Analytical Methods

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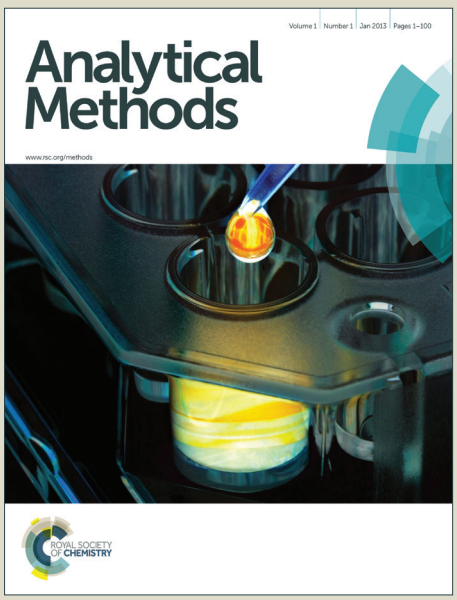

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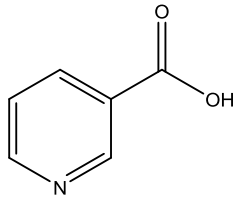
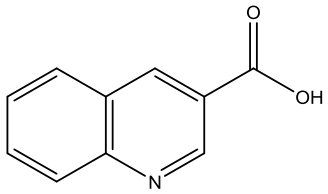
Graphic Abstract

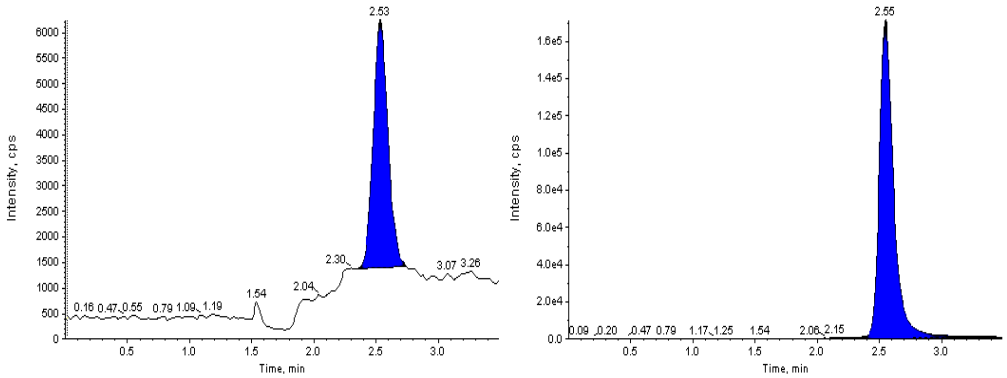


A. Nicotinic acid (NA, MW: 123)

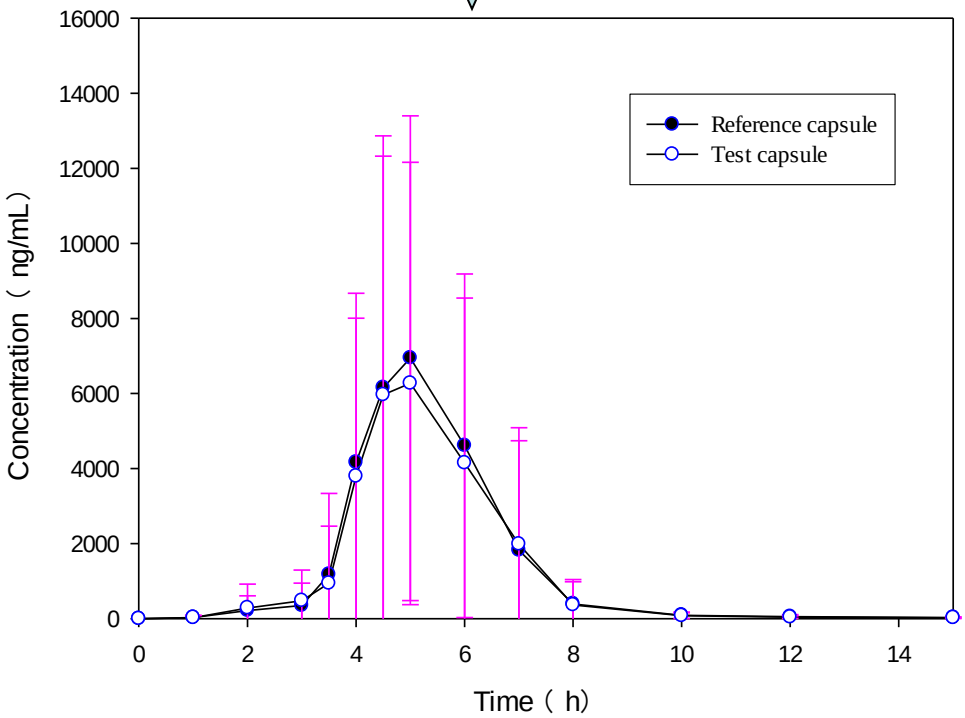


B. Quinoline-3-carboxylic acid (IS, MW:173)

LC-MS/MS analysis



Application for a bioequivalence study



Development and validation of a method for the determination of nicotinic acid in human plasma using liquid chromatography- negative electrospray ionization tandem mass spectrometry and its application to a bioequivalence study

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Abstract

For 60 years, nicotinic acid (NA) has been used as a vitamin potent in milligram doses while NA in gram doses has been administrated as a broad-spectrum lipid drug potent. Obviously, it is critical and important to validate sensitive method for the analysis of NA in human plasma. Accordingly, a simple and sensitive LC-MS/MS method has been developed and validated for the quantification of NA in human plasma using quinoline-3-carboxylic acid as an internal standard (IS). Following liquid-liquid extraction (LLE) with n-butanol, the analytes were separated on a Hypersil-Gold-CN column (4.6 × 150 mm i.d., 5 μm) interfaced with a triple-quadrupole tandem mass spectrometer using negative electrospray ionization. Quantification of NA was conducted by multiple reaction monitoring (MRM) of the transitions at *m/z* 122.0→78.1 for NA and 171.9→127.8 for IS. The lower limit of quantification was 6.57 ng·mL^{-1}, and the assay exhibited a linear range of 6.57-5255 ng·mL^{-1}. The developed method was successfully applied to a bioequivalence (BE) study in healthy volunteers after oral administration of NA.

Keywords

Nicotinic acid; LC-MS/MS; Bioequivalence; Human plasma

1. Introduction

In 1955, nicotinic acid (NA) in gram doses was discovered to lower plasma cholesterol in both normal and hypercholesterolaemic subjects[1]. Generally, NA in milligram is well-known as a kind of water soluble vitamin, which belongs to the vitamin B complex[2]. Moreover, NA was demonstrated clinically to significantly reduce the atherosclerotic cardiovascular complications and total mortality with less side effects[3-6]. NA was regarded as a powerful inhibitor of fat-mobilizing lipolysis in adipose tissue, which resulted in a decrease in plasma free fatty acids and suggested to be a basic mechanism for the lipid effects of NA[7]. With daily doses of 3 to 6g, NA could decrease plasma concentration of free fatty acids, low-density lipoproteins and very low-density lipoproteins[8-11]. NA was marketed as 'the broad-spectrum lipid drug' due to a potent lipid-modifying drug, but its clinical uses had been limited by the side effects[3]. The flush and the increase of uric acid in blood were the regularly occurring side effects with nicotinic acid treatment; the flush occurred rapidly after the dose of NA was administrated, while the other appeared with the treatment of NA for some time[3]. Only 50 mg of oral NA could make the characteristic flush within a few minutes[12, 13]. Therefore, it is critical and important to validate a sensitive method for analysis of NA in human plasma.

Since NA could be rapidly absorbed and metabolized after oral dosing, with an elimination half-life ranging from 20 to 45 min, various sustained-release preparations of NA have been developed in an attempt to prevent the side-effects associated with the rapid drug absorption[14]. To study the hypolipidemic action and investigate the

mechanism for the side-effects of NA, a pharmacokinetic study on NA is crucial and vital. Therefore, the development and validation of a NA analytical method will benefit its pharmacokinetic studies.

In the past decades of NA qualitative and quantitative analysis, a variety of conventional analytical techniques had been used mainly including paper chromatography [15], thin-layer chromatography (TLC) [15-17], high performance thin-layer chromatography (HPTLC) [18-21], gas chromatography (GC) (Limit of detection (LOD): 50.6~90.4 $ng \cdot mL^{-1}$) [22-24] and gas chromatography mass spectrometry (GC/MS) (LOD: 4.6~10.5 $ng \cdot mL^{-1}$) [25-27]. Paper chromatography, TLC and HPTLC are not sensitive enough for quantitative analysis of NA. Since the boiling point of NA is from 234 to 238°C, NA should be transformed to a volatile derivative before GC analysis, adding steps to its analysis [25, 27].

Recently, high performance liquid chromatographic (HPLC) methods had been commonly employed to determine the concentration of NA and its metabolites in human plasma. A high performance liquid chromatographic method with UV detection (HPLC-UV) was developed to quantify NA in human plasma with the lowest concentration of NA at 100 $ng \cdot mL^{-1}$ [14, 28-30]. The HPLC coupled with fluorescence detection method was also validated for the determination of NA after time-consuming derivative procedures so that lower concentrations of NA could be detected in human plasma [31-34]. However, most of these methods suffer by tedious and labor-intensive experimental procedures. Instead, capillary electrophoresis (CE) had been proved to be a powerful technique for the analysis of NA in biological fluids

without tedious clean-up procedures, but the limit of detection was found to be above 100 ng·mL^{-1} [35-41].

HPLC interfaced with mass spectrometry (HPLC-MS) has revolutionized the field of bioanalysis with high sensitivity and short run times. Several liquid chromatography-tandem mass spectrometry had been reported for the quantification of NA in plasma using positive electrospray ionization ((+) ESI) [42-46]. These methods differed mainly by their extraction procedure，limit of quantitation, volume of sample or total run-time from the previous methods. UPLC, which could burden much higher pressure than HPLC, is devoted to increase separation efficiency in short time. Additionally, a column packing material of less than 2 μm small particles could strengthen the resolution, sensitivity and peak areas. However, a common HPLC column could be operated on UPLC system to achieve the analysis of NA in this study if it would satisfy the experiments, since UPLC column is more expensive. In this paper, an LC-MS/MS method was developed and validated with high sensitivity (Lower limit of quantification (LLOQ): 6.57 ng·mL^{-1}), fast run-time (3.5 min) and small volume of sample (0.18 mL) due to the negative electrospray ionization ((-) ESI) optimized. The analytical procedure was fully validated and successfully used to assess the pharmacokinetic study of NA in healthy Chinese subjects.

2. Experimental

2.1. Materials and chemicals

Ammonium formate (purity: 99.5%, batch No.: T20090626) and formic acid (purity: 99.0%, batch No.: 20090517) were purchased from Sinopharm Chemical Reagent Co.,

Ltd (Shanghai, PR China). The hydrochloric acid (purity: 37.5%, batch No.: 200940054) was supplied by Kaixin Chemical Reagent Co., Ltd (Hunan, PR China). The internal standard, quinoline-3-carboxylic acid (IS, batch No.: 177148) was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). The NA (batch No.: 59-67-6-20090218) and tested drug (batch No.: VB3-20090422) were supplied by the Tianjin Zhongrui pharmaceutical Co., Ltd (Tianjin, China). The purities of all compounds were >98%, which were analyzed by ultra-performance liquid chromatography (UPLC) with photo-diode Array (PDA) detection. The structures of NA and IS are shown in Fig.1.

Acetonitrile and methanol for UPLC were purchased from Merck company (Darmstadt, Germany). Deionized water was purified by using a Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA). The centrifuge (Type: Biofuge prime R) was supplied by Heraeus company (Osterode, Germany). Blank human plasma was provided by Xiangya Hospital of Central South University, Xiangya School of Medicine, Central South University (Changsha, China).

2.2. Liquid chromatographic conditions

A Waters Acquity UPLC system (Acquity, Waters, USA) consisting of a vacuum degasser, a binary pump, an autosampler and a column compartment, which was temperature controlled, was used for solvent and sample delivery. An API4000 mass spectrometer from AB Sciex (Concord, Ontario, Canada) was coupled with the UPLC system and the Analyte version 1.4.2 software (Concord, Ontario, Canada) was installed for the chromatographic data system. A Hypersil-Gold-CN column

(Thermofisher Scientific, 4.6 × 150 mm i.d., 5 μm) purchased from Dalian elite analytical instrument Co., Ltd (Dalian, Liaoning, China) was operated for the sample separation at 35°C. A SecurityGuard Cartridges C_{18} column (4.0×3.0 mm i.d., 5 μm, Part No.: AJ0-4287) supplied by Phenomenex Inc. (Torrence, California, USA) was used as a guard column. The mobile phase consisted of acetonitrile and 20 mM ammonium formate with 0.1% formic acid (75:25, v/v) was used for isocratic elution to obtain the baseline separation of both analytes at a flow rate of 0.75 $mL \cdot min^{-1}$.

2.3. Mass spectrometric conditions

The effluent from the chromatographic column was directed into the ESI probe. Mass spectrometric conditions were optimized to obtain maximal sensitivity. The ESI was performed in the negative mode and the optimum conditions for nebulizing gas (GS1) of nitrogen, turbo spray gas (GS2), and curtain gas (CUR) were 40, 45 and 13 psi, respectively. The source temperature of GS2 was set at 550°C, and the ion spray voltage used was -4500 V. Unit resolutions were set for both Q1 and Q3 mass detection, and the collision energy (CE) was set at 17 and 22 V for NA and IS, respectively. Multiple reaction monitoring (MRM) detection was employed using nitrogen as the collision gas with a dwell time of 200 ms for each ion transition. The selection of operating deprotonated ions was shown in Fig.2. The precursor ions $[M\text{-}H]^-$ at *m/z* 122.0 and 171.9 and the product ions at *m/z* 78.1 and 127.8 after collision induced dissociation were used for the quantification of NA and IS, respectively.

2.4. Standard and quality control sample preparation

Stock standard solutions of NA and IS were both prepared in methanol at the concentration of 1051 μg·mL^{-1} and 510 μg·mL^{-1}, respectively. The stock solution of NA was then serially diluted with methanol to provide eight working solutions of desired concentrations at 6.57-5255 ng·mL^{-1}. An IS working solution of 1000 ng·mL^{-1} was obtained by diluting the stock solution of IS with methanol. All the solutions were stored at 4°C until use.

The samples for standard calibration curves were prepared by spiking the blank plasma (180 μL) with 20 μL of the appropriate working solutions to yield the following concentrations: 5255, 2628, 1314, 263, 52.6, 26.2, 13.1 and 6.57 ng·mL^{-1}. Quality control (QC) samples were prepared from blank plasma in a similar procedure for low, medium and high at concentrations of 13.1, 263 and 2628 ng·mL^{-1}.

2.5. Plasma sample preparation

A volume of 180 μL of drug-free human plasma was added to a disposable Eppendorf tube, followed by an addition of 20 μL of the standard working solution and 40 μL of IS working solution, respectively. Then, 50 μL of hydrochloric acid (1 mol/L) was added to the mixture which was subsequently vortexed for 30s using a vortex mixer (IKA VIBR AX, Germany). After that, a single step of liquid-liquid extraction (LLE) was adopted to extract NA and IS from the human plasma. For LLE, 1.0 mL of n-butanol was added to each tube followed by vortex-mixing for 15 min. The well-mixed solutions were then centrifuged at 13,000 rpm for 10 min and 0.7 mL of the upper organic layer was transferred to a new Eppendorf tube and evaporated to dryness set at 40°C in water bath under a gentle stream of nitrogen. The residues were

then reconstituted in 200 μL mobile phase followed by centrifugation at 22,000 rpm for 10 min before analysis. An aliquot of 10 μL was injected into the LC-MS/MS system.

2.6. Method validation

The method was validated with respect to selectivity, linearity, sensitivity, precision, accuracy, extraction recovery, matrix effect, and stability based on the United States Food and Drug Administration (FDA) guidelines [47]. The validation runs were carried out in three consecutive days. The peak area ratios of NA to the IS of QC samples were interpolated from the calibration curves run on the same day to calculate the concentrations of NA. The QC samples were employed to evaluate the precision, accuracy and stability of the method in three consecutive days.

2.6.1. Selectivity

The selectivity was evaluated by comparing the chromatograms of six different blank plasma from six subjects to those of corresponding plasma spiked with NA and IS, and those of plasma samples from drug subjects after oral administration of NA sustained-release capsules.

2.6.2. Linearity and lower limit of quantification

To evaluate the linearity, the calibration standards of NA at eight concentrations covering the range from 6.57 to 5255 $ng \cdot mL^{-1}$ in human plasma were extracted and analyzed in three separate runs. Blank plasma samples were analyzed to confirm the absence of interferences but not used to construct the calibration curves. The peak area ratios for NA and IS were measured and the calibration curves without zero

concentration were constructed by plotting the peak-area ratio of NA to IS versus the concentrations (x) of NA, using weighted least squares linear regression ($1/x$). The LOD (2 ng/mL) is the lowest amount of analyte in a sample that is able to be detected, but not necessarily quantitated. The value of LOD was calculated at a signal-to-noise ratio (S/N) of about 3 from blank plasma samples. The LLOQ for NA was defined as the lowest concentration of the calibration curve and could be quantified with an acceptable precision (relative standard deviation, RSD) and accuracy (relative error, RE). The value of LLOQ was determined at a signal-to-noise ratio (S/N) of about 10. Five replicates were quantitatively determined with acceptable precision (RSD, within 20%) and accuracy (RE, 80–120%) in this assay.

2.6.3. Precision and accuracy

The intra-day precision and accuracy of the method were evaluated by analyzing five replicates of QC samples at three concentration levels of NA (13.1, 263 and 2628 $ng \cdot mL^{-1}$) on the same day. The within-batch (inter-day) precision and accuracy of this method were operated on the same QC samples by analyzing five replicates on three consecutive days (n = 5 series per day). The concentration of each sample was calculated using standard curve prepared and analyzed on the same batch and day. The precision was determined as the RSD and the accuracy as the RE.

2.6.4. Extraction recovery and matrix effect

The extraction recoveries of NA were determined and repeated for six times at three QC concentration levels of 13.1, 263 and 2628 $ng \cdot mL^{-1}$. Recoveries were calculated by comparing the NA/IS peak area ratio (*R1*) obtained from extracted plasma samples

with those (*R2*) from the standard solutions at the same concentration. The matrix effect was measured by comparing the peak response of sample spiked post-extraction (*R2*) with that of pure standard solution containing equivalent amount of the compound (*R3*). The ratio values (*R2*/*R3*×100) % was used to evaluate the matrix effect. The extraction recovery and matrix effect of IS were also evaluated using the same procedure.

2.6.5. Stability

The stability of NA in human plasma was evaluated by analyzing three replicates of plasma samples at concentrations of 13.1, 263 and 2628 $ng \cdot mL^{-1}$ during the sample storage and processing procedures. The freeze-thaw stability was conducted on QC plasma samples at three concentration levels were stored at -40°C for 24 h and thawed at room temperature. When completely thawed, the samples were refrozen for 24h under the same conditions. The freeze-thaw cycles were repeated twice, and the samples were analyzed after three freeze (-40°C) –thaw (room temperature) cycles. The short-term stability was evaluated after the exposure of QC plasma samples at three concentration levels were kept at room temperature for a period that exceeded the routine preparation time of the samples (around 6 h) and the ready-to-inject samples (after extraction, in the mobile phase) at room temperature for 48 h. The long-term stability was estimated by processing QC plasma samples at three concentration levels kept at low temperature (-40°C) for a period of one month. The post-preparative stability was conducted by reanalyzing extracted QC samples kept under autosampler conditions (4°C) for 12 h. The concentrations of QC samples were

calculated by using calibration curves of freshly prepared standards. The stability of stock solutions of NA and the IS in methanol at 4 ºC for two months and at room temperature for 4 h was also assessed. The mean peak areas of three replicates of NA and IS samples were compared with those from freshly prepared solutions at the same concentration.

2.7. Application to pharmacokinetic study

Thirty healthy male subjects (Age: 22.5 ± 3.2 years; Height: 175.5 ± 8.5 cm; Weight: 68.5 ± 5.5 kg.) were enrolled in the study according to the clinical protocol. All the healthy volunteers signed informed consent after the assessments of physical examination, electrocardiogram, medical history and standard laboratory test results including blood cell, urinalysis and biochemical profile.

The method was applied to evaluate the bioequivalence of two sustain-release tablet formulations of NA in healthy subjects. The study was approved by the Human Ethics committee of Hunan Xiangya Hospital. Informed consent was obtained from all subjects after explaining the aims and risks of the study. Thirty healthy male volunteers received a single dose in a two-way randomized crossover design with a week's washout period between doses. An indwelling cannula was placed in one arm for blood sampling. Before sampling, about 0.5 mL blood was discarded, then, 5 mL blood samples were collected in heparinized tubes at the following times on the days of the pharmacokinetic measurements: immediately prior to drug administration (0 h), and at 1, 2, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 10, 12 and 15 h after drug administration, and centrifuged at 3,000 rpm for 10 min to separate the plasma fractions. The collected

plasma samples were stored at -40°C until analysis. Bioequivalence of the two formulations was assessed according to US-FDA methodology. The pharmacokinetic parameters were calculated by model using "Drug and statistics for windows" software (DAS), version 1.0.

The developed method was used to determine the plasma concentrations of NA and the pharmacokinetics parameters were calculated. The maximum plasma concentration (C_{max}) and their time of occurrence (T_{max}) were calculated directly from the measured data. The elimination rate constant (k_e) was calculated by log-linear regression of concentrations observed during the terminal phase of elimination, and the elimination half-life ($T_{1/2}$) was then calculated as $0.693/k_e$. The area under the plasma concentration-time curve (AUC_{0-t}) to the last measurable plasma concentration (C_t) was calculated with the linear trapezoidal rule. The area under the plasma concentration-time curve to time infinity ($AUC_{0-\infty}$) was calculated as $AUC_{0-t} + C_t/k_e$.

3. Results and discussion

3.1. Method development and optimization

3.1.1. Sample preparation

Sample preparation is a critical step for accurate and reliable LC-MS/MS assays. A sample preparation procedure should reduce interferences from the biological sample and be sensitive and robust for the analysis. The most widely employed biological sample preparation methodologies currently are liquid-liquid extraction (LLE) [48], protein precipitation (PPT) [49] and solid-phase extraction (SPE) [50].

In the early stage of method development, a PPT desalting method was employed to separate NA from plasma samples, but strong ion suppression from the endogenous substances in plasma occurred. Although it could be decreased by chromatographic separation, the short run time would be sacrificed. And the SPE method is too expensive to deal with 600 plasma samples. Most methods used deproteinization with acetone or acetone–water [51], n-butanol [35], acetonitrile [46], acetoacetic acid [52] or perchloric acid [53]. It was also reported that acetone was used to remove protein which was followed by extraction with chloroform to remove the endogenous lipophilic components while NA was contained in the aqueous layer [54], but this reported method for sample preparation was complicated. In this study, n-butanol was used to extract NA and 1M hydrochloric acid was added into the plasma to unlock the drug-protein binding [35]. LLE was so advantageous that the technique not only extracted NA and IS with sufficient efficiency and specificity, but also minimized the experimental cost. Quinoline-3-carboxylic acid was chosen as the IS due to its similarity with the analytes in structure, stability, chromatographic and mass spectrographic behaviors.

3.1.2. Optimization of the chromatographic condition

Different types of column were referred in the literature, including C18 and CN columns [31, 32, 34, 37, 55]. Therefore, an Hypersil C_{18} column (4.6 × 150 mm i.d., 5 μm) and a Hypersil-Gold-CN column (4.6 × 150 mm i.d., 5 μm) were tested to obtain optimized response, suitable retention time and good peak shapes for NA and IS. At last, the Hypersil-Gold-CN column was selected for all analysis since it provided symmetrical peak shape and obtained the highest intensity to NA at 35°C. The

separation and ionization of NA and IS could be easily affected by the composition of mobile phase. The mobile phase pH affected not only the retention time, but also the ionization efficiency of NA and IS. The retention time would be longer if the mobile phase pH value was less than 4.2, although it would be shorter if the mobile phase pH value was more than 4.2. The acidity of mobile phase benefited to the ionization of NA and IS. The acidity of mobile would keep the NA neutral and easily negatively charged. The ammonium formate would help to reduce the interference from endogenous substances. Therefore, the mobile phase was employed.

The optimization of the chromatographic conditions was not only focused on chromatographic separation but also on short retention time. The organic solvent compositions and percentage in the mobile phase were investigated for separating NA from other interferences. Finally, a mobile phase consisted of acetonitrile and 20 mM ammonium formate with 0.1% formic acid (pH: 4.2) was used for isocratic elution to separate and accurately determine the concentrations of NA in plasma. Under the optimized conditions, a flow rate of 0.75 $mL \cdot min^{-1}$ with the total run time of 3.5 min for each sample was established.

3.1.3. Optimization of the LC-MS/MS condition

Mass spectrometry operation parameters were accordingly optimized for the determination of NA and IS. The present study aimed to select an appropriate ionization mode in LC-MS/MS analysis. NA and IS were scanned in both ESI and atmospheric pressure ionization (APCI) using injection standard solutions with positive and negative ion modes. Compared to APCI source, ESI was selected because

it offered better efficiency of ionization for NA and IS. In different ionization modes, standard solutions of NA and IS were directly infused along with mobile phase and the signal intensity of negative ions were stronger than positive ions, indicating that the negative mode was much more sensitive. In the precursor ion full-san spectra, the most abundant peaks were quasi-molecular ion $[M-H]^-$ peaks at *m/z* 122.0 and 171.9 for NA and IS, respectively. Therefore, these ions were chosen as parent ions for fragmentation in MRM mode. The product ion spectra of the parent ions revealed that the predominant daughter fragments were at *m/z* 78.1 and 127.8 for NA and IS, respectively (Fig. 2). The collision energy in the product tandem mass spectrometry mode was investigated to optimize the sensitivity, and the optimal values were found to be 17 and 22 V for NA and IS, respectively.

3.2. Method validation

3.2.1. Selectivity

The LC-MS/MS method has a good selectivity due to a better resolution of NA and only ions derived from the analytes of interest were monitored. Fig.3 (A-C) provided the typical MRM chromatograms of a blank plasma, a blank plasma spiked with NA (263 $ng \cdot mL^{-1}$) and IS (1×10^3 $ng \cdot mL^{-1}$) and a plasma sample from a healthy volunteer in 3 h after oral administration of 3g nicotinic acid tablet. All of the ratios of the peak area resolved in the blank sample compared with that resolved in the mobile phase were between 85 and 115%, which indicated that little endogenous substances interfered the analysis of NA and IS in this method. As shown, no interference from endogenous substance at the retention time of NA and IS was observed, which was

approximately 2.51 and 2.56 min for NA and IS, respectively.

3.2.2. Linearity and lower limit of quantification

The calibration curves were prepared daily that showed good linearity in the range 6.57-5255 ng·mL^{-1} for NA. Regularly, the calibration curve covers 10× range and lately extended ones up to 100× range. However, the concentration of NA in human plasma could exceed upper limit of quantification even though the wide test range was validated in this study. Therefore, the test range was confirmed to quantify most of NA concentration in human plasma directly. The concentration of NA in some plasma samples exceeded the concentration range of the constructed calibration curve. During the course of this study, these plasma samples were diluted using blank plasma samples pre-treated following the sample preparation procedure in **2.4.** to make them detected in the concentration range of the constructed calibration curves. The mean regression equation from five replicate calibration curves on different days was: R = (0.00106±0.00006)C, r = 0.9987, where R is the peak-area ratio of NA to IS and C is the concentration of NA in plasma and the linear least-squares regression with a weighing index of 1/C. Results of the calibration curves for NA LC-MS/MS determination are provided in Table 1.

The present method offered an LLOQ of 6.57ng·mL^{-1} for NA using only 0.2 mL plasma (Fig. 4). The RSD (n = 6) was 6.3%, and accuracy was 1.5% at this concentration. Compared with the methods reported using MS/MS detection [45, 46, 53], a higher sensitivity for NA was obtained in this study. The LOD in the reported method was 17 ng/mL [56], while the other referred methods did not report the values of LODs

[45, 46]. In this study, the LOD was 2 ng·mL^{-1}.

3.2.3. Within- and between-batch precision and accuracy

The overall intra- and inter-day precision (RSDs) and accuracy for NA were listed in Table 2. In this assay, the inter-day precision was within 1.81–7.17% and the intraday precision was within 2.30–6.70% or less for each QC level. The accuracy was within ±8.16%. These results indicated that the developed method was precise and accurate.

3.2.4. Extraction recovery and matrix effect

The extraction recovery determined for NA was shown to be consistent, precise and reproducible (Table 3). The extraction recoveries of NA and IS were more than 70%. Matrix effect is due to co-elution of some components present in biological samples. These components may not give a signal in MRM of target analyte but certainly decrease or increase the analyte response dramatically to affect the sensitivity, accuracy and precision of the method, so evaluating matrix effect is essential for an HPLC–MS/MS method. All the calculated ratios between 85% and 115% indicated that no endogenous co-elutes influenced the ionization of NA and IS.

3.2.5. Stability

Table 4 summarizes the freeze-thaw stability, short-term stability, long-term stability and post-preparative stability data of NA. No chemical or biological degradation of NA were observed during sample storage. All the data showed good stability during these tests and there were no stability related problems in the routine analysis of samples for pharmacokinetic, bioavailability or bioequivalence studies.

For the stability of working solutions, the result showed the working solutions were

stable at room temperature (25°C) for 6 hours (RSDs: 1.9–5.2%).

3.3. Clinical applications

The developed method was successfully applied to a pharmacokinetic study of sustain-release capsules. A randomized, one-period and single-dose protocol was adopted. The method described above was successfully applied to a bioequivalence study in which plasma concentrations of NA were determined for 15 h after oral administration a dose of 1500 mg NA sustain-release reference or test capsules. A representative chromatogram of the plasma sample was plotted in Fig. 3 (C), which was collected at 3 h from a subject after oral administration with NA sustain-release capsule. After oral administration of NA, the C_{max} and T_{max} values were similar to those reported in the literature [14]. The variations of the NA concentrations in the plasma as well as the area under the plasma concentration *vs.* time curve (AUC), observed in this study need further research. In addition, the two capsule formulations were found to be equivalent (Fig. 5) with calculated 90% confidence intervals for test/reference ratios of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ within the 80–125% interval required by the US Food and Drug Administration. The calculated pharmacokinetic parameters were summarized in Table 5.

3.4. Comparison with reported methods

The advantages of this method include the use of good sensitivity, high extraction efficiency, less organic solvents consumption and short run time. The developed method is more sensitive than other procedures for determination of NA. Recently, LC-MS/MS methods had been developed to quantify NA in human plasma [43, 46]. It

was reported that the standard curve of NA was linear in the range of 1.25-320 μg·mL^{-1} with a low determination limit of 1.25 μg·mL^{-1} [43], while another method demonstrated that the test range was 2.0-3000 ng·mL^{-1} with a lower limit of detection at 2.0 ng·mL^{-1} [46]. Positive ionization mode was used in both developed methods, while the later report obtained a higher sensitivity [46]. However, the run time of alter method was 9 min, while in this reported method only 3.5 min. The benefit of this shorter run was proven in this study by analyzing more than 600 plasma samples. Although the LLOQ of this method is higher than previous published methods, the linearity range is wider and higher than 3000ng/mL than previous reported. In this study, negative ionization mode was employed to achieve a good sensitivity with LLOQ of 6.57 ng·mL^{-1} and linear in the range of 6.57-5255 ng·mL^{-1} for NA. So, the developed method was useful and sensitive for determination of NA in human plasma.

4. Conclusions

A sensitive, specific, accurate and precise LC-(-) ESI-MS/MS method was validated for the determination of NA in human plasma. The method was fully validated by following FDA guidelines. The proposed method has a good sensitivity for the analyte in different individuals human plasma samples. The short run time of 3.5 min per sample renders the method usefulness in high-throughput bio-analysis. The matrix interference is absent on the evidence of the precision values for the slopes of calibration curves from different individuals human samples. This method has successfully been validated and applied for analyzing samples in pharmacokinetic studies of NA.

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References

1. R. Altschul, A. Hoffer and J. D. Stephen, *Arch. Biochem. Biophys.*, 1955, **54**, 558.
2. E. T. Bodor and S. Offermanns, *Br. J. Pharmacol.*, 2008, **153**, S68.
3. L. A. Carlson, *J. Intern. Med.*, 2005, **258**, 94.
4. V. S. Kamanna, A. Vo and M. L. Kashyap, *Curr. Opin. Cardiol.*, 2008, **23**, 393.
5. A. Rennick, R. Kalakeche, L. Seel and B. Shepler, *Pharmacotherapy*, 2013, **33**, 683.
6. F. Montecucco, A. Quercioli, F. Dallegri, G. L. Viviani and F. Mach, *Expert Rev. Cardiovasc. Ther.*, 2010, **8**, 1457.
7. L. A. Carlson and L. Oro, *Acta Med. Scand.*, 1962, **172**, 641.
8. W. B. Parsons, Jr., R. W. Achor, K. G. Berge, B. F. McKenzie and N. W. Barker, *Proc. Staff Meet. Mayo Clin.*, 1956, **31**, 377.
9. W. B. Parsons, Jr. and J. H. Flinn, *AMA Arch. Intern. Med.*, 1959, **103**, 783.
10. M. Lukasova, J. Hanson, S. Tunaru and S. Offermanns, *Trends Pharmacol. Sci.*, 2011, **32**, 700.
11. L. A. Carlson, *Curr. Opin. Cardiol.*, 2006, **21**, 336.
12. L. Kaijser, B. Eklund, A. G. Olsson and L. A. Carlson, *Med. Biol.*, 1979, **57**, 114.
13. L. A. Carlson, L. G. Ekelund and L. Oro, *Acta Med. Scand.*, 1968, **183**, 423.
14. P. J. Neuvonen, L. Roivas, K. Laine and O. Sundholm, *Br. J. Clin. Pharmacol.*, 1991, **32**, 473.

15. K. K. Reddi and E. Kodicek, *Biochem. J.*, 1953, **53**, 286.

16. A. Pyka, A. Niestroj, A. Szarkowicz and J. Sliwiok, *JPC-J. Planar Chromatogr.*, 2002, **15**, 410.

17. A. Pyka, J. Sliwiok and A. Niestroj, *Acta Pol. Pharm.*, 2003, **60**, 327.

18. A. Pyka and W. Klimuok, *J. Liq. Chromatogr. Relat. Technol.,* 2007, **30**, 2419.

19. U. Hubicka, J. Krzek and J. Luka, *J. AOAC Int.*, 2008, **91**, 1186.

20. W.-B. Shim, M. E. Yakovleva, K.-Y. Kim, B.-R. Nam, E. S. Vylegzhanina, A. A. Komarov, S. A. Eremin and D. H. Chung, *J. Agric. Food Chem.*, 2009, **57**, 791.

21. W. Parys and A. Pyka, *J. Liq. Chromatogr. Relat. Technol.*, 2010, **33**, 1038.

22. B. Becker and K. Hummel, *Arzneimittelforschung*, 1990, **40**, 573.

23. K. Shibata and H. Taguchi, *Chromatogr. Sci. Ser.*, 2000, **84**, 325.

24. Y. Z. Guo and Y. Liu, *Huaxue Yu Nianhe*, 2003, **153**, 155.

25. G. A. Smythe, A. Poljak, S. Bustamante, O. Braga, A. Maxwell, R. Grant and P. Sachdev, *Adv. Exp. Med. Biol.*, 2003, **527**, 705.

26. Z. Z. Cheng, Z. H. Jia, Y. Chen, L. Y. Chen and H. Li, *Chin. Chem. Lett.*, 2006, **17**, 1057.

27. F. L. Zhuang, Q. X. Cui and R. M. Liu, *Lihua Jianyan, Huaxue Fence*, 2010, **46**, 506.

28. S. Talath, P. J. Shirote, W. J. Lough and A. K. Gadad, *Arzneimittel-Forschung Drug Res.*, 2006, **56**, 631.

29. U. Holler, C. Brodhag, A. Knobel, P. Hofmann and V. Spitzer, *J. Pharm.*

Biomed. Anal., 2003, **31**, 151.

30. D. Liang, J. Ma, B. Wei, I. O. Poon, E. C. Bell and T. R. Bates, *J. Chromatogr. B*, 2008, **863**, 172.

31. J. Chen, W. Lu and X. Jiang, *Yaowu Fenxi Zazhi*, 2004, **24**, 349.

32. P. Wang, S. Gu, D. Yang, G. Wu, L. Li and H. Chen, *Zhongguo Yaoshi (Wuhan, China)*, 2005, **8**, 94.

33. R. Lang, E. F. Yagar, R. Eggers and T. Hofmann, *J. Agric. Food Chem.*, 2008, **56**, 11114.

34. M. Ge, L. Wei, K. Fang, L. Huang and W. Li, *Zhongguo Linchuang Yaolixue Zazhi*, 2011, **27**, 118.

35. P. K. Zarzycki, P. Kowalski, J. Nowakowska and H. Lamparczyk, *J. Chromatogr. A*, 1995, **709**, 203.

36. M. Iwaki, E. Murakami and K. Kakehi, *J. Chromatogr. B*, 2000, **747**, 229.

37. M. Iwaki, E. Murakami, M. Kikuchi, A. Wada, T. Ogiso, Y. Oda, K. Kubo and K. Kakehi, *J. chromatogr. B*, 1998, **716**, 335.

38. N. Nuchtavorn and L. Suntornsuk, *J. Chromatogr. Sci.*, 2012, **50**, 151.

39. J. Zhang, U. Chakraborty and J. P. Foley, *Electrophoresis*, 2009, **30**, 3971.

40. A. V. C. Simionato, F. Mauro Lancas and M. Antonio Ruggiero, *J. Liq. Chromatogr. Relat. Technol.*, 2006, **29**, 349.

41. Y. Lu, C. Wu and Z. Yuan, *Fitoterapia*, 2004, **75**, 267.

42. A. Leporati, D. Catellani, M. Suman, R. Andreoli, P. Manini and W. M. A. Niessen, *Anal. Chim. Acta*, 2005, **531**, 87.

43. L. Wang, Y. P. Qin, X. L. Gong, F. Nan, J. Xiang, Q. Yu and M. Z. Liang, *Sichuan DaXue Xue Bao*, 2010, **41**, 1051.

44. R. Mullangi and N. R. Srinivas, *Biomed. Chromatogr.*, 2011, **25**, 218.

45. P. Pfuhl, U. Karcher, N. Haring, A. Baumeister, M. A. Tawab and M. Schubert-Zsilavecz, *J. Pharm. Biomed. Anal.*, 2005, **36**, 1045.

46. M. Liu, D. Zhang, X. Wang, L. Zhang, J. Han, M. Yang, X. Xiao, Y. Zhang and H. Liu, *J. Chromatogr. B*, 2012, **904**, 107.

47. G.F. Industry, Guidance for Industry, Bioanalytical Method Validation (May 2001), http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatory/Information/Guidances/UCM070107.pdf.

48. Y. Q. Tang, N. D. Weng, *Bioanalysis*, 2013, **5**, 1583.

49. M. E. Casas, M. Hansen, K. A. Krogh, B. Styrishave, E. Bjorklund, *J. Chromatogr. B*, 2014, **962**, 109.

50. W. A. W. Ibrahim, L. I. Abd Ali, A. Sulaiman, M. M. Sanagi, H. Y. Aboul-Enein, *Crit. Rev. Anal. Chem.* 2014, **44**, 233.

51. A. C. Li, Y. L. Chen, H. Junga, W. Z. Shou, X. Jiang and W. Naidong, *Chromatographia*, 2003, **58**, 723.

52. V. B. Ravi, R. Mullangi, J. K. Inamadugu, N. R. Pilli, R. Gajula and V. Ponneri, *Biomed. chromatogr.*, 2012, **26**, 1436.

53. M. Pelzer, S. Northcott and G. Hanson, *J. Liq. Chromatogr.*, 1993, **16**, 2563.

54. Y. Tsuruta, K. Kohashi, S. Ishida and Y. Ohkura, *J. chromatogr.*, 1984, **309**,

309.

55. P. Wang, S. Gu, D. Yang, G. Wu, L. Li, C. Hu, C. Wu and H. Chen, *Zhongguo Linchuang Yaolixue Zazhi*, 2004, **20**, 299.

56. Z. Chen, B. Chen and S. Z. Yao, *Anal. Chim. Acta*, 2006, **569**, 169.

Figure legends

Fig.1. Chemical structures of nicotinic acid (NA) and internal standard (IS).

Fig.2. Negative ion mass scan spectra of NA (A) and IS (B).

Fig.3. The typical MRM chromatograms of a blank plasma (A1 and A2); blank plasma spiked with NA (26.3 $ng \cdot mL^{-1}$**) (B1) and IS (**1.0 $\mu g \cdot mL^{-1}$**) (B2); 3h samples after oral administration of 3 g sustained-release capsules of NA (C1) and spiked with IS (**1.0 $\mu g \cdot mL^{-1}$**) (C2).**

Fig.4. The MRM chromatogram calculated for LLOQ of NA (A1) with IS (1.0 $\mu g \cdot mL^{-1}$**) (A2).**

Fig.5. Mean plasma concentration-time profiles of NA in human plasma after oral administration of 1500 mg nicotinic acid sustained-release reference or test capsules, each point and bar represents the mean ± S.D. (n = 30).

Table 1. Results of calibration curves for NA by LC-MS/MS analysis (n = 5, ng·mL^{-1}).

Conc. added [a]	Mean conc. measured ± SD [b]	RSD（%）	Accuracy（%）
6.57	7.22 ± 0.05	0.64	110
13.1	12.8 ± 0.03	0.26	97.4
26.3	24.4 ± 0.39	1.61	92.9
56.6	51.8 ± 0.89	1.72	98.5
263	259 ± 17.6	6.80	98.6
1314	1287 ± 149	11.6	97.9
2628	2833 ± 324	11.5	108
5255	5083 ± 639	12.6	96.7

[a] conc. is the abbreviation of concentration.

[b] SD: standard deviation.

Table 2. Intra- and inter-day precision and accuracy for LC-MS/MS assay of NA in human plasma (ng·mL^{-1}).

Conc. added[a]	Intra-batch (n = 5)			Inter-batch (n = 15)		
	Mean conc. measured±SD[b]	Accuracy (%)	Precision (RSD, %)	Mean conc. measured±SD[b]	Accuracy (%)	Precision (RSD, %)
13.1	13.4 ± 0.96	102	7.17	13.4 ± 0.89	102	6.70
263	252 ± 4.55	95.8	1.81	242 ± 5.57	91.9	2.30
2628	2528 ± 59.9	96.2	2.37	2491 ± 124	94.8	4.97

[a] conc. is the abbreviation of concentration.
[b] SD: standard deviation.

Table 3. Recovery, matrix effect and process efficiency of NA using LLE.

Conc. added[a] (ng/mL)	Recovery (%)	Matrix effect (%)
13.1	70.3 ± 2.58	76.3 ± 5.88
263	71.7 ± 8.92	75.6 ± 3.34
2628	72.4 ± 7.50	87.4 ± 6.25

[a] conc. is the abbreviation of concentration.

Table 4. Stability results for NA in human plasma at different QC levels (n = 5, ng·mL^{-1}).

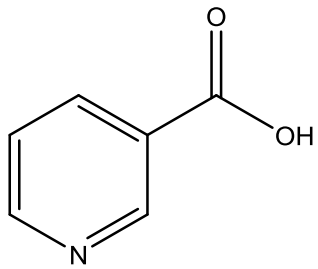
Storage conditions	Conc Added[a] (CA)	Mean conc. measured (MCM)±SD[b]	RSD (%)	RE (%)[c]
	13.1	13.0 ± 1.22	9.35	-0.76
Freeze-thaw (-20°C, three cycles)	263	238 ±5.13	2.20	-9.59
	2628	2474 ± 103	4.15	-5.86
	13.1	12.5 ± 1.62	13.0	-4.79
Long term (-20°C, 60 days)	263	249 ± 4.16	1.67	-5.15
	2628	2635 ± 112	4.26	0.27
	13.1	12.4 ± 1.84	14.8	-5.63
Short-term （6h, 25°C）	263	247 ± 7.29	2.96	-6.20
	2628	2775 ± 110	3.97	5.59
	13.14	13.2 ± 1.37	10.5	0.76
Post-preparative （12h, 4°C）	263	237 ± 2.16	0.91	-9.97
	2628	2470 ± 94.2	3.81	-6.00

[a] conc. is the abbreviation of concentration.
[b] SD: standard deviation.
[c] RE (%) = (MCM/CA × 100) − 100.

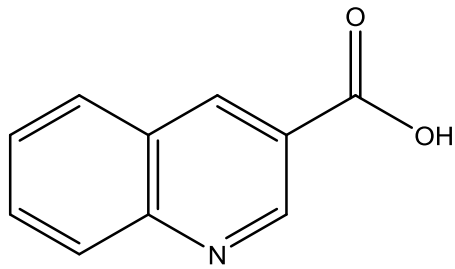
Table 5. The main pharmacokinetic parameter of NA after single oral dose of 3 g to healthy subjects (mean ±SD, n = 30).

Pharmacokinetic parameters	Reference drug	Test drug
C_{max} (ng/mL)	8419 ± 6833	7828 ± 6293
T_{max}	4.32 ± 1.20	4.47 ± 1.33
$T_{1/2}$	4.64 ± 5.10	6.08 ± 11.0
$AUC_{0\text{-}15h}$ (ng·h/mL)	18819 ± 14879	17682 ± 13987
$AUC_{0\text{-}\infty}$ (ng·h/mL)	18984 ± 14731	17857 ± 13836
F (%)	114 ± 58.6	

$T_{1/2}$: half-life of elimination; T_{max}: time of maximum plasma concentration; C_{max}: maximum plasma concentration; AUC: area under the plasma concentration vs time curve.



A. Nicotinic acid (NA, MW: 123)



B. Quinoline-3-carboxylic acid (IS, MW:173)

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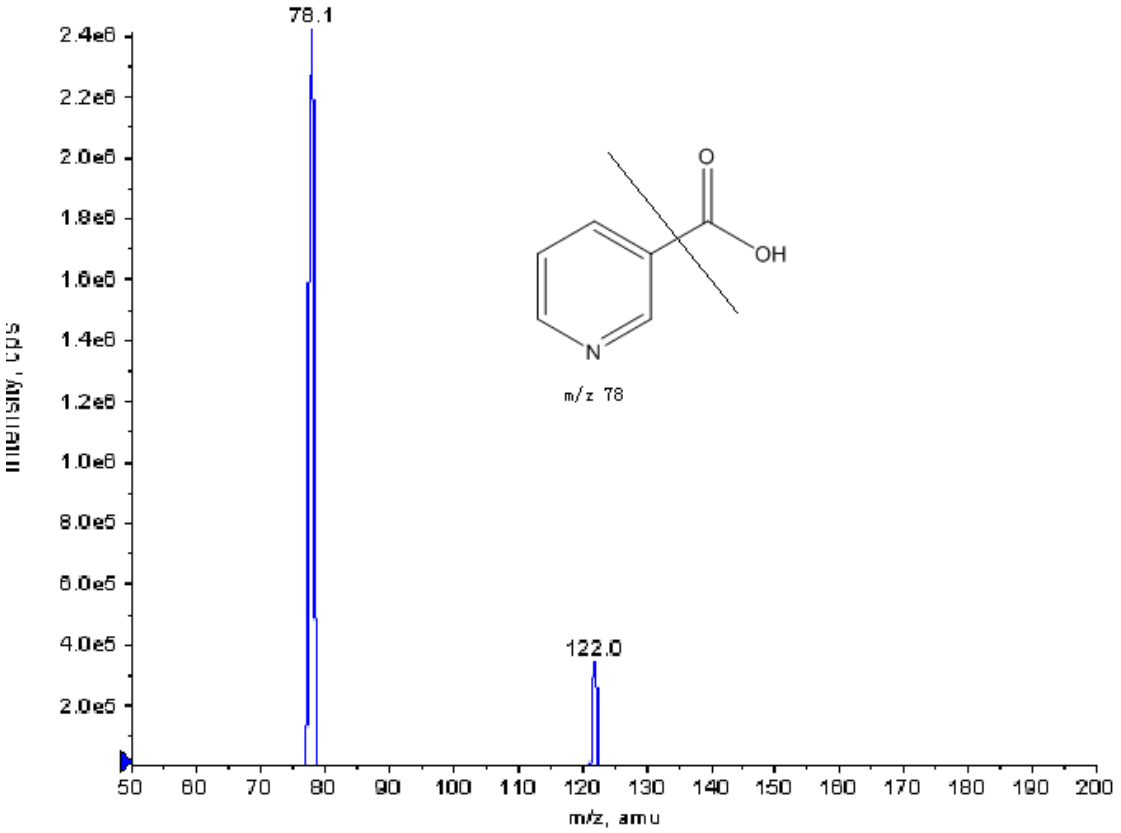
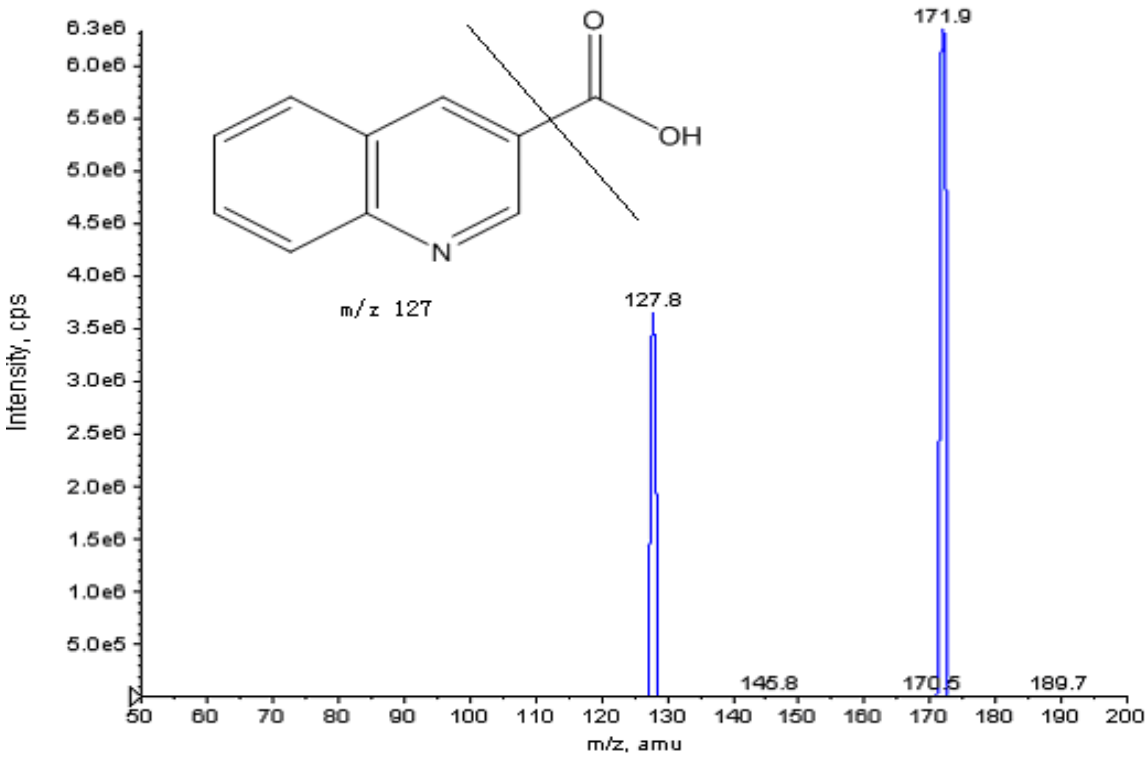
Fig. 1



A: Nicotinic acid (NA, *m/z:* 122.0→*m/z*:78.1)



B: Internal standard (IS, *m/z* :171.9→*m/z*:127.8)

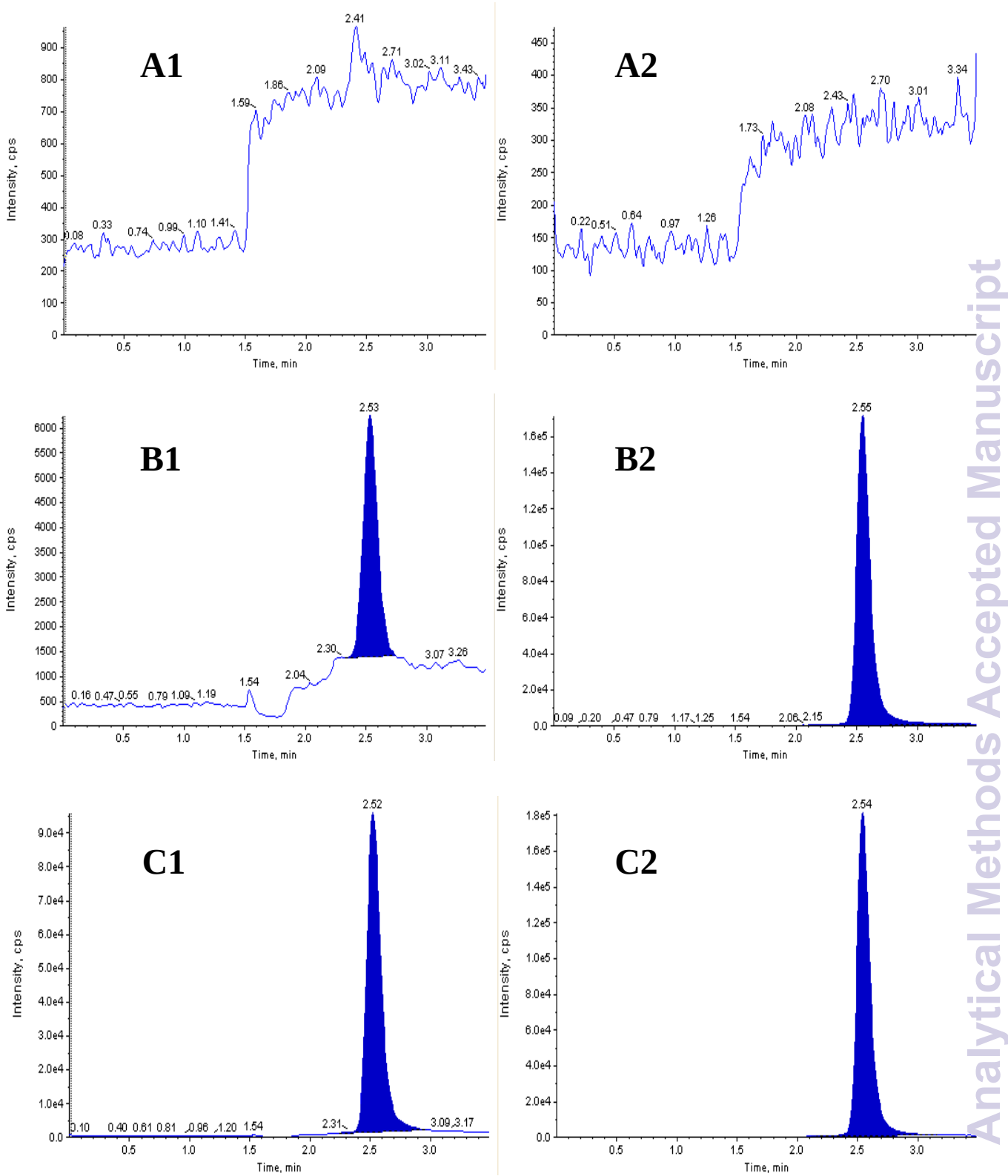


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Fig. 3

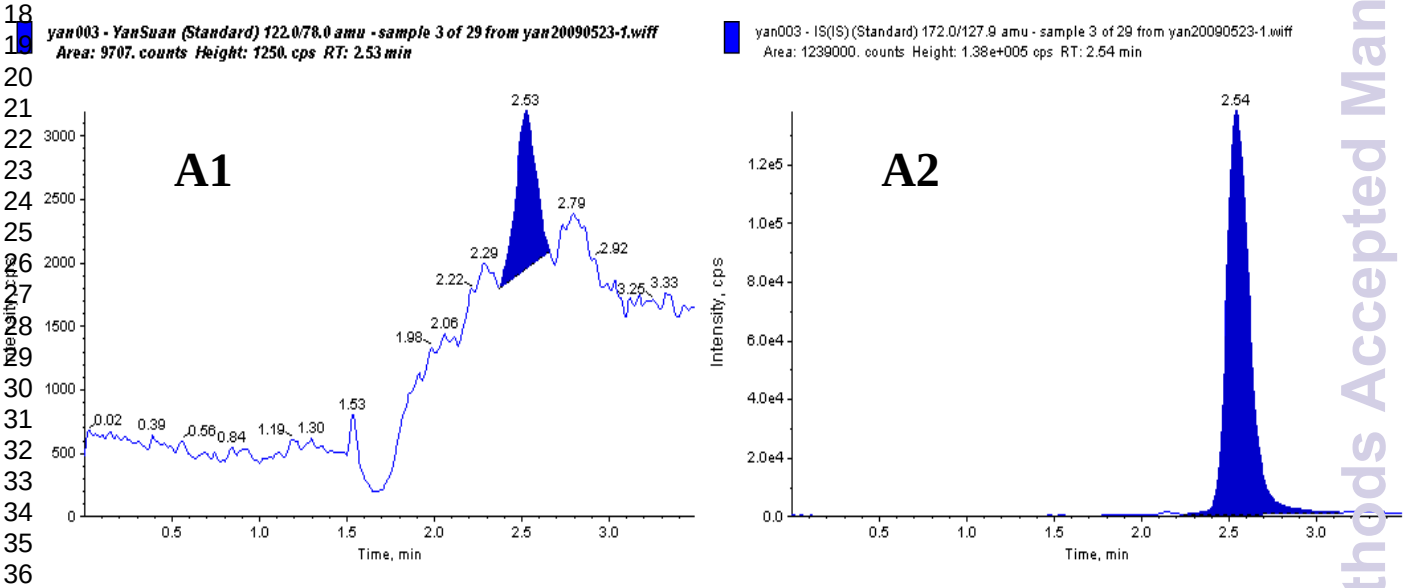

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Fig. 4

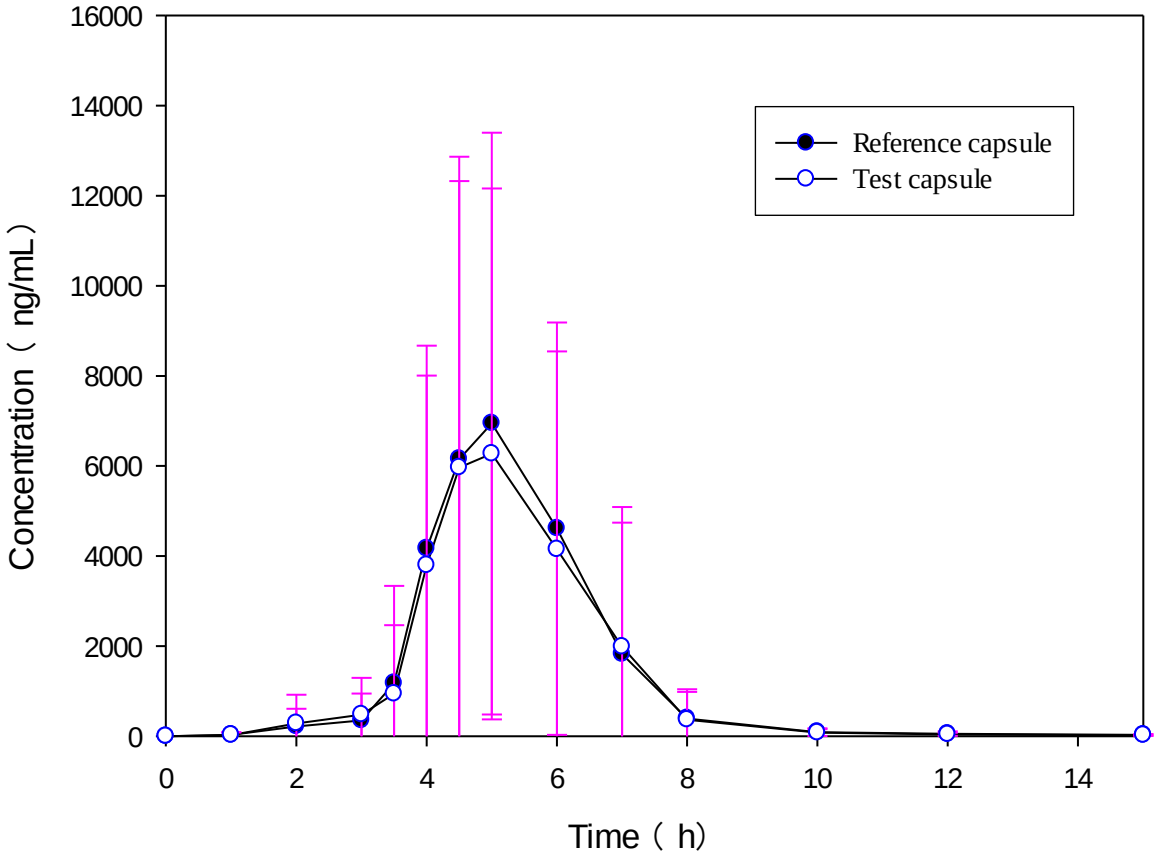

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Fig. 5