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Simultaneous determination of myristyl nicotinate, nicotinic acid, and nicotinamide in rabbit plasma by liquid chromatography–tandem mass spectrometry using methyl ethyl ketone as a deproteinization solvent

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Abstract

Myristyl nicotinate (Nia-114) is an ester prodrug being developed for delivery of nicotinic acid (NIC) into the skin for prevention of actinic keratosis and its progression to skin cancer. To facilitate dermal studies of Nia-114, a novel liquid chromatography–tandem mass spectrometry (LC–MS/MS) method using methyl ethyl ketone (MEK) as a deproteinization solvent was developed and validated for the simultaneous determination of Nia-114, NIC, and nicotinamide (NAM) in rabbit plasma. NAM is the principal metabolite of NIC, which is also expected to have chemopreventive properties. The analytes were chromatographically separated using a Spherisorb Cyano column under isocratic conditions, and detected by multiple reaction monitoring (MRM) in positive-ion electrospray ionization mode with a run time of 9 min. The method utilized a plasma sample volume of 0.2 ml and isotope-labeled D_4 forms of each analyte as internal standards. The method was linear over the concentration range of 2–1000, 8–1000, and 75–1000 ng/ml, for Nia-114, NIC, and NAM, respectively. The intra- and inter-day assay accuracy and precision were within ±15% for all analytes at low, medium, and high quality control standard levels. The relatively high value for the lower limit of quantitation (LLOQ) of NAM was demonstrated to be due to the high level of endogenous NAM in the rabbit plasma (about 350 ng/ml). Endogenous levels of NIC and NAM in human, dog, rat, and mouse plasma were also determined, and mean values ranged from <2 ng/ml NIC and 38.3 ng/ml NAM in human, to 233 ng/ml NIC and 622 ng/ml NAM in mouse. Nia-114 was generally unstable in rabbit plasma, as evidenced by loss of 44–50% at room temperature by 2 h, and loss of 64–70% upon storage at −20 °C for 1 week, whereas it was stable (<7% loss) upon storage at −80 °C for 1 month.
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Keywords: Myristyl nicotinate; Nicotinamide; Nicotinic acid; Cancer chemoprevention; LC–MS/MS; Plasma

1. Introduction

Myristyl nicotinate (Nia-114) is an ester prodrug being developed for delivery of nicotinic acid (NIC) into the skin for prevention of actinic keratosis and its progression to skin cancer. It has been demonstrated that nicotinic acid prevents skin cancer and blocks immune suppression in a mouse model [1]. Nicotinamide (NAM) and nicotinamide adenine dinucleotide (NAD) are well known to modulate the expression of stress proteins and ADP-ribose polymer metabolism in response to DNA damage [1,2]. Thus, raising the cellular concentration of NIC and NAM has been hypothesized to protect tissues from cancer, e.g. topical application of NAM has been shown to reduce photocarcinogenesis in mice [2]. Recently, Nia-114 has been shown to stimulate epidermal differentiation in photodamaged skin, increasing skin NAD content and strengthening the skin barrier of human subjects [3,4].

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To facilitate the preclinical development of Nia-114, a sensitive and specific bioanalytical method for the quantitation of Nia-114, NIC, and NAM in rabbit plasma is required to be used in pharmacokinetic studies of this agent. For practical application to samples generated in the preclinical phase of drug development, the bioanalytical method was designed to meet the following requirements: (1) simultaneous measurement of Nia-114, NIC, and NAM within a run time of <10 min; (2) lower limit of quantitation (LLOQ) of <10 ng/ml for Nia-114 and NIC; (3) linearity to 1000 ng/ml for all three analytes; and (4) performance of assay during validation runs in accordance with current FDA guidelines [5].

Nia-114 is a lipophilic molecule, whereas NIC is highly polar and water soluble, and contains both basic and acidic functionalities. NAM has solubility properties intermediate to Nia-114 and NIC. The different solubility properties of Nia-114 and NIC present difficulties in using a single extraction technique to sufficiently recover all three compounds. Analytical separation of the three compounds within a reasonable run time also poses a challenge using a single chromatographic method.

In a review of chromatographic and capillary electrophoretic (CE) methods for analysis of NIC and/or its metabolites in biological fluids, protein precipitation techniques or solid-phase extraction (SPE) were primarily used [6]. None of the methods demonstrated an extraction technique and analytical separation that would meet the requirements of the present study. This review revealed that protein precipitation techniques utilizing solvents (such as acetone or acetonitrile) or perchloric acid suffered from insufficient sample cleanup, resulting in long chromatographic run times. Many of the HPLC methods used gradient-elution reverse-phase chromatography with ion-pairing agents. This technique is not appropriate due to the long chromatographic run times and incompatibility of the non-volatile agents with liquid chromatography–tandem mass spectrometry (LC–MS/MS). The reported sensitivities by CE or HPLC with UV or fluorescence detection [7–9] are inadequate. One LC–MS/MS method [10] has been reported for measurement of NIC and six metabolites in human plasma using SPE and normal phase chromatography with a silica column, and the linear range was 10–2000 ng/ml for each analyte. However, the methods used in this investigation did not appear to be compatible with the broad range of hydrophobicities of Nia-114, NIC, and NAM.

Therefore, we developed and validated a novel liquid chromatography–tandem mass spectrometry method using methyl ethyl ketone (MEK) as a deproteinization solvent for the simultaneous determination of Nia-114, NIC, and NAM in rabbit plasma. The analytes were chromatographically separated on a Spherisorb Cyano column under isocratic conditions, and detected by multiple reaction monitoring (MRM) in positive-ion electrospray ionization mode. Both the extraction method and analytical method are simple to use, and the assay was shown to perform well in validation studies. Nia-114 was found to be unstable in plasma, except at −80 °C, as demonstrated by stability tests. A probable source of the instability is discussed.

2. Experimental

2.1. Chemicals and reagents

Nia-114, and the D_4-isotope-labeled internal standards of Nia-114 and NAM were provided by Niadyne, Inc. (Tucson, AZ, USA). The D_4-isotope-labeled internal standard of nicotinic acid was purchased from CDN Isotopes (Que., Canada). NIC, NAM, 2-butanone, absolute ethanol, formic acid, sodium fluoride, and phenylmethyl sulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile and methanol were from Burdick & Jackson (Muskegon, MI, USA). All chemicals and solvents were of analytical or HPLC grade unless otherwise noted. All stock drug solutions and HPLC mobile phase were prepared using Milli-Q grade water (Millipore, Bedford, MA, USA). Fisher 344 rat, New Zealand White rabbit, and Beagle dog plasma was obtained from PelFreez, Inc. (Rogers, AR, USA). One lot of rabbit plasma, pre and post charcoal-stripped, was obtained from Biochemed Pharmacologics, Inc. (Winchester, VA, USA). Human plasma was obtained from the Peninsula Blood Bank (Burlingame, CA, USA). All plasma contained EDTA as the anticoagulant, except the pre and post charcoal-stripped rabbit plasma, which contained sodium fluoride as the anticoagulant, selected lots of rabbit plasma from PelFreez that contained heparin, and the human plasma, which contained citrate. All plasma was received frozen and stored at −20 °C prior to use.

2.2. Chromatographic conditions

A Waters 2795 HPLC (Waters Corp, Milford, MA, USA) and a Spherisorb Cyano column (150 mm × 4.6 mm, 5 μm i.d, Waters Corp.) were used for the chromatographic separation of Nia-114, NIC, and NAM. The mobile phase consisted of 56.0/44.0/0.1: acetonitrile/water/formic acid (v/v/v) delivered isocratically at 1.0 ml/min, and split post-column to 0.3 ml/min to the mass spectrometer using a fixed flow splitter (ASI, El Sobrante, CA, USA). The autosampler was maintained at 15 °C, and the column was at ambient temperature. Sample injection volume was 20 μl.

2.3. MS/MS detection

LC detection was obtained using a Micromass Quattro Ultima triple quadrupole mass spectrometer (Waters Corp.) with electrospray ionization operated in positive ion mode. The following mass spectrometric settings were used: capillary voltage 3.0 kV, cone voltage 50 V, source temperature 115 °C, desolvation temperature 275 °C, collision energy 22 eV with argon at 2.3×10^{-3} mbar. Precursor ions of analytes and internal standards were determined from spectra obtained by infusing standard solutions at 10 μl/min into the post-column mobile phase (0.3 ml/min) via a T connector. Each of the precursor ions was subjected to collision-induced dissociation (CID) at varying collision energies to determine product ions. The mass spectrometric conditions were optimized to generate MRM

transitions, and 0.25 s dwell times were used per interval. Q1 and Q3 quadrupoles were operated at unit mass resolution. Data were acquired using the Micromass Masslynx software.

2.4. Sample preparation

Individual 10 mg/ml stock solutions of Nia-114, NIC, and NAM were prepared in 95/5: methanol/water (v/v), 5/95: methanol/water (v/v), and 75/25: methanol/water (v/v), respectively. Individual 1 mg/ml stock solutions of d_4-Nia-114, d_4-NIC, and d_4-NAM were prepared in 95/5: methanol/water (v/v), 5/95: methanol/water (v/v), and 75/25: methanol/water (v/v), respectively, and were used to prepare a mixed solution in 85/15: methanol/water (v/v) for use in the extraction process on the day of analysis. All of the individual stocks were stored at 5 °C and were stable for 3 months. The non-deuterated stocks were used to prepare spiking solutions in 85/15: methanol/water (v/v) for preparation of rabbit plasma calibration standards and quality control samples (QCs). Spiking solutions contained equal concentrations of Nia-114, NIC, and NAM for most calibration standards and QCs. Plasma calibration standards were prepared by spiking 1 part spiking solution to 99 parts plasma (v/v). The following concentrations were used to generate calibration curves in each validation run: Nia-114: 0, 2, 10, 50, 100, 200, 400, 600, 800, and 1000 ng/ml; NIC: 0, 8, 20,100, 200, 400, 600, 800, and 1000 ng/ml; NAM: 0, 75, 100, 200, 400, 600, 800, and 1000 ng/ml. Plasma QCs were prepared at LLOQ, low, medium, high, and dilutional QC values, which were, respectively: Nia-114: 2, 6, 500, 900, and 5000 ng/ml; NIC: 8, 24, 500, 900, and 5000 ng/ml; NAM: 75, 125, 500, 900, and 5000 ng/ml. The plasma calibration standards and QC volumes were 0.2 ml.

The calibration standards and QCs were prepared independently using a single lot of rabbit plasma (lot #13426-B, PellFreez) and stored at −20 °C during the course of the validation runs over 5 days. The calibration standards and QCs were stored under the same conditions and extracted without differences in thaw times. Thus, the resulting accuracy and precision of the QCs from the validation runs reflects the integrity of the bioanalytical method, rather than differences in stability of the calibration standards versus QCs. Dilutional QCs were diluted with blank plasma on the day of extraction just prior to extraction as follows: 0.1 ml of plasma sample was removed and added to a 2.0 ml microcentrifuge tube, and 0.4 ml (for five-fold dilution) or 0.9 ml (for 10-fold dilution) blank plasma added. The tube was then briefly vortexed and 0.2 ml removed to a separate 2.0 ml microcentrifuge tube for analysis.

2.5. Sample extraction

The internal standards d-Nia-114, d-NIC, and d-NAM in 85/15: methanol/water (v/v) were added to MEK, and this spiked MEK solution was used to extract the plasma samples. The MEK/methanol/internal standard mix was prepared on the day of extraction by adding 14 ml of a solution containing 0.1 μg/ml d-Nia-114, 0.5 μg/ml d-NIC, and 0.5 μg/ml d-NAM in 85/15: methanol/water (v/v) to 186 ml MEK.

The steps of the extraction were as follows: to 200 μl of plasma in a 2.0 ml microcentrifuge tube kept frozen in cryovial racks on dry ice was added 1.0 ml MEK containing d-Nia-114, d-NIC, and d-NAM (see above for preparation details). All tubes were then transferred to racks at room temperature for 20 min. The tubes were then vortexed at maximum speed for 20 min on a plate vortexer, centrifuged 5 min at 14,000 × *g*, and 1.0 ml of the supernatant removed to a 1.5-ml microcentrifuge tube. The extracts were then evaporated to dryness overnight using a centrifugal evaporator. Sixty microliters of 90/10/0.1: acetonitrile/water/formic acid (v/v/v) were added to the dried residues, and the tubes were vortexed 20 min at maximum speed, centrifuged 2 min at 14,000 × *g*, and 50 μl of the supernatant transferred to an HPLC vial containing a 150 μl glass insert, for subsequent LC–MS/MS analysis.

2.6. Validation

The validation experiments were designed in accordance with current FDA guidelines on quantitative bioanalysis [5]. Method validation included determination of linearity over the calibration standard range, and intra- and inter-day accuracy (% of nominal concentration) and precision (% R.S.D.) of the QCs. Acceptance criteria were established to be >0.980 correlation coefficient (r^2) for the calibration curve, intra- and inter-day accuracy and precision within ±15% (±15% of nominal concentration, and ≤15% R.S.D.) for low, medium, and high QC levels, and intra-day accuracy and precision within 20% for LLOQ level QCs. Each validation run included duplicate replicates of calibration standards, five replicates of each QC level (LLOQ, low, medium, high, and dilutional), and 10 replicates of blank plasma. The stability of the analytes under different conditions was evaluated as part of the validation.

3. Results and discussion

3.1. Mass spectrometry

Electrospray ionization operated in the positive ion mode was used for the LC–MS/MS MRM analyses, to provide optimum sensitivity and selectivity. Protonated forms of each analyte $[M + H]^+$ were the dominant ions in the Q1 spectra, and were used as the precursor ions to obtain Q3 product ion spectra. Upon CID, the most abundant product ion was selected for the MRM transition. The d_4-internal standards ionized and fragmented predictably relative to the non-deuterated standards, and the resulting MRM transitions (precursor ion $m/z \rightarrow$ product ion m/z) for each analyte were as follows: Nia-114: 320 → 124, d_4-Nia-114: 324 → 128, NIC: 124 → 80, d_4-NIC: 128 → 80, NAM: 123 → 80, d_4-NAM: 127 → 84. Representative CID mass spectra of Nia-114, NIC, and NAM at a collision energy of 22 eV are shown in Fig. 1.

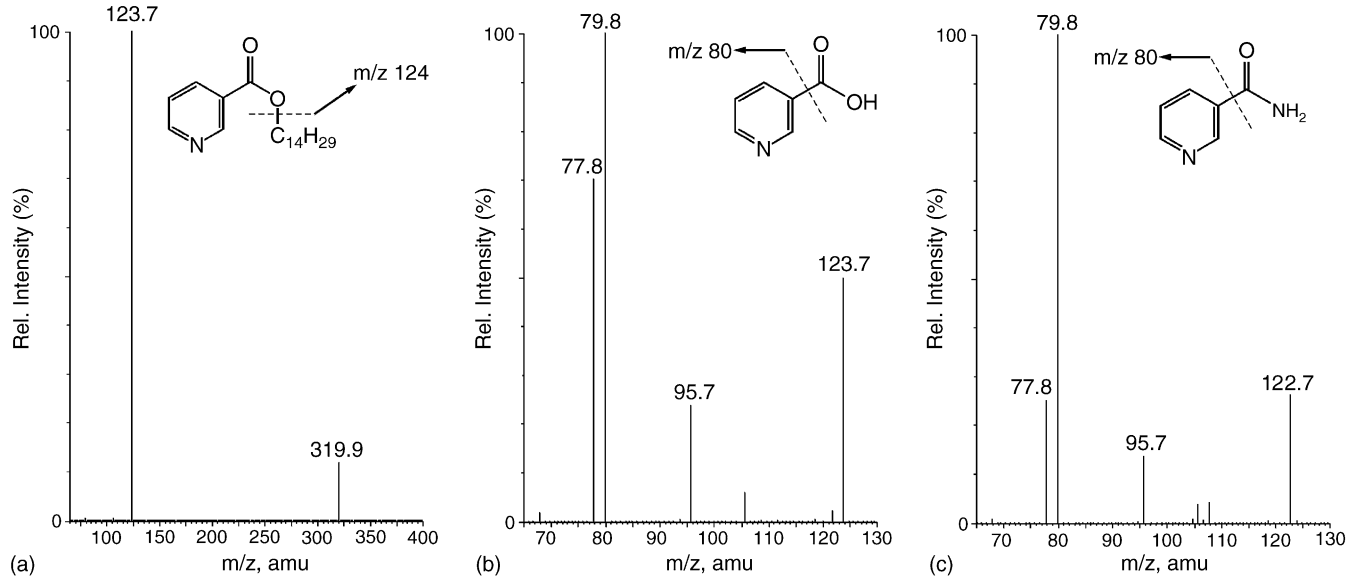

Fig. 1. Representative MS/MS product-ion spectra of Nia-114 (a), NIC (b), and NAM (c). The protonated molecules $[M+H]^+$ of Nia-114, NIC, and NAM were detected at 319.9 *m/z*, 123.7 *m/z*, and 122.7 *m/z*, respectively. The most abundant product ions, at 123.7 *m/z*, 79.8 *m/z*, and 79.8 *m/z*, were chosen to monitor the product ion transitions for Nia-114, NIC, and NAM, respectively.

3.2. Method development

3.2.1. Chromatography

Various HPLC columns were tested to retain and baseline-separate Nia-114, NIC, and NAM without requiring long run times. Due to the hydrophobic property of Nia-114 versus NIC and NAM, and hydrophilic, highly polar properties of NIC at either acidic or basic pH in the mobile phase, conventional reverse-phase columns that were tested (phenyl, C4, C18, including polar-embedded columns designed for retention of both polar and nonpolar analytes) did not adequately retain and separate all three analytes. Normal-phase silica columns were also found to inadequately retain and separate all of the analytes, using either isocratic or gradient elution. Cyano columns can exhibit either reverse-phase or normal phase properties, depending upon the analyte and chromatographic condition. Therefore, Cyano columns expected to retain Nia-114, NIC, and NAM by a combination of the two retention modes were tested, so that a single method could be used for all three analytes. A 150 mm × 4.6 mm, 5 μm Waters Spherisorb Cyano column gave good retention and baseline separation of the three analytes within a 9 min run time using the isocratic chromatographic conditions described earlier. Under these conditions the column demonstrated normal-phase retention characteristics for NIC and NAM, and reverse-phase retention characteristics for Nia-114. This was evidenced by the reproducible change in retention times if the percent acetonitrile in the mobile phase was increased or decreased for a given isocratic run. Specifically, increasing the percentage of organic solvent in the mobile phase increased the retention times of NIC and NAM but decreased the retention times of Nia-114, and decreasing the organic percentage in the mobile phase decreased the retention times of NIC and NAM but increased the retention times of Nia-114. This phenomenon allowed selection of an isocratic condition that resulted in good column retention and separation of the three analytes, without large differences in the retention times.

After analysis of extracted plasma samples during method development, the optimum LC–MS/MS mobile phase was determined to be 56.0/44.0/0.1: acetonitrile/water/formic acid (v/v/v) delivered isocratically at 1.0 ml/min and split post-column to 0.3 ml/min to the mass spectrometer, which yielded retention times of about 4.2, 5.5, and 6.5 min, for NIC, NAM, and Nia-114, respectively. The d_4-internal standards eluted within 0.1 min of the corresponding analyte. The method gave baseline separation of the analytes within a run time of 9 min, and reproducible peak shapes and retention times. Baseline separation of NIC and NAM was needed to prevent peak area contribution from NAM to NIC due to isotope effects, since the precursor-ions for NAM and NIC are 1 amu apart (123 versus 124 *m/z*) and both product-ion settings are at 80 *m/z*.

3.2.2. Plasma extraction

Solid-phase extraction using strong cation exchange (SCX), which has been demonstrated to be effective for NIC and NAM in plasma [10,11] or urine [12] was attempted for simultaneous extraction of Nia-114, NIC, and NAM from rabbit plasma. We found that Nia-114 was poorly recovered (<5%), whereas NIC, and NAM were well recovered (>70%). Thus, it was determined that SPE was not a viable method for recovery of Nia-114, and alternate methods (solvent extraction and plasma protein precipitation) were investigated.

The use of diethyl ether and ethyl acetate as plasma extraction solvents did not recover NIC adequately (<5% recovery). Plasma protein precipitation with acetonitrile, followed by evaporation of the supernatant and reconstitution of the dried extract, provided ≥40% recovery of each analyte, but conditions could not be attained that sufficiently reduced the presence of endogenous material that interfered in the reproducibility and sensitivity of

the LC–MS/MS assay, while maintaining sufficient recovery of the analytes. However, MEK, a volatile (b.p. 80 °C) polar aprotic solvent with partial water solubility (solubility of water in MEK is 12.5% at 25 °C, [13]), was found to be effective in precipitating plasma proteins and extracting Nia-114, NIC, and NAM from rabbit plasma, and the resulting extracts contained much less interfering material.

The use of MEK at a volume ratio of 5/1 to plasma (v/v), with incorporation of 6% methanol in the MEK (from the addition of the deuterated internal standards in 85% methanol) yielded an appropriate balance between optimizing the recovery of Nia-114, NIC, and NAM, and minimizing the co-extraction of interfering material. Recovery results are presented in a later section. The deuterated internal standards were added to the MEK to simplify the extraction process. A reconstitution solvent for the dried extracts was selected that optimized recovery of all 3 analytes. It was determined that 90/10/0.1: acetonitrile/water/formic acid (v/v/v) gave better recovery than the use of solvent mixtures containing a lower percent acetonitrile, while not adversely affecting the peak shapes or retention times.

3.3. Validation

3.3.1. Selectivity

For Nia-114 and NIC, a small but measurable analyte peak was present in plasma blanks after extraction using MEK containing the deuterated internal standards. These small analyte peaks appear to be primarily from non-deuterated analyte impurities in the deuterated internal standards. This can be seen in Fig. 2. Fig. 2a is a representative blank plasma sample extracted without addition of the deuterated internal standards. Fig. 2b is a representative blank plasma sample extracted with MEK containing the deuterated internal standards. Fig. 2c and d are representative chromatograms of QC-low and QC-high samples, and demonstrate no significant changes in the peak shape or retention times at these concentrations. As can be seen, a measurable Nia-114 peak is not present in Fig. 2a, but is present in Fig. 2b. A small endogenous NIC peak is present in Fig. 2a, but is larger in Fig. 2b. For NAM, a large endogenous analyte peak is consistently present in plasma. Due to the consistent presence of Nia-114, NIC and NAM peaks in the blanks after extraction with MEK containing the deuterated internal standards, ten plasma blanks were analyzed in each analysis, and the mean background response (analyte/d-analyte peak area ratio) for Nia-114, NIC, and NAM subtracted from all corresponding standards and QCs within that run. This provided the best calibration standard curve fit and QC sample results in the validation runs. The % R.S.D. of the mean blank response for each analyte used for background subtraction was <15% in all cases.

To compare the differences in endogenous levels of NIC and NAM in rabbit plasma, a selectivity test was performed in which nine different lots of New Zealand White rabbit plasma were analyzed. It was previously found that charcoal-stripping rabbit plasma removed essentially all NIC and NAM, and so charcoal-stripped rabbit plasma was used to prepare calibration standards and QCs for this selectivity test, to allow quantitation of the level of endogenous NIC and NAM in untreated non charcoal-stripped lots of rabbit plasma. The charcoal-stripped rabbit plasma, for which sodium fluoride was used as the anticoagulant, was also provided in the pre-charcoal-stripped form and included in the run.

For comparison, untreated human, dog, rat, and mouse plasma were also analyzed. Four lots of human plasma, and three lots each of dog, rat, and mouse plasma were used. Each lot of plasma was extracted in quadruplicate replicates and measured for analytes using the methods described earlier. It was assumed that the NIC and NAM recovery was equivalent in the different sources of plasma. The detection limit was set at 2.00 ng/ml for each analyte, based on back-calculated accuracy and precision being within ±15% for the 2.00 ng/ml charcoal-stripped plasma standards used in the calibration curve. The charcoal-stripped plasma standards contained equal levels of NIC and NAM at 0, 2, 5, 10, 20, 50, 100, 200, 400, 600, 800, and 1000 ng/ml, and were extracted in duplicate at each concentration.

The mean levels of NIC and NAM in the nine lots of rabbit plasma were 12.0 and 359 ng/ml, respectively (Table 1). The NIC levels ranged from 2.16 to 21.7 ng/ml, and NAM ranged from 273 to 529 ng/ml. The levels did not appear to correlate to the type of anticoagulant used in preparing the plasma. Lot #13426-B rabbit plasma from the selectivity test was selected for preparation of calibration standards, QCs and stability samples in the validation runs, since this lot yielded levels of NIC and NAM (12.5 and 328 ng/ml, respectively) comparable to the mean levels from the nine lots tested.

The NIC and NAM levels in human plasma were much lower than in rabbit plasma (Table 1). The mean NIC concentration was below the detection limit (2.00 ng/ml), and the mean NAM level was 38.3 ng/ml, ranging from 26.6 to 49.8 ng/ml in the different lots. In comparison, the mean NIC and NAM levels in dog, rat, and mouse plasma were 3.22 and 159 ng/ml, 77.1 and 381 ng/ml, and 233 and 622 ng/ml, respectively. Although there was variability in the mean levels of NIC and NAM per species for the lots tested, particularly in rat and mouse, a trend was observed in which higher levels were found in non-human species, with the highest levels in mouse. The trend of highest to lowest levels was as follows: mouse > rat and rabbit > dog > human.

A probable explanation for the much higher level of NIC and NAM in rabbit plasma compared to human plasma is that intrinsic differences in the niacin status of various species exist, e.g. dietary intake differences or other factors that affect the uptake, distribution and metabolism. The finding that NIC and NAM plasma concentrations vary substantially from humans to mice supports this hypothesis. Further investigation would be required to determine more accurately the typical inter-and intra-species differences in endogenous levels. The endogenous level of NAM in mouse plasma has been reported to be approximately 10 nmol/ml [14], which equates to 1220 ng/ml. In comparison, the mean NAM levels in the three lots of mouse plasma we analyzed were 861, 301, and 705 ng/ml. To the authors' knowledge, no other published values for NIC and NAM in plasma of non-human species have been reported in the literature. NIC and NAM concentrations in human plasma have been reported that are similar to those measured in the current study [80 nM (9.84 ng/ml) and 340 nM (41.5 ng/ml) NIC and NAM,

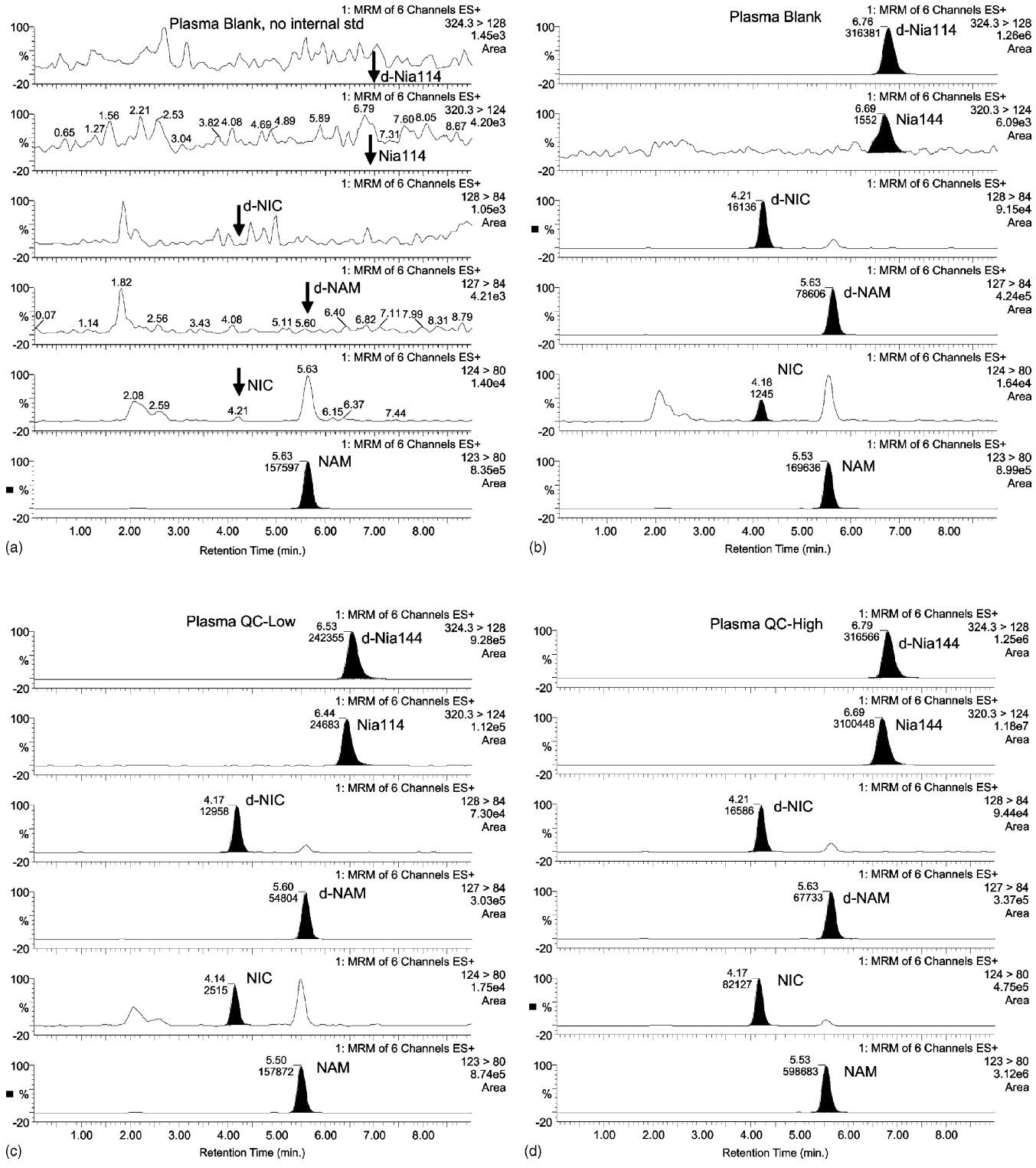

Fig. 2. Representative chromatogram of a blank rabbit plasma sample extracted with MEK without addition of the deuterated internal standards (a), and representative chromatograms of blank (b), QC-low (c), and QC-high (d) plasma samples extracted with MEK containing the deuterated internal standards.

respectively [15] versus mean levels of <2.00 ng/ml (NIC), and 38.3 ng/ml (NAM) observed here (Table 1)],

QC samples of NIC and NAM at 5, 10, and 900 ng/ml prepared from the charcoal-stripped rabbit plasma were included in the selectivity run described above, to demonstrate that if there is a low level of endogenous NAM in the plasma, a low QC quantitation limit is attained. QCs were analyzed at five replicates per concentration. The resulting accuracy and precision of NIC and NAM were 110.1 ± 15.7% and 94.4 ± 6.24% at 5 ng/ml, 101.4 ± 11.7% and 95.8 ± 12.0% at 10 ng/ml, and 111.4 ± 4.39% and 94.4 ± 6.18% at 900 ng/ml, respectively. The accuracy and precision of NAM was within ±12% at 5

Table 1
Endogenous levels of NIC and NAM in blank rabbit, human, dog, rat, and mouse plasma

Species	Anticoagulant	Plasma lot #	Mean NIC concentration (ng/ml)	% R.S.D.	Mean NAM concentration (ng/ml)	% R.S.D.
Rabbit	EDTA	00826	2.16	11.10	273.23	4.71
Rabbit	EDTA	19025	14.66	11.35	412.01	4.91
Rabbit	EDTA	21725	12.60	17.05	430.64	6.94
Rabbit	Heparin	13426-A	8.31	12.21	312.11	3.59
Rabbit	Heparin	13426-B	12.46	16.42	328.45	11.14
Rabbit	Heparin	13426-C	21.72	14.13	286.88	6.91
Rabbit	Heparin	007026	7.05	19.76	386.04	9.13
Rabbit	Heparin	21025	17.54	7.20	268.97	12.71
Rabbit	Heparin	006325	11.10	12.49	528.98	3.89
		Mean value of 9 lots	11.96		358.59	
Rabbit	Sodium fluoride	S-47793	5.20	10.61	412.30	7.36
Rabbit	Sodium fluoride	S-47793-C.S.[a]	ND		ND	
Human	Citrate	T8969	ND		35.61	6.82
Human	Citrate	V65803	ND		49.75	1.32
Human	Citrate	503174	ND		41.09	8.14
Human	Citrate	V65779	ND		26.55	3.58
		Mean value of 4 lots	ND		38.25	
Dog	EDTA	01525	ND		114.88	4.75
Dog	EDTA	23821	5.12	13.04	174.76	3.88
Dog	EDTA	20426	4.53	11.47	187.96	1.38
		Mean value of 3 lots	3.22		159.20	
Rat	EDTA	25425	20.90	9.48	285.31	2.35
Rat	EDTA	21726	13.95	6.65	581.50	3.92
Rat	EDTA	01526	196.43	5.79	275.63	3.99
		Mean value of 3 lots	77.09		380.81	
Mouse	EDTA	31215E	215.85	1.48	861.13	5.41
Mouse	EDTA	00523	345.26	3.08	300.85	1.29
Mouse	EDTA	27921	139.08	3.36	705.40	0.62
		Mean value of 3 lots	233.40		622.46	

ND: not detected (<2.00 ng/ml). A value of 0 ng/ml is used for ND in calculating means.
[a] C.S.: charcoal-stripped plasma.

or 10 ng/ml, demonstrating a low QC quantitation limit in this matrix. For comparison, the LLOQ for NAM in the non charcoal-stripped plasma used in the validation runs was 75 ng/ml. Thus, it was demonstrated that the LLOQ for NAM is dependant on the level of endogenous NAM in the matrix, and it is expected that the method would provide much better sensitivity for NAM, and equivalent or better sensitivity for NIC, if validated in human plasma.

3.3.2. Linearity

Due to the levels of endogenous NIC and NAM in the rabbit plasma, the best calibration curve fit was attained by subtracting the mean blank plasma response of the analyte from the calibration standards response, to generate a non-weighted linear regression forced through the origin. This fit was used for each analyte and provided optimum QC results. The resulting linear range (lower limit of quantitation to upper limit of quantitation) for each analyte was as follows: Nia-114: 2.00–1000 ng/ml NIC: 8.00–1000 ng/ml, and NAM: 75.0–1000 ng/ml. A summary of the correlation coefficients (r^2) and slopes from each standard curve from the validation runs is shown in Table 2. The correlation coefficients, r^2 varied from 0.983 to 0.998. Each calibration curve in each validation run met the current FDA guidelines for bioanalytical method validation, in that >67% of the back-calculated calibration standards within a run were within ±15% of nominal concentration, and ±20% at LLOQ. The decreasing slopes in the calibration curves of Nia-114 in Table 2 were determined to be due to short-term instability of Nia-114 in rabbit plasma at −20 °C. The validation runs were performed over a 7 day period (run i.d. #1, 2, 3, and 4 = day # 1, 2, 7, and 3, respectively).

3.3.3. Accuracy and precision

To assess the accuracy and precision of the bioanalytical method for Nia-114, NIC, and NAM in rabbit plasma, QC samples at five concentrations (LLOQ, low, medium, high, and dilutional QC) were processed on three separate days of analysis. Five replicates were used per concentration. As seen in

Table 2
Summary of calibration curves results for Nia-114, NIC, and NAM in rabbit plasma from validation runs

Run ID	Calibration curve results	Nia-114	NIC	NAM
1	Slope	0.02808	0.00520	0.00804
	r^2	0.986	0.998	0.985
2	Slope	0.01914	0.00512	0.00733
	r^2	0.987	0.995	0.990
3	Slope	0.00786	0.00574	0.00764
	r^2	0.983	0.995	0.989
4[a]	Slope	0.01590	0.00533	0.00714
	r^2	0.998	0.992	0.986

[a] Validation run # 4 was performed using calibration standards and QCs that were thawed to RT 20 min rather than remaining frozen before the addition of the MEK/internal standard mix.

Table 3, the inter-day accuracy and precision of each analyte in the runs was within ±15% at low, medium, and high QC concentrations. LLOQ QCs were within ±20% for each analyte, except for NAM, which gave accuracy within ±20% in each run, but precision of >20% in one of the three runs (run #1, 22.1% R.S.D.). Dilutional QCs were within ±15% accuracy and precision in each run, except for run #3, which gave 119% accuracy for NAM. The intra-day accuracy and precision of each analyte in runs # 1–3 were within ±15% at low, medium, high, and dilutional QC concentrations. LLOQ QCs were within ±20% accuracy and precision for each analyte.

A fourth validation run was performed using a modified step in the extraction method; the plasma standards and QCs were thawed to room temperature for 20 min instead of remaining frozen before addition of the MEK/internal standard mix. The resulting accuracy was within ±13%, and precision within ±15%, for all analytes at all QC levels tested, except for NIC at the LLOQ level, which had 17% R.S.D. This run demonstrated that equivalent validation results can be attained if the samples are thawed briefly for an equal time prior to extraction.

Overall, the method accuracy and precision results met acceptance criteria for analysis of Nia-114, NIC, and NAM in rabbit plasma at levels ranging from 2 to 1000 ng/ml, 8 to 1000 ng/ml, and 75 to 1000 ng/ml, respectively. Additionally, five-fold dilutions of plasma containing the analytes at 5000 ng/ml were shown to provide accurate and precise results (within ±15%).

3.3.4. Recovery

The recovery of Nia-114, NIC, and NAM was determined for high-QCs (900 ng/ml) by spiking triplicate post-extracted blanks with neat Nia-114, NIC, and NAM to generate analyte concentrations at levels of 100% theoretical recovery, and then comparing the resulting concentrations to the QC-high mean concentrations measured within the same run. This technique provides a more realistic determination of analyte recovery than comparison of analyte response to neat standards. The resulting percent recoveries, and corresponding % R.S.D., were as follows: Nia-114: 73.7 ± 2.36%, NIC: 36.7 ± 4.23%, and NAM: 95.1 ± 13.1%. Though the recovery of NIC is lower than for Nia-144 and NAM, it appears to be consistent across the linear range of the method, as evidenced by the consistent QC results in each validation run.

3.3.5. Freeze/thaw stability

The freeze/thaw stability of Nia-114, NIC, and NAM in rabbit plasma was determined using triplicate replicates of low and high plasma QCs. The QCs were put through three freeze/thaw cycles, in which the QCs were thawed unassisted for 20 min during each thaw cycle. These QCs were then analyzed in the same run as $n = 5$ replicates of untreated low and high QCs. The resulting difference in the measured concentration for the freeze/thaw QCs versus the untreated QCs is summarized in Table 4. As seen in the table, both low and high QCs yielded lower Nia-114 (16–19% loss) after the 3 freeze/thaw cycles. NIC and NAM yielded variable results and no obvious trend of an increase or decrease in concentration. While the percent loss of NAM at the QC low level was calculated to be 22.4%, the decrease was not considered significant due to the relative variability (large standard deviations) in the measured concentrations.

3.3.6. Benchtop stability

The benchtop stability of Nia-114, NIC, and NAM in rabbit plasma was determined using triplicate replicates of low and high plasma QCs. The QCs were kept at room temperature for 2 or 24 h prior to analysis. These QCs were analyzed in the same run as $n = 5$ replicates of untreated low and high QCs. The results are summarized in Table 5.

As seen in the table, there was 44–50% loss of Nia-144 in rabbit plasma after 2 h at room temperature. By 24 h, less than 1% Nia-144 was measured in either the low or high QCs, and there was an increase in NIC concentration in both QCs, and at both timepoints. The increase in NIC concentration likely arises from hydrolysis of the Nia-114 in the QC samples. At 24 h, there was a 48%, and 23% increase in NIC concentrations for the low QC and high QCs, respectively. No significant trend of an increase or decrease in NAM concentration was seen for the 2 or 24-h benchtop QCs, and the percent change for all NAM QCs was within ±20%.

3.3.7. Benchtop stability with enzyme inhibitor

A stability test was performed to determine if the addition of sodium fluoride or phenylmethyl sulfonyl fluoride (PMSF) to rabbit plasma inhibits the loss of Nia-114 in plasma at room temperature or 5 °C. The structure of Nia-114 indicates it may be susceptible to hydrolysis by esterases. Sodium fluoride is a concentration-dependent reversible inhibitor of plasma esterases [16], whereas PMSF reacts with sulfhydryl groups in esterases and amidases to inhibit their activities irreversibly [17].

High-QCs were prepared fresh using rabbit plasma which had either 50 mM (final concentration) sodium fluoride added 30 min earlier, or 1 mM (final concentration) PMSF added 30 min earlier, or no inhibitor added (control group). The enzyme inhibitors were freshly prepared in water (sodium fluoride) or ethanol (PMSF) before addition to the plasma at volume ratios of 1 part inhibitor to 99 part plasma (v/v), followed by immediate vortexing. A batch of QCs was prepared for each of the three groups of plasma, and quadruplicate

Table 3
Intra- and inter-day accuracy (% of nominal) and precision (% R.S.D.) of Nia-114, NIC, and NAM in rabbit plasma

QC i.d.	QC nominal concentration (ng/ml)	Validation run i.d. #	Analyte: Nia-114				Analyte: NIC				Analyte: NAM			
			Intra-day results		Inter-day results		Intra-day results		Inter-day results		Intra-day results		Inter-day results	
			Mean % accuracy	% R.S.D	Mean % accuracy	% R.S.D.	Mean % accuracy	% R.S.D.	Mean % accuracy	% R.S.D.	Mean % accuracy	% R.S.D.	Mean % accuracy	% R.S.D.
	2.00 (Nia-114)	1	101.34	9.80			101.95	11.69			107.06	22.00		
LLOQ	8.00 (NIC)	2	93.83	9.05			108.83	6.50			111.80	18.73		
	75.0 (NAM)	3	112.52	8.24	102.50	11.22	116.18	12.62	109.00	11.35	96.35	13.76	105.07	18.53
	6.00 (Nia-114)	1	91.18	9.16			106.34	12.14			96.78	13.84		
Low	24.0 (NIC)	2	111.44	12.14			89.42	6.29			103.19	9.39		
	125 (NAM)	3	88.50	6.78	97.04	14.43	105.63	9.61	100.46	12.32	92.21	13.22	97.39	12.25
		1	94.68	1.74			96.82	3.07			98.54	4.04		
Medium	500	2	95.96	4.04			101.77	2.72			104.50	2.11		
		3	93.48	7.24	94.71	4.63	97.78	5.34	98.79	4.20	103.30	7.85	102.11	5.53
		1	85.10	9.63			102.90	3.99			96.43	2.75		
High	900	2	98.37	3.96			105.56	1.49			105.91	11.17		
		3	102.85	11.22	95.44	11.59	96.46	4.78	101.64	5.12	104.41	5.37	102.25	7.99
		1	84.84	2.08			107.95	1.75			108.23	2.54		
Dilutional	5000	2	111.75	6.37	98.30	15.26	112.41	7.14	109.93	5.11	111.29	6.85	109.76	5.14
		3[a]	104.71	2.85			102.39	2.02			118.95	3.10		

Inter-day accuracy and precision calculated for validation runs # 1–3.

[a] In validation run # 3, dilutional QCs diluted 1 to 10 (to 500 ng/ml) instead of 1–5 on day of analysis. Between-day accuracy and precision of dilutional QCs calculated for validation runs # 1–2.

Table 4
Freeze/thaw stability of Nia-114, NIC, and NAM in plasma QCs

Analyte	QC nominal concentration (ng/ml)	Measured concentration within run	After three freeze/thaw cycles	% Change
Nia-114	6	6.60 ± 0.69	5.52 ± 0.16	−16.4
	900	956.62 ± 35.92	773.47 ± 54.18	−19.2
NIC	24	23.05 ± 2.57	22.37 ± 3.11	−3.0
	900	892.62 ± 39.43	902.96 ± 80.42	1.2
NAM	125	131.73 ± 18.02	102.23 ± 36.99	−22.4
	900	845.22 ± 76.52	858.97 ± 66.63	1.6

aliquots incubated at room temperature for 0, 20, 60, or 120 min after addition of Nia-114, NIC, and NAM. Aliquots were also kept at 5 °C for 60 or 120 min. At the end of each incubation time the designated aliquots were frozen on dry ice and stored frozen at −70 °C until the rest of the samples were collected, at which point all samples were extracted in a single run and analyzed for Nia-114, NIC, and NAM using the same method as for the validation runs. Blank plasma aliquots from each group were saved before Nia-114, NIC, and NAM were spiked into the plasma, and also analyzed in the run. Neither sodium fluoride nor PMSF caused interference in the LC–MS/MS method.

The resulting percent change in analyte concentration and % R.S.D. at each time point versus the $T = 0$ mean level is shown in Table 6. As seen in the table, both sodium fluoride and PMSF were only slightly effective at inhibiting the rate of loss of Nia-114 in plasma over time. The percent decrease in Nia-114 concentration in rabbit plasma at 20 min was 11% for the control group, 9% for the sodium fluoride group, and 8% for the PMSF group. The percent decrease at 1 h was 36% for the control group, 22% for the sodium fluoride group, and 23% for the PMSF group. The percent decrease at 2 h was 58% for the control group, 36% for the sodium fluoride group, and 39% for the PMSF group. As seen in the table, keeping the plasma at 5 °C instead of room temperature helped improved the Nia-114 stability, but not dramatically; at 1 h there was a 26% decrease for the control group versus 36% at room temperature.

The stability test revealed a trend of NIC levels increasing slightly as Nia-114 levels decreased in plasma at room temperature. The percent increase in NIC concentration in rabbit plasma at 1 h was 7% for the control group, 11% for the sodium fluoride group, and 10% for the PMSF group. The percent increase at 2 h was 16% for the control group, 10% for the sodium fluoride group, and 21% for the PMSF group. The stability test showed a minor trend of the percent NAM increasing in the control group at later time points as Nia-114 levels decreased (for example, at 2 h the NAM percent increase was 16%), however no such trend was observed for the sodium fluoride or PMSF groups.

The stability test with enzyme inhibitor was repeated to confirm the results at 1 h using a different lot of rabbit plasma. The plasma was lot # 19025, which contained EDTA as the anticoagulant instead of Heparin. As seen in Table 6, the percent decrease in Nia-114 concentration in rabbit plasma at 1 h was 39% for the control group, 19% for the sodium fluoride group, and 31% for the PMSF group, which are similar results as observed for the other lot of plasma. No clear trend was observed in the NIC and NAM levels at 1 h, although the percent increase in NIC at 1 h was 15% for the control group.

The results of these stability tests reveal that addition of sodium fluoride or PMSF to rabbit plasma helped slow the rate of loss of Nia-114 only marginally, thus indicating that plasma esterases are not the primary cause of hydrolysis of Nia-114 in rabbit plasma. It has been reported that human serum albumin has esterase-like activity towards a series of esters of nicotinic acid [18], and our stability results are in keeping with this proposed mechanism of catalytic hydrolysis by albumin, occurring concomitantly with hydrolysis by plasma esterases.

Table 5
Benchtop stability of Nia-114, NIC, and NAM in plasma QCs

Analyte	QC nominal concentration (ng/ml)	Time at RT (h)	Measured concentration within run	After Benchtop RT	% Change
Nia-114	6	2	6.60 ± 0.69	3.67 ± 0.45	−44.4
	900	2	956.62 ± 35.92	482.38 ± 27.95	−49.6
Nia-114	6	24	6.60 ± 0.69	0.06 ± 0.03	−99.1
	900	24	956.62 ± 35.92	4.90 ± 0.45	−99.5
NIC	24	2	23.05 ± 2.57	24.78 ± 1.80	7.5
	900	2	892.62 ± 39.43	975.91 ± 24.53	9.3
NIC	24	24	23.05 ± 2.57	34.06 ± 1.37	47.8
	900	24	892.62 ± 39.43	1100.21 ± 24.33	23.3
NAM	125	2	131.73 ± 18.02	119.08 ± 26.00	−9.6
	900	2	845.22 ± 76.52	808.39 ± 78.48	−4.4
NAM	125	24	131.73 ± 18.02	107.61 ± 12.40	−18.3
	900	24	845.22 ± 76.52	814.65 ± 80.59	−3.6

Table 6
Benchtop stability of Nia-114 in rabbit plasma containing an enzyme inhibitor

Analyte: Nia-114			No inhibitor		NaF		PMSF	
Time (min)	Temperature	Plasma lot	% Change	% R.S.D.	% Change	% R.S.D.	% Change	% R.S.D.
0	RT	1	NA	4.4	NA	3.1	NA	6.1
20	RT	1	−11.4	2.8	−8.6	1.5	−8.2	5.9
60	RT	1	−35.9	1.5	−22.2	3.5	−23.3	5.4
120	RT	1	−58.1	4.1	−35.6	6.0	−39.4	3.8
60	5 °C	1	−26.2	1.7	−18.4	3.2	−21.5	2.1
120	5 °C	1	−29.8	4.4	−18.7	2.5	−30.8	5.0
0	RT	2	NA	4.5	NA	3.3	NA	4.6
60	RT	2	−39.0	2.4	−18.8	2.9	−30.6	6.9
Analyte: NIC								
0	RT	1	NA	5.7	NA	5.0	NA	5.2
20	RT	1	0.6	6.3	1.8	7.6	4.4	6.6
60	RT	1	7.1	4.8	10.5	2.1	9.6	9.1
120	RT	1	15.6	4.4	9.9	3.8	20.6	7.4
60	5 °C	1	11.9	7.1	3.9	5.4	10.6	6.3
120	5 °C	1	15.5	4.1	6.8	6.1	12.0	2.5
0	RT	2	NA	10.3	NA	2.2	NA	4.5
60	RT	2	15.2	6.8	−0.8	5.1	4.3	3.4
Analyte: NAM								
0	RT	1	NA	13.4	NA	7.3	NA	14.5
20	RT	1	4.1	17.0	3.9	11.3	2.3	15.3
60	RT	1	6.1	10.9	−5.1	3.2	−10.6	14.8
120	RT	1	15.9	8.4	−5.8	7.0	−3.5	8.3
60	5 °C	1	10.7	6.0	−4.3	6.6	−0.8	10.4
120	5 °C	1	10.8	10.4	0.7	6.9	−9.7	8.7
0	RT	2	NA	7.8	NA	12.7	NA	8.6
60	RT	2	−10.9	7.5	4.8	18.5	9.3	12.3

Percent change in analyte concentration is versus 0 min. NA: not applicable.

3.3.8. *Freezer storage stability*

The freezer storage stability of Nia-114, NIC, and NAM in rabbit plasma was determined using quadruplicate replicates of low and high plasma QCs. The QCs were kept at −20 °C for one week or at −80 °C for one month prior to analysis. These QCs were analyzed in the same run as five replicates each of freshly prepared low and high QCs. The resulting differences in the measured concentration for the freezer storage stability QCs versus the fresh QCs are summarized in Table 7.

Table 7
Freezer storage stability of Nia-114, NIC, and NAM in plasma QCs

Analyte	QC nominal concentration (ng/ml)	Storage condition	Measured concentration within run	% Change
Nia-114	6	Fresh	5.08 ± 0.06	
	6	−80 C 1 month	5.29 ± 0.06	4.1
	6	−20 C 1 week	1.81 ± 0.51	−64.4
Nia-114	900	Fresh	772.72 ± 12.10	
	900	−80 C 1 month	726.35 ± 14.05	−6.0
	900	−20 C 1 week	230.68 ± 44.80	−70.2
NIC	24	Fresh	24.95 ± 2.93	
	24	−80 C 1 month	24.47 ± 1.18	−1.9
	24	−20 C 1 week	21.60 ± 0.77	−13.4
NIC	900	Fresh	772.60 ± 26.53	
	900	−80 C 1 month	685.10 ± 22.37	−11.3
	900	−20 C 1 week	728.57 ± 33.30	−5.7
NAM	125	Fresh	157.95 ± 7.21	
	125	−80 C 1 month	155.17 ± 10.25	−1.8
	125	−20 C 1 week	131.54 ± 28.06	−16.7
NAM	900	Fresh	797.89 ± 25.43	
	900	−80 C 1 month	692.81 ± 81.32	−13.2
	900	−20 C 1 week	726.98 ± 146.09	−8.9

Table 8
Autosampler stability of processed plasma QCs

Analyte	QC nominal concentration (ng/ml)	Measured concentration within run	Measured concentration 48 h later	% Change
Nia-114	6	6.60 ± 0.69	5.95 ± 0.27	−9.8
	900	956.62 ± 35.92	919.66 ± 71.48	−3.9
NIC	24	23.05 ± 2.57	21.90 ± 2.21	−5.0
	900	892.62 ± 39.43	888.28 ± 55.46	−0.5
NAM	125	131.730 ± 18.02	127.94 ± 18.58	−2.9
	900	845.22 ± 76.52	900.03 ± 33.85	6.5

As seen in the table, there was 64–70% loss of Nia-114 in the low and high QCs after storage at −20 °C for one week, whereas <7% loss was measured after storage of the QC's at −80 °C for one month. NIC and NAM yielded variable results and no obvious trend of an increase or decrease in concentration for a given freezer storage condition, with the resulting % change <15% in all cases except one. Due to the instability of Nia-114 at −20 °C versus −80 °C, it is recommended that plasma containing Nia-114 be stored at −80 °C.

3.3.9. Autosampler stability

The stability of Nia-114, NIC, and NAM was determined in extracted rabbit plasma QCs after residence in the HPLC autosampler for 2 days at 15 °C. The tests were performed using five replicates each of low and high plasma QCs. The resulting change in analyte response was found to be within ±10% of the original value, for each analyte, as seen in Table 8. The absolute peak areas of all analytes did not show evidence of decreasing after 2 days. Thus, there was no significant instability of the analytes in the autosampler at low and high plasma QC concentrations over 2 days at 15 °C.

4. Conclusions

A bioanalytical method was successfully developed and validated for the determination of Nia-114, NIC, and NAM in rabbit plasma, and is appropriate for use in support of rabbit dermal studies of Nia-114. The linear range of the method was 2–1000, 8–1000, and 75–1000 ng/ml for Nia-114, NIC, and NAM, respectively. The relatively high value for the LLOQ of NAM was due to the high level of endogenous NAM in the rabbit plasma. The use of MEK as a deproteinization solvent provided a simple, single method to extract the analytes with good recovery, and the Spherisorb Cyano column provided isocratic chromatographic separation of the analytes with a run time of 9 min, to allow simultaneous analysis by LC–MS/MS. The validation results demonstrated good sensitivity, specificity, linearity, accuracy, and precision of the method for each analyte.

Nia-114 was unstable in rabbit plasma, as evidenced by loss of 44–50% at room temperature by 2 h, and loss of 64–70% upon storage at −20 °C for 1 week, whereas it was stable (<7% loss) upon storage at −80 °C for 1 month. It is recommended that plasma samples containing Nia-114 be stored at −80 °C. Due to the instability of Nia-114 in rabbit plasma, care must be taken to minimize and normalize the duration plasma calibration standards, QCs and samples are at room temperature before extraction. We have demonstrated that MEK can be added to plasma samples while they are frozen, or after they are thawed 20 min, and appropriate validation results attained.

Because NIC and NAM are endogenous intermediary metabolites in mammals, it is important that the plasma used to prepare calibration standards and QCs be from the same species as the experimental samples, and to analyze matrix blanks in each run. From selectivity tests measuring plasma levels of NIC and NAM in different lots of rabbit and human plasma, the endogenous levels of NIC and NAM were much higher in rabbit plasma than in human plasma (359 ng/ml versus 38.3 ng/ml for mean NAM, and 12.0 ng/ml versus <2.00 ng/ml for mean NIC, in rabbit and human plasma, respectively). Due to the differences in the endogenous levels in rabbit and human, it is expected that the method would provide much better sensitivity for NAM, and equivalent or better sensitivity for NIC, if validated in human plasma.

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