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Simultaneous determination of niacin, niacinamide and nicotinuric acid in human plasma

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Abstract

A sensitive, specific, accurate, and reproducible HPLC/MS-method for the simultaneous quantitative determination of niacin (NA) and its main metabolites niacinamide (NAM) and nicotinuric acid (NUR) in human plasma using chinolin-3-carboxylic acid as an internal standard was developed and validated according to international guidelines for method validation.

All analytes and the internal standard were separated from acidified plasma by solid phase extraction. Afterwards the extracted samples were analyzed by HPLC/MS in the positive electrospray ionization mode (ESI) and selected ion monitoring (SIM). The total run time was 7 min between injections. The assay had a lower limit of quantification of 50.0 ng/mL for each analyte using 1 mL of plasma. The calibration curves were linear in the measured range between 50.0 and 750 ng/mL plasma. The overall precision and accuracy for all concentrations of quality controls and standards was better than 15%. No indications were found for possible instabilities of niacin, niacinamide and nicotinuric acid in plasma at −20 °C, in the extraction solvent or after repeated thawing/freezing cycles. In stabilities were observed in whole blood and in plasma at room temperature. The recovery of the extraction method ranged from 86 to 89% for the three analytes.

Keywords: Niacin; Niacinamide; Nicotinuric acid; Human plasma; Quantitative determination; LC/MS

1. Introduction

Niacin (nicotinic acid, 3-pyridine-carboxylic acid) (NA) is a water soluble vitamin and belongs to the vitamin B complex. Its amide derivative niacinamide (nicotinamide) (NAM) is a component of NAD (nicotinamide adenine dinucleotide), an essential coenzyme for many cellular oxidation–reduction reactions [1].

Niacin in pharmacological doses (1–6 g/day) represents an important therapeutic option for the treatment of hyperlipidemia [2]. It is the only agent currently available that favorably alters all major lipid subfractions. Niacin is able to significantly reduce levels of total cholesterol, low density lipoprotein (LDL), triglycerides and lipoprotein (a), while it increases high density lipoprotein (HDL) [3]. Alone or in combination, it promotes regression of coronary artery diseases, decreases coronary events, stroke and total mortality [4]. When administered orally NA is either metabolized to nicotinuric acid (NUR) by glycine conjugation or to NAM, which is utilized in NAD synthesis [1].

For the determination of NA and its metabolites in human plasma a gas chromatographic [5] and many HPLC methods using either UV or flourimetric detection have been published [6–16]. By UV absorbance NA could be detected down to 0.100 μg/mL [14]. Only by using flourimetric detection after time-consuming derivatization procedures could lower concentrations of NA and NAM be determined [9,16]. However,

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many of these assays are complicated by the use of tedious and labour intensive extraction procedures, since NA and its metabolites differ in their physico-chemical properties as well as in their polarity, which make their simultaneous analysis very difficult. Most methods use deproteinization with acetone or acetone–water [9,14], acetonitrile [12] or perchloric acid [10]. In some methods deproteinization with acetone is followed by extraction with chloroform [6,9] in order to remove the endogenous lipophilic components still present in the aquous layer containing NA. Takikawa et al. pointed out that the aqueous layer obtained by these time-consuming extraction procedures was still not sufficiently cleaned up for the HPLC analysis [8]. Solid-phase extraction procedures (SPE) are also used to extract NA and its metabolites from biological matrices [7,13,15]. But SPE cannot be applied to all metabolites of NA because of their different pK_as and hydrophobic properties [1].

Recently capillary electrophoresis (CE) has been revealed to be a powerful technique for the simultaneous analysis of NA and its metabolites in biological fluids without tedious clean-up procedures [14,17]. However, the detection limit was found to be 1.00 μg/mL.

These days high performance liquid chromatography (HPLC) together with mass spectrometry (MS) has revolutionized the field of bioanalysis. It is becoming increasingly the method of choice for the determination of biomolecules, drugs and their metabolites in biological fluids. In view of this trend the aim of this investigation was to develop and validate a sensitive and selective HPLC/MS method, with appropriate extraction and runtimes, allowing the simultaneous analysis of trace amounts of NA as well as its main metabolites in human plasma.

2. Experimental

2.1. Chemicals and reagents

Niacin (Lot No.069H0741), niacinamide (Lot No.128H1168) as well as nicotinuric acid (Lot No.068F0329) were purchased from Sigma (Deisenhofen, Germany) and chinolin-3-carboxylic acid from Fluka (Buchs, Switzerland). All solvents used were of analytical grade or better quality. Methanol, acetonitrile, *n*-hexane as well as water were obtained from Merck (Darmstadt, Germany). Ammonia solution was purchased from J.T. Baker (Gross Gerau, Germany) and formic acid from Fluka. Isolute SCX SPE-colums (500 mg, 3 mL) from Separtis GmbH (Grenzach-Wyhlen, Germany) were used for solid phase extraction (Fig. 1).

2.2. Stock solutions

Concentrated stock solutions of NA, NAM and NUR were prepared by dissolving 10.0 mg of each in 100 mL of methanol to give 100 ng/μL stock solutions (SS1). This procedure was repeated for each analyte to obtain a second set of stock solutions (SS2).

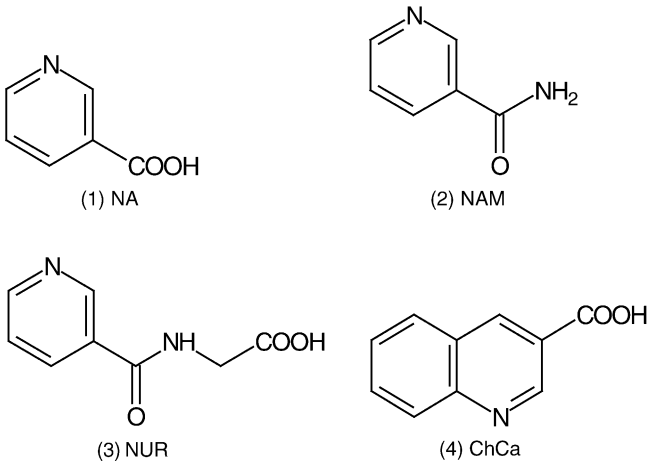

Fig. 1. Structures of: (1) niacin (NA), (2) niacinamide (NAM), (3) nicotinuric acid (NUR) and (4) chinoline-3-carboxylic acid (ChCa) as internal standard.

For the preparation of the internal standard stock solution, 10.0 mg of chinoline-3 carboxylic acid were dissolved in 100 mL methanol to give a 100 ng/mL stock solution.

2.3. Preparation of calibration standards, quality control samples and internal standard working solution

Different working solutions containing NA, NAM and NUR were obtained by diluting the stock solutions SS1 with methanol. Calibration standards were prepared daily by spiking 1 mL of blank plasma with 25 μL of the appropriate working solution resulting in concentrations of 50.0, 75.0, 125, 150, 250, 500 and 750 ng of NA, NAM and NUR per mL plasma.

Three different concentrations of quality control samples (100, 300 and 600 ng per mL of plasma) were prepared by spiking 1 mL aliquots of blank plasma with 25 μL of spiking solutions freshly diluted from the stock solutions SS2.

A 10.0 ng/μL working solution of the internal standard was prepared by diluting the stock solution with methanol. Plasma samples were spiked with 25 μL of this working solution yielding a concentration of 250 ng internal standard per mL plasma.

All solutions were stored in a refrigerator at $5 \pm 3\,^{\circ}C$.

2.4. Extraction procedure

One millilitre aliquots of blank plasma were spiked with 250 ng of internal standard as well as NA, NAM and NUR in the concentrations mentioned above. After addition of 1 mL of 1% formic acid, the samples were extracted using Isolute SCX SPE-columns, which were pre-conditioned with 2 mL of methanol and 1 mL of 1% formic acid. Then the plasma samples were added to the columns. After washing with 2 mL of acetonitrile, 2 mL of methanol and 2 mL of hexane the columns were aspirated to dryness. Finally, the samples were

eluted twice with 2 mL of methanol, containing 2% (v/v) ammonia solution, into a glass tube.

The organic phase was then evaporated to dryness in a gentle stream of nitrogen at 45 °C and the residue was reconstituted in 30 μL water.

Finally a volume of about 3 μL of the extract was subjected to HPLC/MS.

2.5. Measurement

HPLC was performed out using an equipment of Agilent Technologies (Palo Alto, USA) consisting of an Agilent 1100 HPLC pump and an Agilent 1100 thermostatted autosampler. A Grom Hypersil CPS column 5 μm (250 × 2 mm) (Grom Analytik und HPLC GmbH, Rottenburg-Hailfingen, Germany) was used for liquid separation of the analytes at a flow rate of 0.2 mL/min. The optimal composition of the mobile phase was determined to be: acetonitrile: methanol: water: formic acid (700/190/110/1, v/v/v/v).

The MS analysis was performed in the positive electrospray ionization mode on a Finnigan LCQ (ThermoFinnigan, San Jose, USA) equipped with an ion trap operating in the selected ion monitoring (SIM) detection mode.

Quantitation of NA, NAM, NUR and chinolin-3-carboxylic acid was achieved by monitoring the ions at *m/z* 124.3, 123.3, 181.0 and 174.3 respectively. Ion sampling was performed in one time segment with four scan events. Data acquisition and integration of the peak areas were conducted using the standard Finnigan selected ion recording software "Navigator".

A typical chromatogram obtained by the described method can bee seen in Fig. 2.

2.6. Evaluation

Analyte concentrations were evaluated using the internal standard method. The standard curves $y = a + bx$ (a = intercept, b = slope) were calculated from the peak area ratios of analyte/internal standard and the nominal analyte concentrations using linear regression with $1/x^2$ weighting.

For calculating accuracy (expressed as bias) and precision (expressed as coefficient of variation CV) the following formulae were used:

$$\text{bias}\,(\%) = 100 \times \frac{\text{concentration found} - \text{concentration added}}{\text{concentration added}}$$

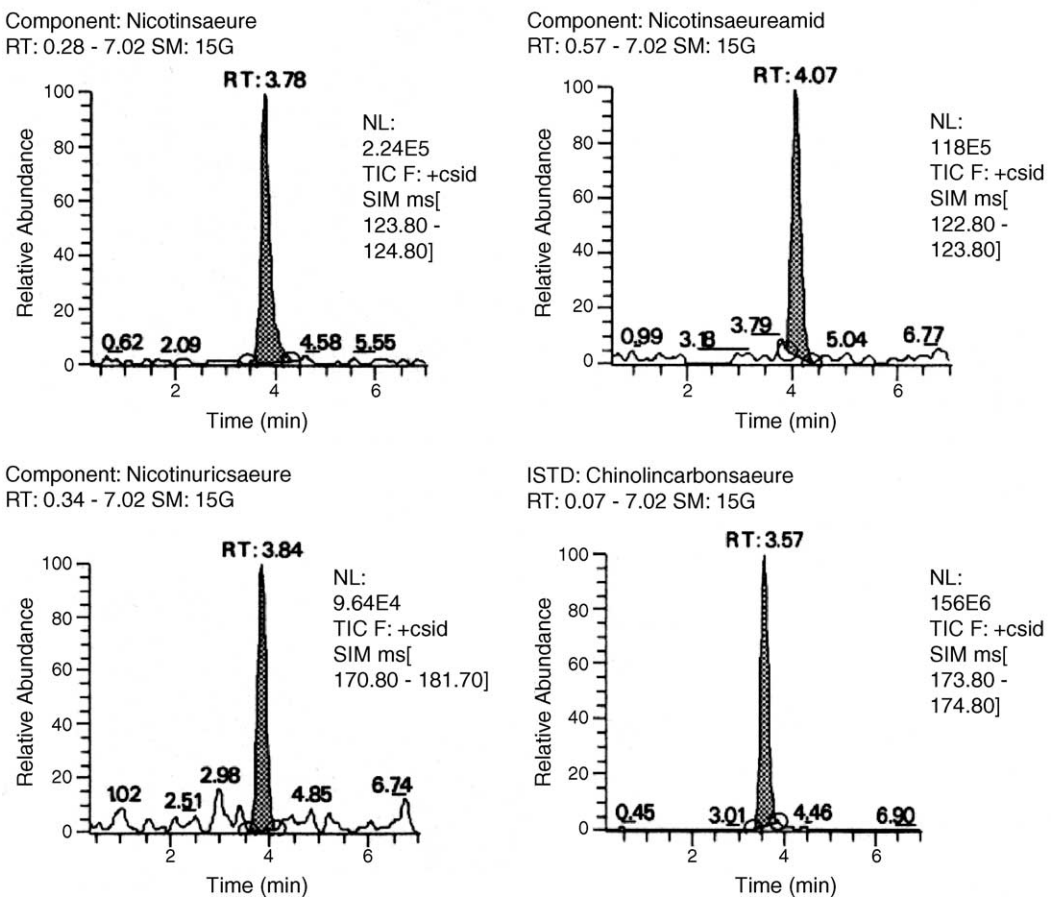

Fig. 2. SIM-chromatogram of a calibration standard 75 ng/mL of NA (r_t = 3.78 min), NAM (r_t = 4.07 min), NUR (r_t = 3.84 min) and chinoline-3-carboxylic acid (internal standard r_t = 3.57 min).

$$\mathrm{CV}\,(\%) = 100 \times \frac{\text{standard deviation}}{\text{mean}}$$

2.7. Validation

2.7.1. Selectivity

Selectivity was confirmed by injection of the extracts of six blank plasma samples from different indviduals, which neither contained NA, NAM, NUR nor the internal standard. For blood donors no limitations were made in regard to lifestyle habits such as smoking.

Additionally, six blank plasma samples were measured after spiking them with the internal standard only.

2.7.2. Blood withdrawal systems

Different blood withdrawal systems and two sorts of plasma storage tubes were tested for possible interference peaks or adsorption phenomena. The tested systems were:

Glass tube		VWR (Munich)
Polypropylene tube		Sarstedt
Vacutainer	Li–Heparin	Becton–Dickinson
Vacutainer	K–EDTA	Becton–Dickinson
Vacutainer	Na–Heparin	Becton–Dickinson
Monovette	K–EDTA	Sarstedt

Four quality control samples of the same concentration were pipetted into each of the six different tubes. The samples were shaken for 20 min and then pipetted into the glass-extraction tubes. These samples were then extracted, measured and calculated with a freshly prepared standard curve.

2.7.3. Inter-day precision and accuracy

The inter-day precision and accuracy was verified by measuring different standard curves ($n = 6$) including blank and zero standard at different days. Two sets of quality control samples ($n = 12$) were analyzed with each of the standard curves.

2.7.4. Intra-day precision and accuracy

The intra day data (repeatability) reflects the precision and accuracy of the method under the same conditions within one day. For repeatability, five different quality control sample sets were analyzed together with one standard curve in one analytical run.

2.7.5. Linearity–overall precision and accuracy

Linearity was confirmed using 10 standard curves and 20 sets of quality control samples.

To check the appropriate weighting model, standard curves were calculated without weighting as well as with $1/x$ and $1/x^2$ weighting.

2.7.6. Sensitivity/lower limit of quantitation

For validation of the LLOQ, six different lots of plasma were spiked with the analytes at the lowest concentration level (50.0 ng/mL) and recalculated with a freshly prepared standard curve.

2.7.7. Stability tests

All stability tests were examined by extracting and measuring four sets of quality control samples (each set consisting of three concentration levels: 100, 300 and 600 ng/mL for each of the three analytes). After the incubation period, the samples were processed and analyzed with a freshly prepared standard curve and compared with their nominal values. According to the international acceptance criteria [18] the measured samples may be assumed stable if the statistical data (CV for precision and bias for accuracy) are better than 15%.

The stability in plasma was determined after storage at room temperature for 12 and 24 h, after three freeze and thaw cycles and after storage at $-20 \pm 5\,^{\circ}\mathrm{C}$ for 64 days

Furthermore the stability of the three analytes was assessed in processed samples after storage at room temperature, after storage in the refrigerator at $5 \pm 3\,^{\circ}\mathrm{C}$ and after storage in the autosampler for 72 h respectively.

To test the stability of NA, NAM and NUR during sample collection and processing, whole blood was spiked with 300 ng/mL of each analyte. The whole blood was aliquoted and stored at room temperature for 0, 30 and 60 min. After processing the aliquots into plasma by centrifugation they were, extracted and measured as mentioned above.

2.7.8. Dilution of out-of-range specimen

Two out-of-range QC pools were prepared with a concentration of 1125 and 2250 ng NA, NAM and NUR per mL plasma. Four samples from each pool were diluted with blank plasma by a factor of two (DF = 2) and four (DF = 4) to give 562.5 ng NA, NAM and NUR per mL plasma.

All these samples were analyzed and calculated with a freshly prepared, undiluted standard curve.

This procedure is necessary to show that samples with concentration above the upper limit of quantitation (ULQ) can be diluted in a linear way.

2.7.9. Recovery

The recovery of the extraction was examined by comparing two sets of spiked plasma samples:

a) each four blank plasma samples were spiked with 100, 300 and 600 ng of NA, NAM and NUR. All 12 samples were spiked additionally with the internal standard and extracted as usual.
b) 12 blank plasma samples were spiked with the internal standard and extracted as usual. Each four extracted samples were spiked with 100, 300 and 600 ng of NA, NAM and NUR respectively. The mesasured peak area ratios

Table 1
Overall precision and accuracy data-standards ($n = 10$)

Nominal concentration (ng/mL)	Measured concentration (niacin)			Measured concentration (niacinamide)			Measured concentration (nicotinuric acid)		
	Mean (ng/mL)	CV (%)	Bias (%)	Mean (ng/mL)	CV (%)	Bias (%)	Mean (ng/mL)	CV (%)	Bias (%)
50.0	49.7	7.1	−0.7	51.2	3.9	2.4	49.8	5.4	−0.4
75.0	73.2	10.7	−2.4	70.9	6.7	−5.5	77.6	7.1	3.5
125	132	6.8	5.6	126	10.0	0.5	122	8.7	−2.4
150	152	6.5	1.5	150	5.7	0.3	145	9.7	−3.5
250	246	4.4	−1.6	251	6.6	0.6	251	4.9	0.5
500	504	7.8	0.8	505	6.6	1.0	517	7.2	3.4
750	727	5.8	−3.1	747	7.6	−0.4	745	3.9	−0.6

of these samples were taken as a 100% reference for the three analytes

The recovery of the extracted samples from a) were then calculated using the following equation:

$$\text{recovery}(\%) = \frac{\bar{x}_{\text{extr.}}}{\bar{x}_{\text{ref.}}} \times 100$$

2.8. Results

All results were compared to international acceptance criteria [18].

For calculation of precision and accuracy the evaluation shown in Section 2.6 was applied.

2.8.1. Selectivity

Selectivity could be confirmed since no significant interference peaks could be observed at the retention times of the three analytes in blank samples as well as in the samples spiked only with the internal standard.

2.8.2. Blood withdrawal systems

The only withdrawal system found to be suitable was the Vacutainer Li–Heparin system. All other systems were unsuitable as blood withdrawal systems because significant changes in concentration were observed.

For storage only glass tubes should be used because polypropylene is not suitable because significant changes in the concentration of Nicotinamide were observed.

2.8.3. Inter-day precision and accuracy

The international acceptance criteria (CV for precision and bias for accuracy better than 15%) were met [18] (Tables 1 and 2).

2.8.4. Intra-day precision and accuracy

The statistical data of this experiment fulfill at the international acceptance criteria (CV better than 15% for precision and bias better than 15% for accuracy) for intra-day experiments [18].

2.8.5. Linearity–overall precision and accuracy

Calculations without weighting could not be used due to unacceptable relative deviations in the lower concentration range. The comparison of the two weighting models, $1/x$ and $1/x^2$ revealed, that the use of $1/x^2$ weighting gave better results for accuracy and precision in the low concentration range.

By using the recommended $1/x^2$ model values for r^2 were obtained which indicate linearity over the whole calibration range for NA, NAM and NUR. In addition the overall regression parameters, mean value for $r^2 > 0.98$ and CV for the slope of 11.5% are a further indication for the suitability of this analytical method. The statistical data for the standards and quality control samples are summarized in Tables 1 and 2.

2.8.6. Sensitivity/lower limit of quantitation

All the values for accuracy (bias <7.5%) and precision (CV < 8.0%) of the method at LLOQ of 50.0 ng/mL for all three analytes are acceptable and fulfill the requirements of bioanalytical studies.

2.8.7. Stability tests

All three analytes were stable in plasma after storage frozen freezing at $-20^{\circ} \pm 5\,^{\circ}\text{C}$ for 64 days and after three thawing/freezing cycles. During the stability test of samples containing the three analytes in plasma at room temperature the data obtained for NUR and NAM were acceptable, but NA showed a possible instability at the lowest QC-range (bias = −27.7%) after 24 h. Until further investigations are performed all samples should be kept frozen until extraction.

Table 2
Overall precision and accuracy data-quality control samples ($n = 20$)

Nominal concentration (ng/mL)	Measured concentration (niacin)			Measured concentration (niacinamide)			Measured concentration (nicotinuric acid)		
	Mean (ng/mL)	CV (%)	Bias (%)	Mean (ng/mL)	CV (%)	Bias (%)	Mean (ng/mL)	CV (%)	Bias (%)
100	101	7.4	0.5	98.8	10.6	−1.2	100	10.3	0.0
300	303	9.6	1.0	289	7.8	−3.7	312	6.6	4.0
600	614	9.8	2.3	603	10.3	0.5	634	7.4	5.7

Table 3
stability data during sample collection

Stability test	Nominal conccentration (ng/mL)	Measured concentration (niacin)			Measured concentration (niacinamide)			Measured concentration (nicotinuric acid)		
		Mean (ng/mL)	CV (%)	Bias (%)	Mean (ng/mL)	CV (%)	Bias (%)	Mean (ng/mL)	CV (%)	Bias (%)
Whole blood $t = 0$ min	300	204	23.3	−32.0	209	12.4	−30.2	359	21.4	19.8
Whole blood $t = 30$ min	300	132	25.2	−56.1	276	10.1	−8.2	476	8.1	58.8
Whole blood $t = 60$ min	300	121	10.4	−59.7	333	10.6	11.1	503	16.0	67.7

All analytes were stable in the extraction solvent on storage at room temperature and in the autosampler as well as in the refrigerator at 5° ± 3° C after 72 h storage.

Problems were observed during the process of sample collection. The results presented in Table 3 show significant changes in the concentrations of NA, NUR and NAM, so that it can be assumed that these compounds are not stable in human whole blood at the tested conditions. To avoid rapid and significant changes in concentrations of NA, NUR and NAM during sample collection it is advisable to cool down and centrifuge the whole blood sample as soon as possible after the blood withdrawal and to freeze the plasma at −20 °C immediately after preparation.

A detailed summary of all stability tests is given in Table 4.

2.8.8. Dilution of out-of-range specimen

The recalculated concentrations of the diluted samples fit their original nominal concentrations (cv better than 8.5%, bias better than 6.5%), which shows the ability to dilute samples up to a dilution factor of four in a linear fashion.

2.8.9. Recovery

The extraction recoveries determined were found to be between 86.4 and 89.1%. As can be seen in Table 5 the precision and accuracy of the recovery at each concentration level was better than ±15%. Due to this good recovery it was not necessary to improve the extraction procedure described above.

2.8.10. Measurement of human plasma samples

Human plasma samples obtained from a clinical pharmacokinetic study, using 1000 mg extended release tablets were measured with the method described in this paper. The validated concentration ranges and the selectivity was sufficient to determine the analytes and to obtain resolved results. *C*, *t*-profiles of the concentrations of the three analytes can be seen in Fig. 3. As expected the concentration levels of NA were mostly below, the LLOQ of 50.0 ng/mL caused by its quick metabolism in blood. NAM was detected with C_{max} concentrations about 500 ng/mL about 5 h after dosing. NUR showed the highest concentrations by 2 h post dose. The C_{max} was found at concentrations up to 1500 ng/mL, so these sam-

Table 4
Summary of the validation results of the simultaneous determination of NA, NAM and NUR in human plasma

Validated parameter	Results (niacin)	Results (niacinamide)	Results (nicotinuric acid)
Calibration range	50.0–750 ng/mL	50.0–750 ng/mL	50.0–750 ng/mL
LOQ	50.0 ng/mL	50.0 ng/mL	50.0 ng/mL
r^2 (Overall mean)	0.98586	0.98828	0.98770
Inter-day precision[a]	CV = 7.0%	CV = 10.9%	CV = 10.3%
Inter-day accuracy[a]	Bias = −2.9%	Bias = −1.7%	Bias = −1.5%
Intra-day precision[a]	CV = 10.1%	CV = 5.8%	CV = 14.4%
Intra-day accuracy[a]	Bias = −2.2%	Bias = −1.3%	Bias = −0.5%
Stability of samples after three thawing/freezing cycles	No problems observed	No problems observed	No problems observed
Stability of extracted samples at room temperature	At least 72 h	At least 72 h	At least 72 h
Stability of extracted samples in the refrigerator (5 °C ± 3 °C)	At least 72 h	At least 72 h	At least 72 h
Stability in fresh whole blood	No	No	No
Stability in matrix at room temperature	At least 12 h	At least 24 h	At least 24 h
Stability in matrix at −20 °C ± 5 °C	At least 64 days	At least 64 days	At least 64 days
Stability of extracated samples in autosampler at room temperature	At least 72 h	At least 72 h	At least 72 h
Stability in blood withdrawal	Only vacutainer Li–Hep is suitable	Only vacutainer Li–Hep is suitable	Only vacutainer Li–Hep is suitable
Matrix storage tubes	No problems observed	Polypropylen not suitable	No problems observed
Recovery	87.4	86.4	89.1
Dilution procedures	No problems observed	No problems observed	No problems observed

[a] At the lowest QC level.

Table 5
Recovery of NA, NAM and NUR

Niacin		Niacinamide		Nicotinuric acid	
Concentration (ng/mL)	Recovery (%)	Concentration (ng/mL)	Recovery (%)	Concentration (ng/mL)	Recovery (%)
100	87.0	100	84.7	100	92.1
300	84.7	300	86.9	300	87.1
600	90.4	600	87.5	600	88.8
Mean	87.4	Mean	86.4	Mean	89.1

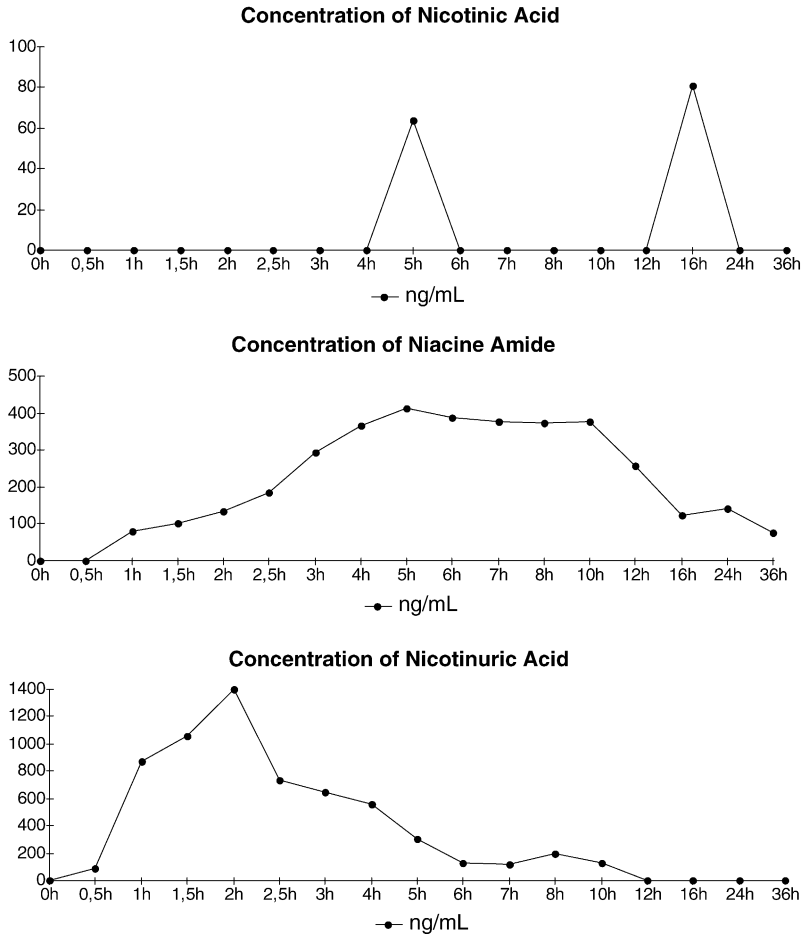

Fig. 3. *C–t* profiles for nicotinic acid, niacine amide and nicotinuric acid after oral administration of a 1000 mg tablet.

ples had to be measured with dilution before extraction because the validated calibration range was 50.0–750 ng/mL.

3. Conclusion

It has been shown that HPLC/MS provides the potential to quantify NA, NAM and NUR simultaneously with high specificity, sensitivity, accuracy, precision and reproducability in the concentration ranges of 50.0–750 ng/mL in very short time. Compared to previously developed methods no labour intensive extraction procedures are required. All three analytes were extracted from plasma by introducing a single solid phase extraction step prior to HPLC analysis, thus obviating the need for time-consuming liquid–liquid-extractions and derivatization procedures. Validation of this analytical method was carried out according to current guidelines for method validation. All the results obtained during the validation procedure fulfill the requirements and recommendations generally accepted for bioanalytical studies [18]. A summary of the validation results is given in Table 5.

Within the frame of a clinical pharmacokinetic study performed using 1000 mg extended release tablets consistent plasma concentration curves were obtained for NA, NAM and NUR. The validated calibration range was sufficient to determine all three analytes in the subject samples.

Thus HPLC/MS is shown to be a powerful technique for the simultaneous quantitative analysis of niacin and its main

metabolites in human plasma and can be used efficiently in pharmacokinetic and toxicokinetic studies.

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