Simultaneous quantification of niacin and its three main metabolites in human plasma by LC–MS/MS

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ABSTRACT

A sensitive and specific LC–MS/MS method for the simultaneous quantification of niacin (NA) and its three main metabolites nicotinamide (NAM), nicotinuric acid (NUA) and N-methyl-2-pyridone-5-carboxamide (2-Pyr) in human plasma has been developed and validated. Plasma samples (200 μL) were prepared by deproteinization with acetonitrile (500 μL), then the supernatant after centrifugation was evaporated and reconstituted. Chromatography was performed on a phenomenex synergi hydro-RP column with an isocratic elution of methanol-0.1% formic acid (5:95, v/v). The full separation of all analytes was achieved within 9 min. Multiple-reaction monitoring (MRM) using the fragmentation transitions of m/z $124.1 \rightarrow 80.1$, $123.1 \rightarrow 80.0$, $181.0 \rightarrow 79.0$ and $153.1 \rightarrow 110.2$ in positive electrospray ionization (ESI) mode was performed to quantify NA, NAM, NUA and 2-Pyr, respectively. The calibration curves were linear over the concentration range of 2.0–3000 ng/mL for NA and NUA, 10.0–1600 ng/mL for NAM and 50.0–5000 ng/mL for 2-Pyr. This method has been validated in accordance with the US FDA guidelines for bioanalytical method development and applied to the determination of NA and its three main metabolites in Chinese subjects following a single oral dose of niacin extended-release and simvastatin 1000 mg/20 mg. In particular, because of the endogenous NAM and 2-Pyr in human plasma, the concentrations of NAM and 2-Pyr in human plasma after dosing were determined by subtracting blank values of them.

1. Introduction

Niacin (nicotinic acid, NA, Fig. 1), a water-soluble vitamin and belongs to the vitamin B complex, is essential for the metabolism of carbohydrates, fats and many other substances in the body. It shows hypolipemic activity at high doses [1] and it possesses vasodilating and fabrinolytic properties [2,3]. NA reduces levels of total cholesterol, low density lipoprotein, triglycerides and lipoprotein-A; and increases high-density lipoprotein level [4].

NA is metabolized through two pathways. The first is the metabolic route to nicotinuric acid (NUA, Fig. 1) through glycine conjugation by nicotinyl-CoA, and the second is that to nicotinamide (NAM, Fig. 1). NAM is further transformed to pyridone derivatives, i.e. N-methyl-2-pyridone-5-carboxamide (2-Pyr, Fig. 1, major metabolite) and N-methyl-4-pyridone-5-carboxamide (4-Pyr, minor metabolite), through methylation and oxidation. The tablet of niacin extended-release and simvastatin, which contained NA 1000 mg and simvastatin 20 mg, was developed recently in China. It is necessary to investigate the pharmacokinetics of NA in Chinese subjects following a single oral dose of niacin extended-release and simvastatin 1000 mg/20 mg. Moreover, the two metabolic pathways were thought to be the mechanism responsible for the flushing side effect and hepatotoxicity of NA in human [5–7]. So, it is of paramount importance to develop a method for simultaneous quantification of NA and its three main metabolites in human plasma.

Previous publications have described several methods for determination of NA or NA along with its metabolites in plasma [8–22], including many HPLC-UV/FD methods [8–17] and a few LC–MS/MS methods [18–22]. Pfuhl et al. [20] have discussed the drawbacks of the various methods reported and established an LC–MS/MS method. However, this method utilized a complicated solid-phase extraction (SPE) process for the extraction of analytes from 1 mL plasma.
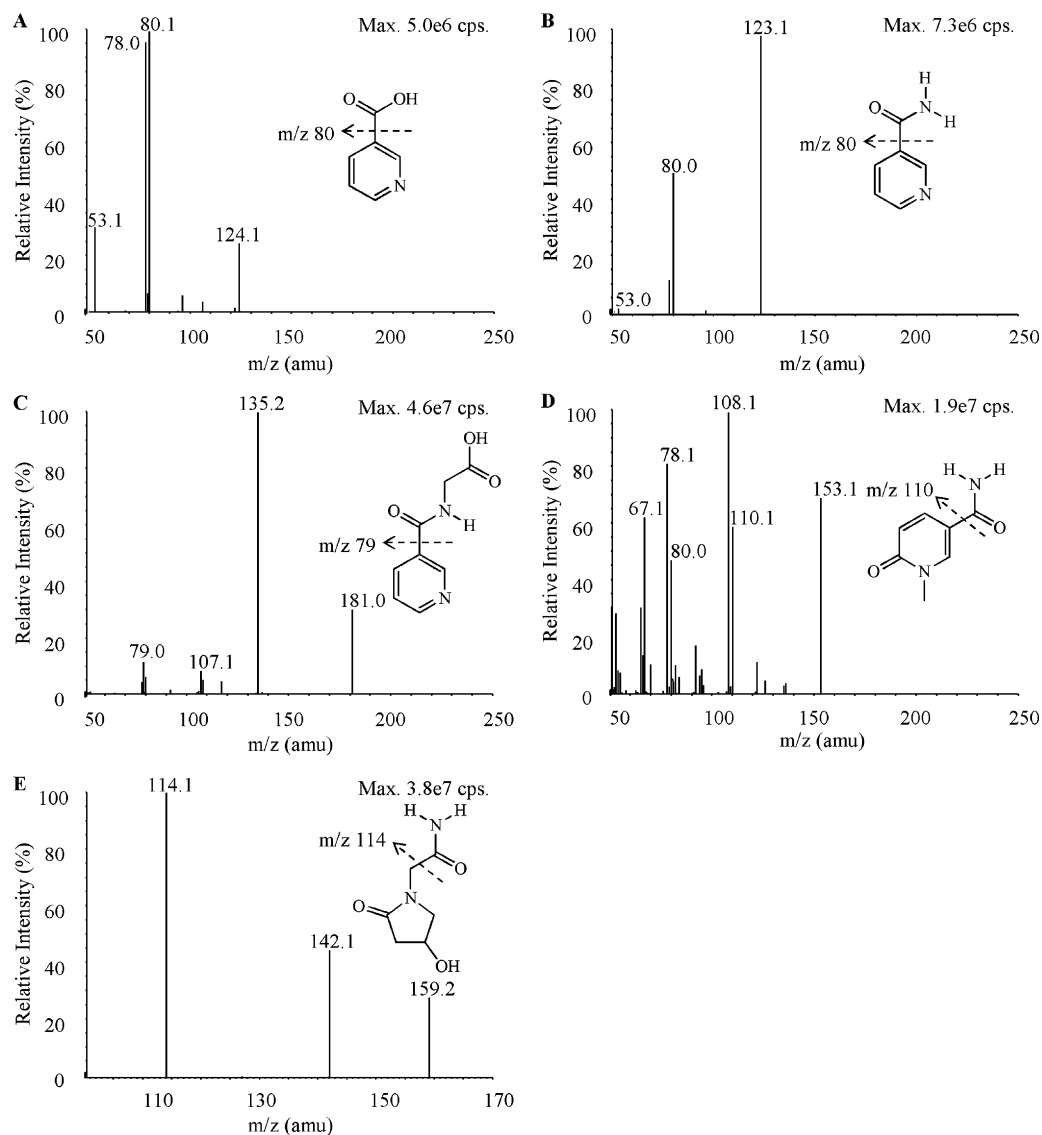
Fig. 1. Structures and full-scan product ion spectra of [M+H]⁺ for (A) NA, (B) NAM, (C) NUA, (D) 2-Pyr and (E) OXI (IS).

Because of the different physico-chemical properties and polarities of NA and its three main metabolites, the simultaneous determination of the four analytes remains difficult using single mode of separation. It was reported that the polar and zwitterionic character of NA made the development of a liquid–liquid extraction (LLE) sample preparation difficult [18,23]. Recently, one method was developed with protein precipitation (PPT), which simultaneously quantified NA and its metabolites including NAM, MNA, 2-Pyr and 4-Pyr. However, the main metabolite NUA was not quantified and the method was only applied to rat plasma [22]. Hitherto to the best of our knowledge there was one LC–MS/MS method reported for the simultaneous quantification of NA, NAM, NUA and 2-Pyr in human plasma [21], the samples were prepared with LLE, which has been reported not applicable to NA [18,23]. Moreover, endogenous NAM and 2-Pyr were not found in human blank plasma in that article.

In this paper, we present a sensitive and specific LC–MS/MS method for simultaneous quantification of NA, NAM, NUA and 2-Py in human plasma using a simple PPT process. The validated method was successfully applied to a pharmacokinetic study in Chinese subjects following a single oral dose of niacin extended-release and simvastatin 1000 mg/20 mg.

2. Experimental

2.1. Chemicals and materials

NA (99.9%) was purchased from SUPELCO Analytical (USA); NUA (98.0%) was purchased from Toronto Research Chemicals (Canada); NAM (100%) and 2-Pyr (99.8%) were purchased from TLC PharmaChem (Canada); Oxiracetam (OXI, 99.9%, internal standard (IS)) was purchased from Hebei Institute for Drug Control (China). HPLC grade methanol and acetonitrile were purchased from Fisher Scientific (USA). Distilled water used throughout the study was prepared from deionized water. All other chemicals and solvents were commercially available analytical grade materials used without further purification. Blank (drug free) human plasma was obtained from Aerospace Center Hospital (China). The tablets of niacin extended-release and simvastatin 1000 mg/20 mg were provided by RedSun Pharmaceutical Co., Ltd. (China).

2.2. Apparatus and chromatographic conditions

The HPLC system was a Shimadzu Prominence-20A series (Shimadzu, Kyoto, Japan) consisting of two LC-20AD solvent delivery

Table 1
MRM transitions and correlative optimized DP and CE used for the detection.

Analyte	Transition		DP (V)	CE (eV)
NA	For quantitation	*m/z* 124.1 → 80.1	51	31
	For qualitation	*m/z* 124.1 → 53.0	51	53
NAM	For quantitation	*m/z* 123.1 → 80.0	44	28
	For qualitation	*m/z* 123.1 → 78.0	44	35
NUA	For quantitation	*m/z* 181.0 → 79.0	22	51
	For qualitation	*m/z* 181.0 → 135.2	22	21
2-Pyr	For quantitation	*m/z* 153.1 → 110.2	54	29
	For qualitation	*m/z* 153.1 → 108.1	54	29
OXI	For quantitation	*m/z* 159.2 → 114.1	28	17
	For qualitation	*m/z* 159.2 → 142.1	28	12

modules, a DGU-20A3 on-line degasser, a SIL-20AHT autosampler, a CTO-20A column oven, and a CBM-20A system controller. Chromatography was carried out on a C_{18} analytical column (Synergi Hydro-RP 80A, 150 mm × 2.0 mm i.d., 4 μm, Phenomenex, USA) fitted with a C_{18} guard column (AQ, 4.0 mm × 2.0 mm i.d., 5 μm, Phenomenex, USA). The mobile phase consisted of methanol-0.1% formic acid (5:95, v/v). The flow rate was 0.25 mL/min with column temperature maintained at 35 °C. Total run time for each injection was 9 min. An API 3200 triple-quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Concord, Ontario, Canada) equipped with an electrospray ionization (ESI) source was used for mass analysis and detection. Analyst software (Applied Biosystems/MDS SCIEX, version 1.4.2) was used for data acquisition and processing. The mass spectrometer was operated in the positive ion mode with the MRM transitions and correlative optimized declustering potential (DP) and collision energy (CE) listed in Table 1. Other parameters were as follows: collision gas, curtain gas, gas 1 and gas 2 (all nitrogen) 3, 20, 55 and 50 psi, respectively; dwell time 150 ms; IonSpray voltage 5500 V; source temperature 550 °C. Unit resolution was used for Q1 and Q3 mass detection.

2.3. Preparation of calibration standard (CS) and quality control (QC) samples

Standard stock solutions of NA, NAM, NUA, 2-Pyr and OXI were prepared in methanol at concentrations of 4.0, 2.0, 0.50, 2.0 and 1.0 mg/mL, respectively. The IS stock solution was diluted with methanol–water (5:95, v/v) to a working concentration of 100 μg/mL. All solutions were subsequently aliquoted and stored at −40 °C, with aliquots used in each analytical run. In each analytical run, aliquots of separate standard stock solution were used to spike blank plasma to give a series of CS samples freshly. Lower limit of quantification (LLOQ), low QC, medium QC, high QC and ultra-high

QC samples were prepared independently using the same procedure. All QC samples were prepared in bulk, aliquoted, and frozen at −80 °C, with aliquots thawed and analyzed with each analytical run. The concentrations of the CS samples and QC samples are listed in Table 2.

2.4. Sample preparation

Frozen plasma samples were thawed at 25 °C and vortex-mixed briefly. Aliquots of plasma (200 μL) were transferred to 1.5 mL Eppendorf tubes followed by additions of IS working solution (10 μL) and methanol–water (5:95, v/v) (10 μL). Then aliquots of acetonitrile (500 μL) were added to precipitate the protein. The mixture was extracted by vortex-mixing for 1 min and centrifuged at 10,000 × g for 10 min. The supernatants were transferred to another 1.5 mL Eppendorf tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 150 μL of methanol–water (5:95, v/v). A 10 μL aliquot of the solution was injected into the LC–MS/MS system for analysis. Samples at the concentration higher than the upper limit of quantification (ULOQ), including ultra-high QC samples, should be diluted with the dilution factor of 2 and re-assayed.

2.5. Method validation

The method was fully validated according to US FDA guideline for validation of the bioanalytical method [24]. Because of the presence of endogenous NAM and 2-Pyr in the plasma, blank values of them should be subtracted.

2.5.1. Selectivity

The selectivity was determined by analysis of six blank plasmas from different subjects. The blank plasmas were extracted and

Table 2
Concentrations of combined CS samples and QC samples.

Sample name	Concentration (ng/mL)		Sample name	Concentration (ng/mL)	
	NA	NUA		NAM	2-Pyr
CS 01	2.0	2.0	CS 01	10.0	50.0
CS 02	6.0	6.0	CS 02	30.0	150
CS 03	16.0	16.0	CS 03	80.0	400
CS 04	40.0	40.0	CS 04	200	1000
CS 05	80.0	80.0	CS 05	400	2000
CS 06	400	400	CS 06	1600	5000
CS 07	1000	1000			
CS 08	3000	3000			
Low QC	6.0	6.0	Low QC	30.0	150
Media QC	40.0	40.0	Media QC	200	1000
High QC	320	320	High QC	1280	4000
Ultra-high QC	2400	2400			

analyzed, and the responses were assessed at the retention times of analytes and IS.

2.5.2. Cross-talk

The cross-talk phenomena among MS/MS channels were assessed by injecting four analytes and IS, separately, at the highest concentrations for calibration curve and monitoring the response in the other MS/MS channels.

2.5.3. Carryover

The carryover was evaluated by analyzing a blank sample immediately after the ULOQ sample of the standard curve. The carryover level should <20% of the response observed for the analyte LLOQ and <5% of the response observed for the IS at the working concentration [25].

2.5.4. Extraction recovery

Extraction recoveries were determined at QC concentrations of four analytes and at the working concentration of IS by comparing the peak areas of extracted QC samples with mean peak areas of blank plasma extracts reconstituted with analytes and IS at corresponding concentrations ($n = 6$ in each case). In both cases, the blank plasma/IS peak area ratio of NAM and 2-Pyr was deducted before the percent value of extraction recovery was calculated.

2.5.5. Matrix effect

Matrix effect factors were determined at QC concentrations of four analytes and at the working concentration of IS by comparing the peak areas of post-extraction blank plasma (from six different individuals) spiked with analytes and IS with mean peak areas of solutions containing analytes and IS at corresponding concentrations. The blank plasma/IS peak area ratios of NAM and 2-Pyr should be subtracted from the corresponding matrix.

2.5.6. Linearity and LLOQ

Calibration curves (peak area ratio of NA/NUA to IS versus nominal analyte concentration) were fitted by least square linear regression using $1/x^2$ as weighting factors prepared in triplicate. For NAM and 2-Pyr, blank values of them should be subtracted from each calibration point. Calibration curves were constructed in triplicate by plotting the margin of the analyte/IS peak area ratio between the plasma that spiked standard solutions and the blank plasma with IS as a function of the concentration calibrator. Analyte of blank plasma/IS peak area ratio was set as R_0, and analyte of spiked plasma/IS peak area ratio was set as R_n ($n = 1, 2, 3, 4, 5$ and 6). Calibration curves were then fitted to the equation $y = bx + a$ by weighted linear regression ($1/x^2$), where y corresponds to the peak area ratio difference ($y_n = R_n - R_0$, $n = 1, 2, 3, 4, 5$ and 6) and x refers to nominal analyte concentration.

To assess linearity, the coefficient of correlation (r^2) should be higher than 0.99, and deviations of the calculated concentrations should be within ±15% from nominal concentrations except for the LLOQ level, at which a deviation of ±20% is permitted. The signal to noise (S/N) of each analyte at LLOQ should be at least 10.

For endogenous analytes, the LLOQ values are not fixed measures due to the varying C_0 (concentration of blank plasma) value in biological samples. So the relative lower limit of quantification (rLLOQ) was proposed to evaluate and compare the analytical methods. The rLLOQ is defined as the percentage ratio of C_{LLOQ} to C_0. The smaller the rLLOQ value is, the greater the discriminatory power of the analytical method for the analyte. For different basal concentrations, probable range of LLOQ can be calculated based on the rLLOQ [26].

2.5.7. Precision and accuracy

Precision and accuracy were calculated in terms of relative standard deviation (RSD) and relative error (RE), respectively. They were evaluated by analysis of six replicates of LLOQ and QC samples for three successive days. The intra- and inter-day precision should not exceed 20% for LLOQ, and 15% for QC samples. Accuracy should be within ±20% for LLOQ, and ±15% for QC samples.

2.5.8. Stability

The stability of each analyte in human plasma was investigated by subjecting QC samples to various conditions. Short-term stability was evaluated by placing QC samples at 25 °C for 8 h. For freeze-thaw stability, QC plasma samples were subjected to three cycles from −80 °C to 25 °C. The autosampler stability was assessed by placing processed QC samples in the autosampler at 22 °C for 10 h. Long-term stability was evaluated by freezing QC samples at −80 °C for 30 days. The stability of analytes in the stock solution and IS in the working solution stored at 25 °C for 12 h and at −40 °C for 60 days were also investigated.

2.6. Application of the method

The developed method was applied to a pharmacokinetic study in Chinese subjects following a single oral dose of niacin extended-release and simvastatin 1000 mg/20 mg.

The protocol of the pharmacokinetic study was approved by the Ethics Committee of the Aerospace Center Hospital (China). Each subject gave written informed consent before participating in the study. The subjects were 6 men and 6 women who had the following clinical characteristics: age, 20–28 years (mean, 24 ± 3 years); body mass index (BMI), 19–24 kg/m² (mean, 22 ± 2 kg/m²). The subjects had not donated blood and participated in other drug clinical trials within 3 months prior to dosing. Subjects were in good health as determined by past medical history, physical examination, vital signs, electrocardiogram (ECG) and laboratory tests (hematology, biochemistry and urinalysis) at screening. Subjects were also screened for drugs of abuse, hepatitis B and C, HIV, and pregnancy in female subjects. All subjects confirmed abstinence from other medications, alcohol, tobacco and caffeinated products throughout the study.

Each subject was administered a single dose of niacin extended-release and simvastatin 1000 mg/20 mg with 200 mL of water at approximately 10 p.m. after a low-fat snack. Meals controlled for niacin content were administered at about 8 a.m., noon, 6 p.m. and 9:30 p.m. daily. Blood samples were obtained prior to dosing and 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16 and 24 h after dosing. Plasma was obtained by centrifugation (3000 × g for 5 min at 4 °C) and stored immediately at −80 °C until analysis. All samples were analyzed within 30 days.

3. Results and discussion

3.1. Optimization of LC–MS/MS conditions

The precursor ions of NA and NAM are 1 amu apart (m/z 124 versus 123) and both product ion settings are at m/z 80, so baseline separation of them was needed to prevent interference resulted from isotope effect. 2-Pyr and 4-Pyr have some product ions in common, therefore there are isotopic interference between them. According to Li et al. [22], the transition of m/z 153 → 110 instead of 153 → 108 for 2-Pyr was selected for avoiding the interference, as the product ion at m/z 110 is unique to 2-Pyr. For NUA, the transition of m/z 181 → 79 was chosen for its lower baseline and less interference. The product ion mass spectra of analytes and IS are shown in Fig. 1.
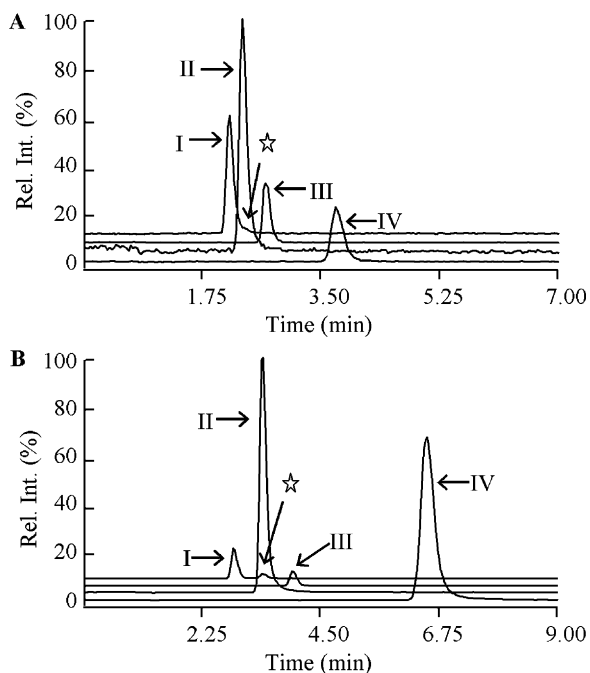
Fig. 2. MRM chromatograms of the mixed standard solution, using aqueous mobile phase with (A) 10% water and (B) 5% water. Peak I, NA; Peak II, NAM; Peak III, NUA; Peak IV, 2-Pyr.

Various HPLC columns were tested, the Phenomenex Synergi Hydro-RP 80 A (150 mm × 2.0 mm i.d., 4 μm) analytical column could give good retention. Different mobile phases were also investigated, the signal intensities of the analytes were increased three times when methanol used as the organic phase compared with acetonitrile. The presence of a low amount of formic acid in the mobile phase improved the sensitivity of the analytes. As shown in Fig. 2, the mobile phase proportion affected the separation of NA and NAM, the baseline separation could be obtained until the proportion of aqueous was reduced to 5%. Finally, a mobile phase consisting of methanol and 0.1% formic acid (5:95, v/v) was used in the study.

3.2. Optimization of extraction

NA is zwitterionic nature, whereas its metabolites, NUA is acidic, NAM is basic and 2-Py is neutral compound. Hence NA and its metabolites display complexities in their physico-chemical properties that may have to be carefully addressed during the sample clean-up process for an optimal/efficient recovery of all the analytes. For the plasma samples clean-up process, various extraction techniques, PPT, LLE and SPE have been reported [18–23,27]. In the present study, the LLE was not feasible due to the low extraction recovery. Simple PPT followed by evaporation of the supernatant and reconstitution of the dried extract was proved to be the most effective (the extraction recovery of the four analytes ≥88.5%).

3.3. Method validation

3.3.1. Selectivity
Fig. 3 shows the typical chromatograms of blank plasma sample, LLOQ sample plus IS, and plasma sample from a healthy Chinese subject 4 h after a single oral dose of niacin extended-release and simvastatin 1000 mg/20 mg. No significant interference from endogenous substances was observed at the retention times of NA, NUA and IS. But for the endogenous NAM and 2-Pyr, the corresponding blank values of them should be subtracted from the plasma sample.

3.3.2. Cross-talk
There was no cross-talk phenomena of NUA, 2-Pyr and IS was observed among any other MS/MS channels. However, the obvious cross-talk phenomenon of NA and NAM was found between the two MS/MS channels, so baseline separation of NA and NAM was needed in the study.

3.3.3. Carryover
No peak was observed at the retention times of any analyte or IS in the chromatogram of a blank sample analyzed after the injection of ULOQ sample, indicating the absence of carryover.

3.3.4. Extraction recovery and matrix effect
The results of extraction recoveries and matrix effects factors are summarized in Table 3. The extraction recoveries were high and
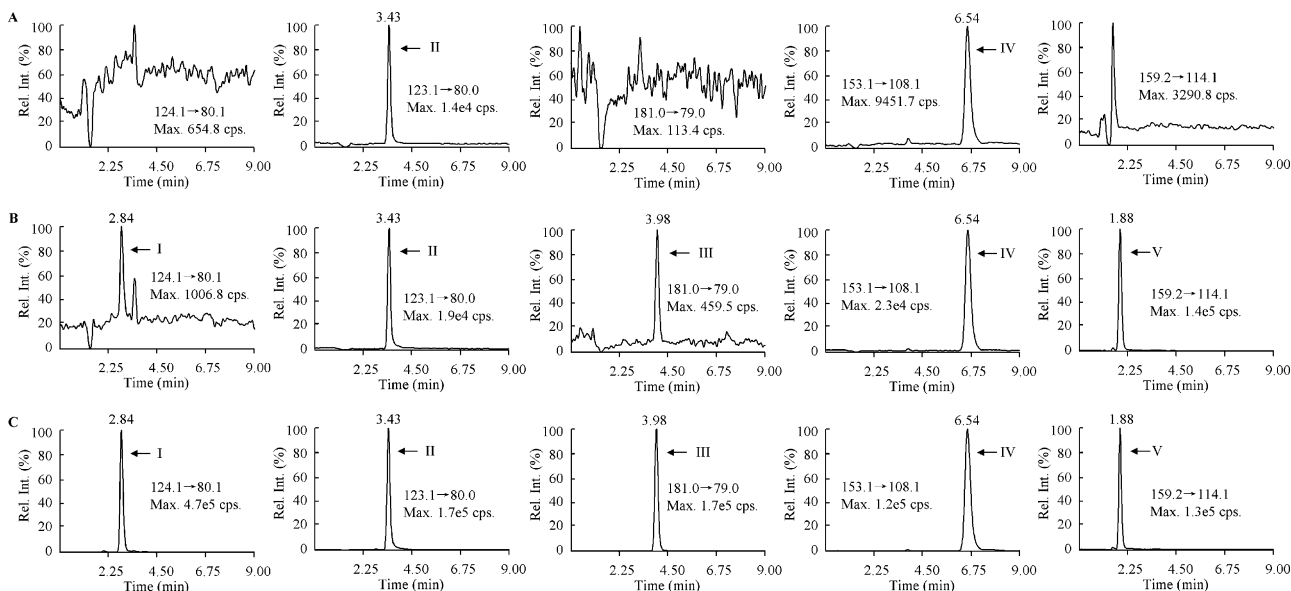

Fig. 3. Representative MRM chromatograms of (A) blank plasma, (B) LLOQ sample plus IS, and (C) plasma sample from a healthy subject 4 h after a single oral dose of niacin extended-release and simvastatin 1000 mg/20 mg plus IS. Peak I, NA; Peak II, NAM; Peak III, NUA; Peak IV, 2-Pyr; Peak V, OXI (IS).

Table 3
Extraction recoveries and matrix effects factors for the determination of four analytes at QC concentrations ($n = 6$ at each concentration) and of IS at the working concentration ($n = 18$).

Analyte	Concentration (ng/mL)	Extraction recovery (%)	Matrix effects factor (%)
NA	6.0	90.3 ± 3.1	63.7 ± 1.9
	40.0	88.5 ± 1.6	63.5 ± 0.6
	320	91.4 ± 1.3	63.5 ± 1.3
	2400	90.8 ± 1.1	67.1 ± 0.6
NAM	30.0	97.6 ± 3.3	56.6 ± 6.6
	200	93.0 ± 1.1	56.9 ± 6.2
	1280	95.6 ± 1.0	61.7 ± 6.6
NUA	6.0	90.4 ± 3.3	86.7 ± 2.2
	40.0	89.6 ± 1.7	82.1 ± 2.1
	320	91.9 ± 0.8	81.4 ± 1.9
	2400	89.5 ± 1.6	82.6 ± 1.5
2-Pyr	150	93.0 ± 1.3	81.5 ± 2.5
	1000	93.2 ± 0.7	85.1 ± 0.8
	4000	96.2 ± 0.9	88.3 ± 1.1
OXI	100,000	83.1 ± 1.0	32.1 ± 2.8

reproducible. Co-eluting endogenous substances did not influence the ionization of NUA and 2-Pyr, and inhibited the ionization of NA, NAM and IS. However, the low variability (<12%) indicated no influence of matrix effect on the determination.

3.3.5. Linearity and LLOQ

Due to the varying basal concentrations of NAM and 2-Pyr in plasma coming from different human, all CS and QC samples used for this method validation were prepared from the same pooled plasma to assure the same basal levels of NAM and 2-Pyr. The calibration curves were linear over the concentration ranges of 2.0–3000 ng/mL for NA, 10.0–1600 ng/mL for NAM, 2.0–3000 ng/mL for NUA, and 50.0–5000 ng/mL for 2-Pyr. Good linearity with a coefficient of determination r exceeding 0.9975 was observed for each analyte. The representative regression equations were $y = 0.00137x + 0.000481$ for NA, $y = 0.00221x - 0.00124$ for NAM, $y = 0.00107x - 0.0000955$ for NUA, and $y = 0.000981x + 0.00691$ for 2-Pyr. The slopes of the regression equations were consistent for the calibration curves prepared on 3 separate days.

The LLOQs were 2.0 ng/mL for NA, 10.0 ng/mL for NAM, 2.0 ng/mL for NUA and 50.0 ng/mL for 2-Pyr in plasma, respectively. The concentrations of NAM and 2-Pyr were 34.4 ng/mL and

103 ng/mL in the blank plasma used for the method validation. So, the rLLOQ of endogenous NAM and 2-Pyr calculated as the percentage ratio of C_{LLOQ} to C_0 was 29.1% and 48.5%, respectively.

3.3.6. Precision and accuracy

Table 4 summarizes the intra- and inter-day precision and accuracy for each analyte from LLOQ and QC samples. The precision and accuracy were acceptable, with RSD below 15% and RE within 15%.

3.3.7. Stability

The four analytes were found stable in following conditions: in plasma at 25 °C for 8 h, in plasma after three freeze-thaw cycles, in autosampler at 22 °C for 10 h, in plasma at −80 °C for 30 days. The data for stability tests of the four analytes are presented in Table 5. All RE values between post-storage and initial QC samples were within ±15%. All analytes in the stock solution and IS in the working solution were also stable stored at 25 °C for 12 h and at −40 °C for 60 days.

3.4. Clinical application of the method

In the present study, we evaluated the pharmacokinetics of NA and its main metabolites in 12 healthy Chinese subjects

Table 4
Precision and accuracy for the determination of four analytes in human plasma (data are based on assay of six replicate samples at the LLOQ and QC concentrations on three different days).

Analyte	Nominal concentration (ng/mL)	Measured concentration (ng/mL)	Intra-day RSD (%)	Inter-day RSD (%)	RE (%)
NA	2.0	1.94 ± 0.15	9.2	12.0	−3.0
	6.0	6.12 ± 0.28	4.5	4.6	2.0
	40.0	40.7 ± 1.2	2.8	4.3	1.8
	320	316 ± 8	1.8	5.6	−1.3
	2400	2427 ± 47	1.3	4.3	1.1
NAM	10.0	9.53 ± 0.62	2.9	2.8	−1.7
	30.0	29.5 ± 0.9	2.5	6.6	0.0
	200	200 ± 7	1.7	2.9	−1.9
	1280	1256 ± 24	7.1	6.7	−4.7
NUA	2.0	1.99 ± 0.18	9.9	6.3	−0.5
	6.0	6.03 ± 0.16	2.8	1.9	0.5
	40.0	40.0 ± 1.0	2.3	3.6	0.0
	320	318 ± 6	1.6	3.2	−0.6
	2400	2308 ± 48	1.6	4.0	−3.8
2-Pyr	50.0	44.9 ± 3.4	9.5	13.8	−10.2
	150	154 ± 6	3.2	8.5	2.7
	1000	992 ± 22	1.8	4.3	−0.8
	4000	3962 ± 76	1.4	4.0	−1.0

Table 5
Results of stability studies of four analytes under various storage conditions ($n = 3$).

Analyte	Nominal concentration (ng/mL)	Storage in human plasma at 25 °C for 8 h		Storage in human plasma after 3 freeze-thaw cycles		Storage in the autosampler at 22 °C for 10 h		Storage in human plasma at −80 °C for 30 days	
		Measured concentration (ng/mL)	RE (%)	Measured concentration (ng/mL)	RE (%)	Measured concentration (ng/mL)	RE (%)	Measured concentration (ng/mL)	RE (%)
NA	6.0	6.28 ± 0.19	4.7	5.96 ± 0.29	−0.7	5.86 ± 0.24	−2.3	5.89 ± 0.12	−1.8
	40.0	41.9 ± 0.5	4.8	40.0 ± 0.5	0.0	40.9 ± 1.2	2.3	41.9 ± 2.0	4.7
	320	325 ± 4	1.7	314 ± 8	−2.0	328 ± 2	2.6	323 ± 8	0.9
	2400	2450 ± 10	2.1	2397 ± 15	−0.1	2450 ± 89	2.1	2402 ± 84	0.1
NAM	30.0	29.8 ± 0.3	−0.8	29.1 ± 0.7	−2.9	31.0 ± 1.1	3.4	30.2 ± 1.1	0.6
	200	202 ± 3	1.0	195 ± 2	−2.3	208 ± 9	3.8	205 ± 9	2.5
	1280	1297 ± 5	1.3	1250 ± 31	−2.3	1280 ± 17	0.0	1289 ± 22	0.7
NUA	6.0	6.14 ± 0.24	2.3	5.99 ± 0.39	−0.2	6.42 ± 0.19	6.9	6.0 ± 0.18	0.1
	40.0	40.1 ± 0.3	0.3	38.7 ± 0.2	−3.3	40.3 ± 0.9	0.7	41.2 ± 0.9	3.1
	320	324 ± 4	1.3	314 ± 8	−2.0	318 ± 3	−0.7	325 ± 2	1.7
	2400	2290 ± 10	−4.6	2263 ± 32	−5.7	2277 ± 61	−5.1	2343 ± 93	−2.4
2-Pyr	150	163 ± 2	8.4	155 ± 2	3.1	162 ± 2	8.2	155 ± 7	3.1
	1000	1020 ± 20	2.0	963 ± 12	−3.7	1015 ± 15	1.5	1017 ± 24	1.7
	4000	4117 ± 27	2.9	3944 ± 135	−1.4	4108 ± 21	2.7	4052 ± 115	1.3
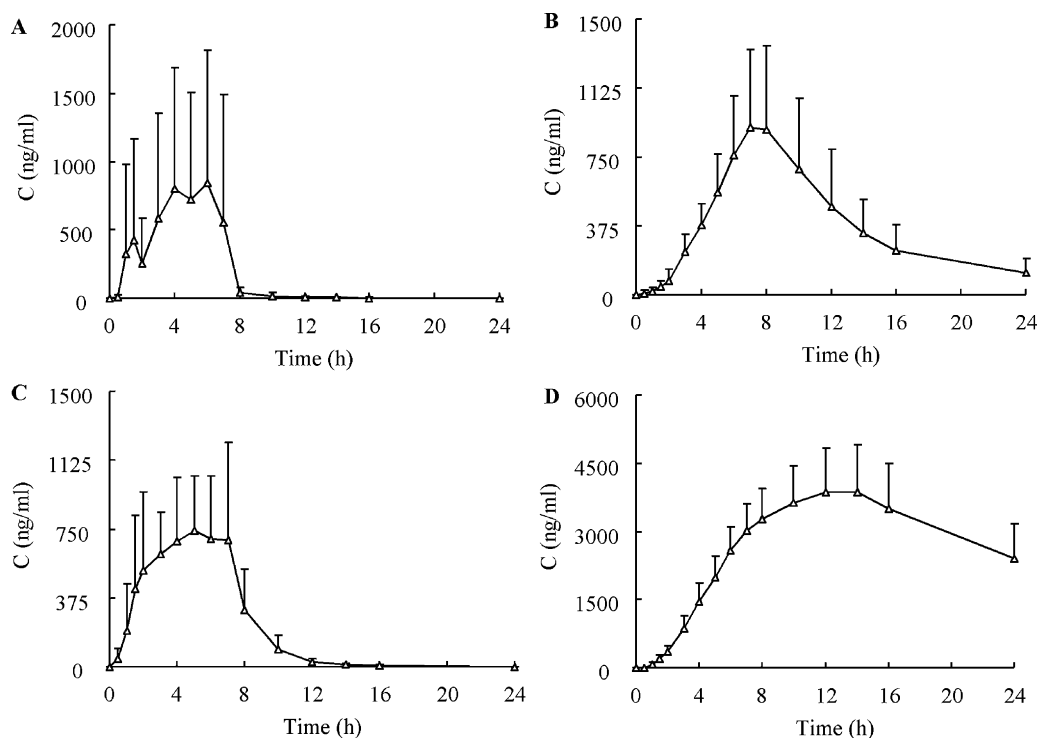
Fig. 4. Mean plasma concentration–time profiles of NA (A), NAM (B), NUA (C) and 2-Pyr (D) in healthy Chinese subjects following a single oral dose of niacin extended-release and simvastatin 1000 mg/20 mg ($n = 12$).

following a single oral dose of niacin extended-release and simvastatin 1000 mg/20 mg.

The mean plasma concentration–time profiles of NA and its three main metabolites are displayed in Fig. 4. For endogenous analytes, the plasma concentrations of NAM and 2-Pyr in 12 healthy Chinese subjects after dosing were calculated by the formula: $C = C_D - C_P$. In which, the C_D represents the concentrations of NAM or 2-Pyr in subjects after dosing; the C_P represents the concentrations of NAM or 2-Pyr in subjects prior to dosing, and C_P was defined to zero when lower than LLOQ concentrations.

4. Conclusion

A sensitive and specific LC–ESI-MS/MS method for the simultaneous quantification of NA and its three main metabolites in human plasma was developed and validated. The assay was reproducible, accurate and precise, and has been successfully applied to a

clinical pharmacokinetic study in healthy Chinese subjects following a single oral dose of niacin extended-release and simvastatin 1000 mg/20 mg.

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