | 100.62 | 11.21 | 98.03 | 9.83 | 100.07 | 7.96 | 109.55 | 9.42 |
| Low | 40 | 98.25 | 9.52 | 100.47 | 9.00 | 95.82 | 3.54 | 103.30 | 7.90 | 105.35 | 6.00 |
| Medium | 400 | 100.43 | 4.42 | 97.98 | 4.34 | 101.42 | 5.27 | 97.97 | 5.33 | 99.85 | 4.11 |
| High | 1800 | 95.93 | 3.03 | 97.43 | 6.71 | 102.07 | 7.58 | 104.52 | 6.21 | 102.41 | 6.75 |

**Table 5**
Post-preparative stability (12 and 48 h) of NicA, NA, MNA, M2PY and M4PY.

| Analyte | QC nominal concentration (ng/mL) | Measured concentration within run ± SD | | %Change | |
|---|---|---|---|---|---|
| | | After 12 h in 10 °C | After 48 h in 10 °C | After 12 h in 10 °C | After 48 h in 10 °C |
| NicA | 10 | 10.07 ± 0.57 | n/a | +0.07 | n/a |
| | 40 | 40.83 ± 4.13 | 9.02 ± 0.61 | +2.07 | −77.44 |
| | 400 | 403.04 ± 19.55 | 136.04 ± 9.56 | +0.76 | −66.00 |
| | 1800 | 1731.07 ± 36.04 | 639.54 ± 60.18 | −3.83 | −64.47 |
| NA | 10 | 10.54 ± 0.86 | n/a | +0.54 | n/a |
| | 40 | 40.75 ± 3.64 | 19.37 ± 1.96 | +1.87 | -51.57 |
| | 400 | 381.32 ± 13.58 | 249.52 ± 16.14 | -4.67 | -37.62 |
| | 1800 | 1707.37 ± 98.75 | 1181.16 ± 81.26 | -5.14 | -34.38 |
| MNA | 10 | 9.86 ± 1.07 | 9.24 ± 1.43 | -1.40 | -7.57 |
| | 40 | 38.33 ± 1.33 | 41.80 ± 1.96 | -4.17 | +4.49 |
| | 400 | 389.27 ± 16.31 | 388.80 ± 23.25 | -2.68 | -2.8 |
| | 1800 | 1732.66 ± 46.49 | 1832.76 ± 57.91 | -3.74 | +1.82 |
| M2PY | 10 | 9.91 ± 0.30 | n/a | -0.01 | n/a |
| | 40 | 40.30 ± 2.96 | 18.54 ± 1.8 | +0.75 | −53.66 |
| | 400 | 390.89 ± 16.64 | 223.08 ± 32.28 | −2.27 | −44.23 |
| | 1800 | 1961.14 ± 119.48 | 921.24 ± 41.18 | +8.89 | −48.82 |
| M4PY | 10 | 10.76 ± 1.15 | n/a | +0.76 | n/a |
| | 40 | 42.14 ± 2.61 | 33.64 ± 4.01 | +5.35 | −15.89 |
| | 400 | 403.91 ± 18.25 | 301.12 ± 13.61 | +0.97 | −24.72 |
| | 1800 | 1890.89 ± 137.05 | 1184.22 ± 29.25 | +5.05 | −34.21 |

detect changes in plasma concentration of MNA and its metabolites in MNA-treated rats. As shown in Fig. 4 basal levels of MNA, M2PY and M4PY were 135.49 ± 31.7, 69.7 ± 9.7 and 233.27 ± 85.11 ng/mL respectively. We also detected that treatment with MNA (100 mg/kg) given in drinking water resulted in nearly 10-fold elevation of plasma MNA concentration and concomitant increase in the concentration of major MNA metabolite such as M2PY. Interestingly M4PY did not increase significantly, suggesting preferable synthesis of M2PY from MNA in rats exposed to exogenous MNA.

Moreover we analyzed changes in the concentration of endogenous MNA along with the development of hypertriglyceridemia induced by fructose-high diet (60%) [12]. As shown in Fig. 5, there was a significant increase in NA and MNA concentration in plasma in the early phase of the development of hypertriglyceridemia within first 2 weeks upon the introduction of the high-fructose diet, that faded away afterwards. Pharmacological activity of MNA includes anti-thrombotic effect and triglycerides lowering effect that was described previously [2,12]. Accordingly early activation of NA–MNA pathways by hypertriglicerydemia detected in the present work for the first time may have a compensatory role to limit the detrimental effect of high-fructose diet on the development of hypertriglicerydemia and endothelial dysfunction.

In previous reports various absolute levels of endogenous MNA in plasma were reported in humans (from 6 to 116 ng/mL) and there is limited number of reports for measurements in rat plasma to compare with [13,14]. Still, our data seem compatible with previous work and basal plasma concentration of MNA stays in low micromolar range. However, further studies are warranted to understand better nutritional, functional and genetic factors that determine the basal plasma concentration of MNA and its metabolites and their changes in various physiological and pathological conditions. This seems worthwhile taking into the consideration the novel method developed in the present work and recently described biological activity of MNA [1,2,3,11].

## 5. Conclusion

In this manuscript, we described a sensitive and selective high performance liquid chromatography–tandem mass spectrometry method for the simultaneous determination of nicotinic acid and

its metabolites NA, MNA, M2PY and M4PY in rat plasma. Validation of the method in specific conditions, taking into account the endogenous nature of all analyzed compounds, shows that it is selective and precise with linear response of mass spectrometer for all analytes. The simple sample preparation procedure produced clean chromatograms and sufficient and reproducible recovery.

The method has been successfully employed to support various applications. It proved useful for detection of the basal plasma levels of NA and its metabolites MNA, M2PY and M4PY in rats and changes in their concentration in MNA-treated rats. Furthermore, using this method we were able to uncover changes in the activity of endogenous NA–MNA pathways in response to the fructose-induced hypertriglicerydemia.

## References

[1] J. Gebicki, A. Sysa-Jedrzejowska, J. Adamus, A. Wozniacka, M. Rybak, J. Zielonka, Pol. J. Pharmacol. 55 (2003) 109.
[2] S. Chlopicki, J. Swies, A. Mogielnicki, W. Buczko, M. Bartus, M. Lomnicka, J. Adamus, J. Gebicki, Br. J. Pharmacol. 152 (2007) 230.
[3] K. Bryniarski, R. Biedron, A. Jakubowski, S. Chlopicki, J. Marcinkiewicz, Eur. J. Pharmacol. 578 (2008) 332.
[4] P. Catz, W. Shinn, I.M. Kapetanovic, H. Kim, M. Kim, E.L. Jacobson, M.K. Jacobson, C.E. Green, J. Chromatogr. B 829 (2005) 123.
[5] K. Yamada, N. Hara, T. Shibata, H. Osago, M. Tsuchiya, Anal. Biochem. 352 (2006) 282.
[6] E.M. Slominska, P. Adamski, M. Lipinski, J. Swierczynski, R.T. Smolenski, Nucleosides Nucleotides Nucleic Acids 25 (2006) 1245.
[7] A.C. Li, Y.L. Chen, H. Junga, W.Z. Shou, X. Jiang, W. Naidong, Chromatographia 58 (2003) 723.
[8] A. Kloepfer, J.B. Quintana, T. Reemtsma, J Chromatogr. A 1067 (2005) 153.
[9] P.J. Taylor, Clin. Biochem. 38 (2005) 328.
[10] B.K. Matuszewski, J. Chromatogr. B 830 (2006) 293.
[11] D. Tsikas, J. Chromatogr. B 877 (2009) 2244.
[12] M. Bartus, M. Lomnicka, R.B. Kostogrys, P. Kazmierczak, C. Watala, E.M. Slominska, R.T. Smolenski, P.M. Pisulewski, J. Adamus, J. Gebicki, S. Chlopicki, Pharmacol. Rep. 60 (2008) 127.
[13] S. Ringeissen, S.C. Connor, H.R. Brown, B.C. Sweatman, M.P. Hodson, S.P. Kenny, R.I. Haworth, P. McGill, M.A. Price, M.C. Aylott, D.J. Nunez, J.N. Haselden, C.J. Waterfield, Biomarkers 8 (2003) 240.
[14] A. Somogyi, D. Siebert, F. Bochner, Anal. Biochem. 187 (1990) 160.