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The determination of nicotinic acid in plasma by mixed-mode liquid chromatography–tandem mass spectrometry following ion exchange solid phase extraction

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Abstract

An assay for nicotinic acid in plasma samples has been developed using ion exchange solid phase extraction in 96-well format followed by mixed-mode ion exchange/reversed-phase liquid chromatography with positive ion tandem mass spectrometry detection. The assay avoids the need for time-consuming derivatisation procedures or involatile ion-pair chromatography reagents. The assay is linear over the wide range 0.05–20 μg/mL, based on a 100 μL sample (correlation coefficient >0.99). The assay is accurate and precise (bias and coefficient of variation <18%) over this calibration range.

Keywords: Nicotinic acid; Mixed-mode liquid chromatography–tandem mass spectrometry; Ion exchange solid phase extraction

1. Introduction

Nicotinic acid (Niacin, Fig. 1) is a B-complex Vitamin that has use, alone or in combination with other therapy, as a lipid-altering agent [1]. It is often used as a model compound in drug discovery studies to comparatively test the effects of new therapeutic agents that may be potential lipid-altering agents.

As a consequence, there is a need to quantify nicotinic acid in plasma samples. Many methods have been reported for this assay, but most involve either ion-pair chromatography, e.g., Refs. [2–6], or derivatization, e.g., Refs. [7–10], following protein precipitation or solid phase extraction, often with subsequent pre-chromatographic derivatisation. In the ion-pair chromatography assays for nicotinic acid, the reagents used are often involatile and, thus, incompatible with mass spectrometry interfaces. Secondly, the inclusion of a derivatisation process into the sample preparation procedure can be time-consuming and, in some cases, complicated. The modern drug discovery bioanalytical laboratory is set up for fast generic methods of quantification of potential candidate drugs in biological fluids, most often by liquid chromatography coupled to tandem mass spectrometry (LC–MSMS). In many laboratories such generic assays are capable of successfully performing the bioanalysis of >95% of the new chemical entities that undergo pharmacokinetic evaluation. The reported ion-pair or derivatisation assays, therefore, represent a substantial deviation from the practice and experience of many of today's pharmaceutical bioanalysts with the potential for a greater number of errors unless substantial training is carried out.

There is, therefore, a need for a simple, routine, LC–MSMS assay for the determination of nicotinic acid in plasma. Recently, Pfuhl et al. [11] have reported an assay using liquid chromatography coupled to selective ion monitoring (SIM) mass spectrometry, following extraction from plasma by ion exchange solid phase extraction. The authors report a limit of quantification of 50 ng/mL, but this sensitivity is based on a 1 mL sample volume which, while often applicable to clinical samples, is a volume not usually attainable in small animal studies. Further, the reported assay has a narrow linear calibration range of 50–750 ng/mL and while the authors show the ability to dilute samples linearly by

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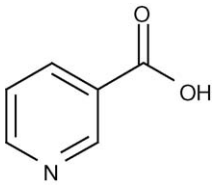

Fig. 1. Structure of nicotinic acid.

up to a factor of 4, frequently plasma nicotinic acid concentrations exceed this effective upper limit of 3 μg/mL. It has been reported [12] that following a 1 g oral dose (a dosage typical for lipid modifying activity with nicotinic acid), peak plasma nicotinic acid concentrations of approximately 25 μg/mL are achieved 30–60 min post dose.

In the current paper, an assay for nicotinic acid in plasma based on liquid chromatography with multiple reaction monitoring (MRM) mass spectrometry detection (LC–MSMS) following solid phase extraction is reported. The assay is based on a small, 100 μL sample volume and is linear, accurate and precise over a wide calibration range (0.05–20 μg/mL) that is applicable to the quantification of samples derived from comparative pharmacological and pharmacokinetic animal studies in drug discovery programmes. With further validation, the wide calibration range means the assay can also bring benefits in clinical situations. It is acknowledged that a significant reduction in throughput compared to a typical generic drug discovery plasma assay has been necessary, but the simplicity of the assay and the avoidance of non-routine procedures mean that the presented assay has proved to be of great benefit.

2. Experimental

2.1. Chemicals

Nicotinic acid, sodium salt and d4-nicotinic acid (for use as internal standard) were obtained from the Aldrich Chemical Company (Gillingham, Dorset, UK). Ammonium acetate, formic acid, water (HPLC grade), methanol (HPLC grade) and acetonitrile (HPLC grade) were obtained from Fisher Scientific (Loughborough, Leics., UK).

2.2. Preparation of standard solutions

Approximately 10 mg of nicotinic acid was weighed accurately and dissolved in an appropriate volume of 50:50 acetonitrile:water to provide a 10 mg/mL solution. Subsequent dilutions were then made in water to provide solutions for spiking control plasma (rat plasma was used in the intra-assay validation experiment detailed below) to generate calibration samples. Approximately 10 mg d4-nicotinic acid was accurately weighed and dissolved in a volume of 50:50 MeCN:H_2O to provide a 1 mg/mL solution. A 0.1 mL aliquot of this solution was diluted to 10 mL to provide a 10 μg/mL solution. This was termed the working internal standard (IS) solution.

Table 1
Gradient profile for the analysis of nicotinic acid by mixed-mode ion exchange/reversed-phase LC–MSMS

Time (min)	%A	%B
0	100	0
2	100	0
8	0	100
11	0	100
11.1	100	0
25	100	0

Mobile phase A: 0.1% formic acid in water; mobile phase B: 50 mM ammonium acetate in 50:50 acetonitrile:water; flow rate 0.5 mL/min; column 100 mm × 2.1 mm 5 μm Oasis MCX; n.b. retention time of nicotinic acid = 9.9 min.

2.3. Chromatography

The determination of nicotinic acid was carried out on a 100 mm × 2.1 mm Oasis MCX 5 μm column (Waters Inc., Milford, MA, USA). A simple linear reversed-phase gradient was employed, using 0.1% aqueous formic acid (A) and 50 mM ammonium acetate in 50:50 acetonitrile:water (B). The mobile phase composition was held at 0% B for the initial 2 min. Thereafter the percentage of solvent B was programmed to increase from 0% to 100% in a further 6 min. The composition was maintained at 100% B for 3 min then rapidly returned to 100% A by 11.1 min. The system was then allowed to re-equilibrate in 100% up to a total time of 25 min. The flow rate was 500 μL/min without splitting prior to entering the mass spectrometer. The first 2 min of flow were diverted to waste post-column. The gradient profile is summarised in Table 1.

2.4. Mass spectrometry

All mass spectrometry was performed on an API-365 (Sciex, Concord, Ont., Canada) which had received an Ionics EP10+ modification (Ionics, Toronto, Ont., Canada) and was fitted with a Turbo Ionspray interface operating in positive ion mode at 400 °C with nitrogen as both the nebuliser and curtain gas. Nitrogen was also used as the collision gas at a setting of 2, with a collision energy of 30 V. Nicotinic acid and the internal standard (d4-nicotinic acid) were detected by tandem mass spectrometry (MS/MS) using selected reaction monitoring (SRM) of the transitions m/z 124 → 80 and 128 → 84, respectively, with a dwell time of 200 ms per transition.

2.5. Solid phase extraction (SPE) of nicotinic acid from plasma samples

The extraction of nicotinic acid from plasma was achieved using Strata-X-C 33 μm cation mixed-mode polymer 96-well solid phase extraction plates, filled with 10 mg sorbent per well (Phenomenex, Macclesfield, UK). The plates were conditioned with 0.4 mL of methanol, 0.4 mL of 50:50 methanol:50% aqueous acetic acid and, finally, 0.4 mL 50% aqueous acetic acid. To each 100 μL sample aliquot was added 10 μL of the working

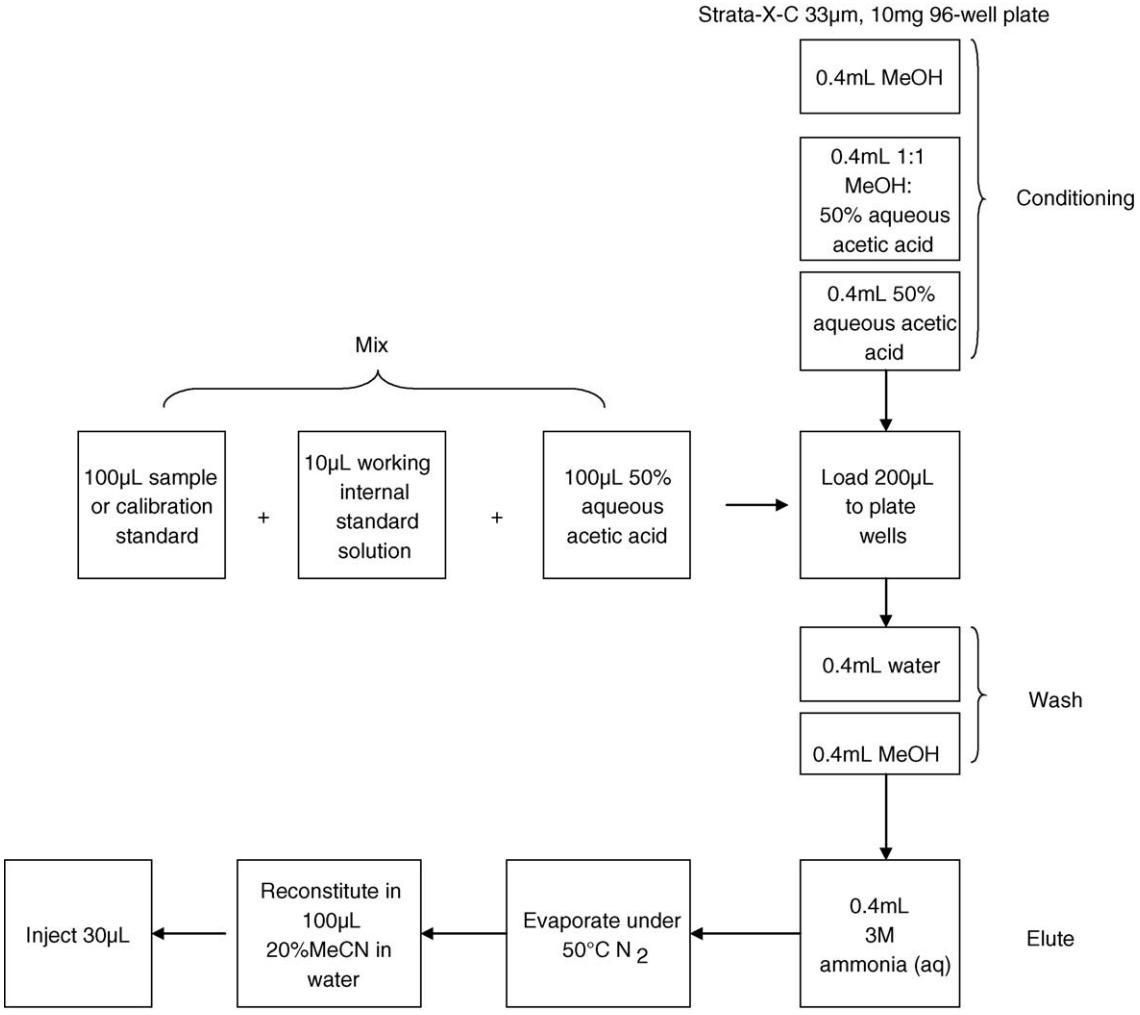

Fig. 2. Schematic representation of the sample preparation process for the assay of nicotinic acid in plasma.

internal standard solution and 100 μL of 50% aqueous acetic acid. The samples were then vortex-mixed and 200 μL aliquots were added to the extraction plate. Following sample addition, the wells were washed with 0.4 mL water and 0.4 mL methanol, before elution was effected using 0.4 mL 3 M aqueous ammonia solution. Samples were evaporated to dryness under nitrogen at 50 °C and reconstituted in 100 μL of 20% acetonitrile in water. Thirty-microliter aliquots were injected into the chromatograph. A schematic of the sample preparation process is given in Fig. 2.

2.6. Calibration lines and intra-assay precision and accuracy

Six-fold replicates of calibration standards were analysed as described above. The first and last sets were nominated as calibrants and the middle four sets were considered as intra-assay validation controls. Peaks were integrated by the Analyst software, Version 1.3. The calibration curve was constructed by plotting the peak area ratios of the analyte to internal standard against the nominal concentration using a weighted ($1/x^2$) linear regression model. Concentrations of the analyte in the samples were subsequently interpolated from the curves. The intra-assay variability of the method was determined using the coefficient of variation of replicate analyses for each concentration in the calibration range on a single occasion. The intra-assay accuracy of the method was determined by comparing the mean measured concentrations with the nominal concentrations.

3. Results and discussion

3.1. General

A simple, robust assay is detailed which has a limit of quantification for nicotinic acid in plasma samples of 0.05 μg/mL, based on cation exchange solid phase extraction followed by mixed-mode ion exchange/reversed-phase chromatography with liquid chromatography–tandem mass spectrometry (LC–MSMS) detection. Example chromatograms of a blank plasma extract, a 0.05 and a 10 μg/mL calibration standard are given in Fig. 3. A representation of the regression line formed from all six sets of replicates is shown in Fig. 4. As can be seen from Fig. 3, due to some degree of interference (possibly due to autosampler carry-over or endogenous nicotinic acid) the signal:noise ratio for the 0.05 μg/mL calibrant is approximately 2× that for the "blank",

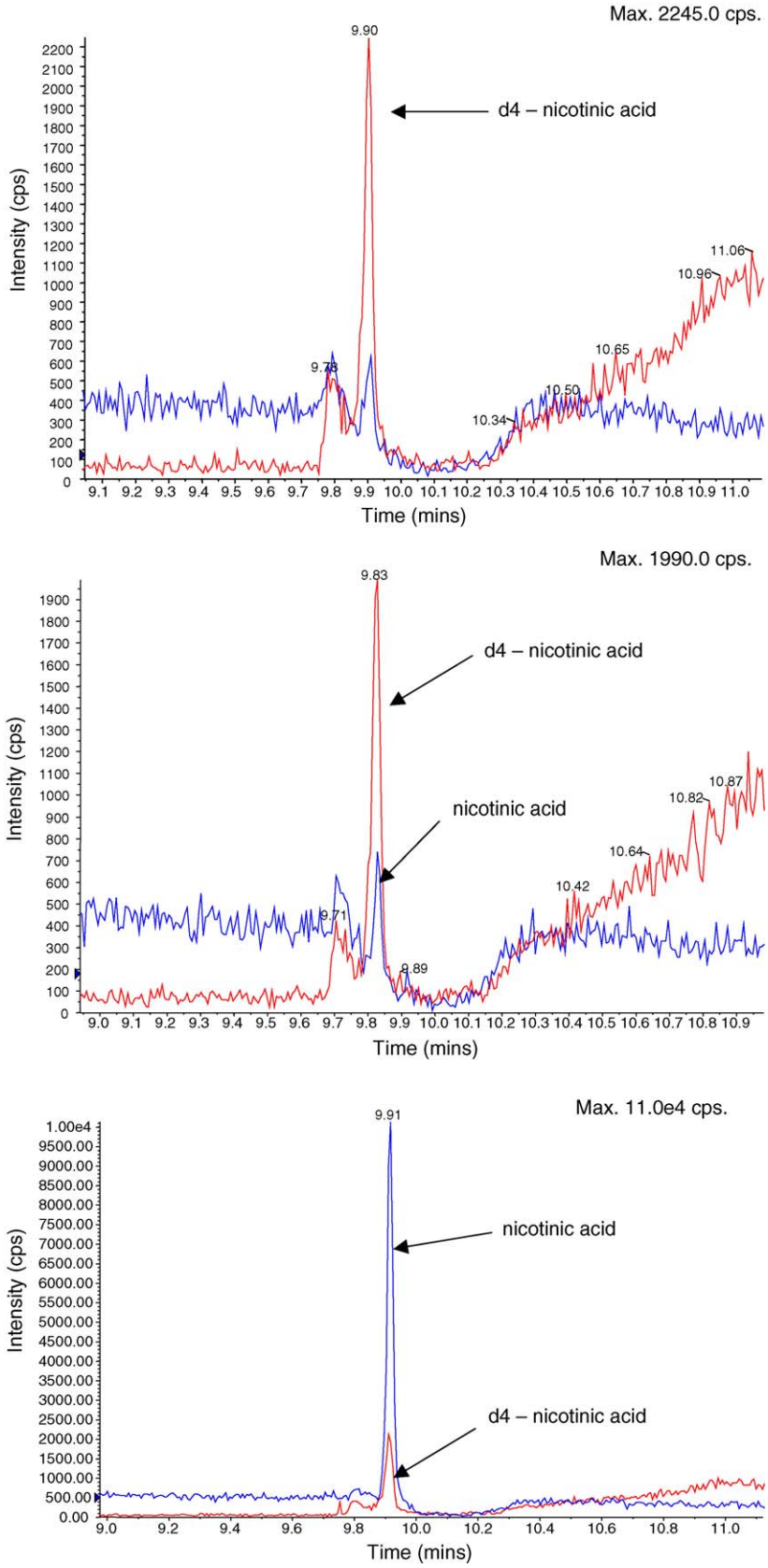

Fig. 3. (a) Cation exchange SPE/mixed-mode ion exchange/reversed-phase LC–MSMS analysis of an extract of blank plasma, spiked with d4-nicotinic acid as internal standard. (b) Cation exchange SPE/mixed-mode ion exchange/reversed-phase LC–MSMS analysis of an extract of a 0.05 μg/mL nicotinic acid spiked in plasma sample, spiked also with d4-nicotinic acid as internal standard. (c) Cation exchange SPE/mixed-mode ion exchange/reversed-phase LC–MSMS analysis of an extract of a 10 μg/mL nicotinic acid spiked in plasma sample, spiked also with d4-nicotinic acid as internal standard.

Table 2
Intra-assay accuracy and precision data for the analysis of nicotinic acid in plasma in the range 0.05–20 μg/mL by cation exchange SPE followed by mixed-mode ion exchange/reversed-phase LC–MSMS

Nominal concentration (μg/mL)	0.02	0.05	0.10	0.20	0.50	1.00	5.00	10	20
Replicate 2	0.025	0.046	0.098	0.23	0.61	1.16	5.68	11.0	19.7
Replicate 3	0.014	0.054	0.083	0.21	0.39	1.17	4.42	9.13	14.5
Replicate 4	0.020	0.053	0.084	0.17	0.51	1.03	4.45	8.19	18.4
Replicate 5	0.024	0.060	0.091	0.13*	0.50	1.05	5.02	8.78	11.7*
Mean	0.021	0.053	0.089	0.21	0.50	1.10	4.89	9.28	17.5
S.D.	0.005	0.006	0.007	0.029	0.090	0.073	0.59	1.21	2.71
%CV	24.1	10.8	7.8	14.2	17.9	6.6	12.1	13.1	15.5
%Bias	3.8	6.5	−11.0	2.5	0.4	10.0	−2.2	−7.2	−12.5

* Anomalous value omitted from statistical calculations due to poor peak shape; calibration range 0.05–20 μg/mL; 0.02 μg/mL data included here to illustrate the poor precision at this concentration.

which is less than the ideal 3× differentiation that would be desired. However, interpolated sample concentration results at this spiked concentration are more than acceptable—see Table 2 for the summary data for an intra-accuracy and precision analysis batch. The assay has been widely applied in our laboratory to the analysis of samples from many animal species and example plasma concentration versus time chromatograms and profiles for nicotinic acid following oral dosing at 100 mg/kg to rats are shown in Figs. 5 and 6. The measured concentrations fit well with those observed before in rats dosed orally with nicotinic acid [13] giving further evidence of the accuracy of the method reported here.

3.2. Method development

During the method development process, nicotinic acid was, not surprisingly, found to be very poorly retained under common reversed-phase conditions, even with an extremely shallow gradient profile, e.g., 0–5% organic modifier over a 15 min period. Hydrophilic interaction liquid chromatography (HILIC) conditions were tried but also found to yield surprisingly poor retention. Successful chromatography of nicotinic acid was only achieved on a mixed-mode column, in which not only the organic modifier percentage but also the ionic strength is raised over the gradient profile. The retention issue could have been resolved by the use of ion-pairing reagents, but the majority of commonly used ion-pair reagents are involatile and thus incompatible with mass spectrometry interfaces.

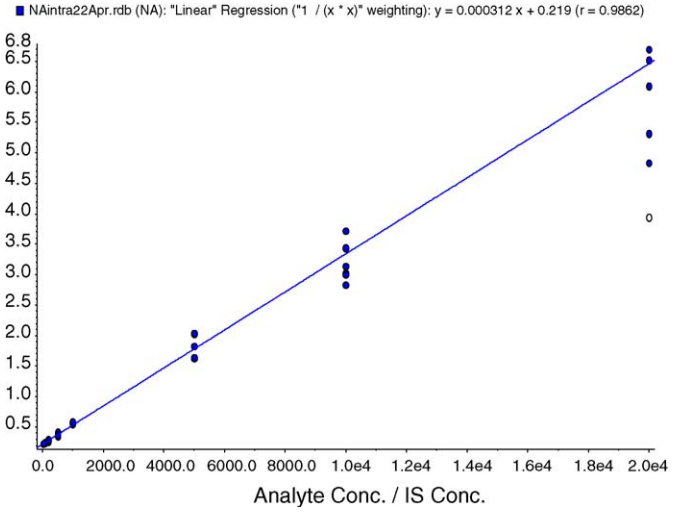

Fig. 4. Regression line of all concentration data from an intra-assay accuracy and precision batch of samples for the analysis of nicotinic acid in plasma by cation exchange solid phase extraction followed by mixed-mode ion exchange/reversed-phase LC-positive ion MSMS.

It was found that simple protein precipitation procedures commonly adopted for pharmaceutical bioanalysis did not lead to extracts of sufficient "cleanliness" for successful quantification, presumably due to these crude extracts containing many endogenous compounds which could contribute to significant interference. Further, the zwitterionic nature of nicotinic acid (at high pH it is negatively charged at the carboxylic acid function, while at low pH it is positively charged at the pyridinyl nitrogen) made it not readily achievable to define conditions under which it would be extracted from plasma into typical liquid–liquid extraction solvents, which are generally relatively non-polar. Some of these issues may have been resolvable by performing a derivatisation procedure following extraction but it was not desired to add further steps and complication to the assay procedure.

Fortunately, ion exchange solid phase extraction was found to achieve sufficient cleanliness of extract and sufficient recovery to permit the quantification of nicotinic acid from plasma samples on standard LC–MSMS equipment at a sensitivity which was adequate.

3.3. Sensitivity

The present assay has a limit of quantification of 0.05 μg/mL based on a 0.1 mL sample volume which makes the assay suitable for use in support of small animal studies, as well has having a wide calibration range which could allow the assay to be further validated for clinical use. The limit of quantification is adequate for the determination of plasma nicotinic acid concentrations in animal model studies or in the clinic.

However, it is higher than for most modern LC–MSMS drug bioanalyses, probably as a result of the observed higher background noise experienced at the low molecular mass of the analyte (MW = 123) due to it being in the mass range of many compounds endogenous to plasma, coupled with the high salt concentration in the elution solvent. That said, the assay does display acceptable intra-assay accuracy and precision over the range 0.05–20 μg/mL for nicotinic acid in plasma, (%coeffi-

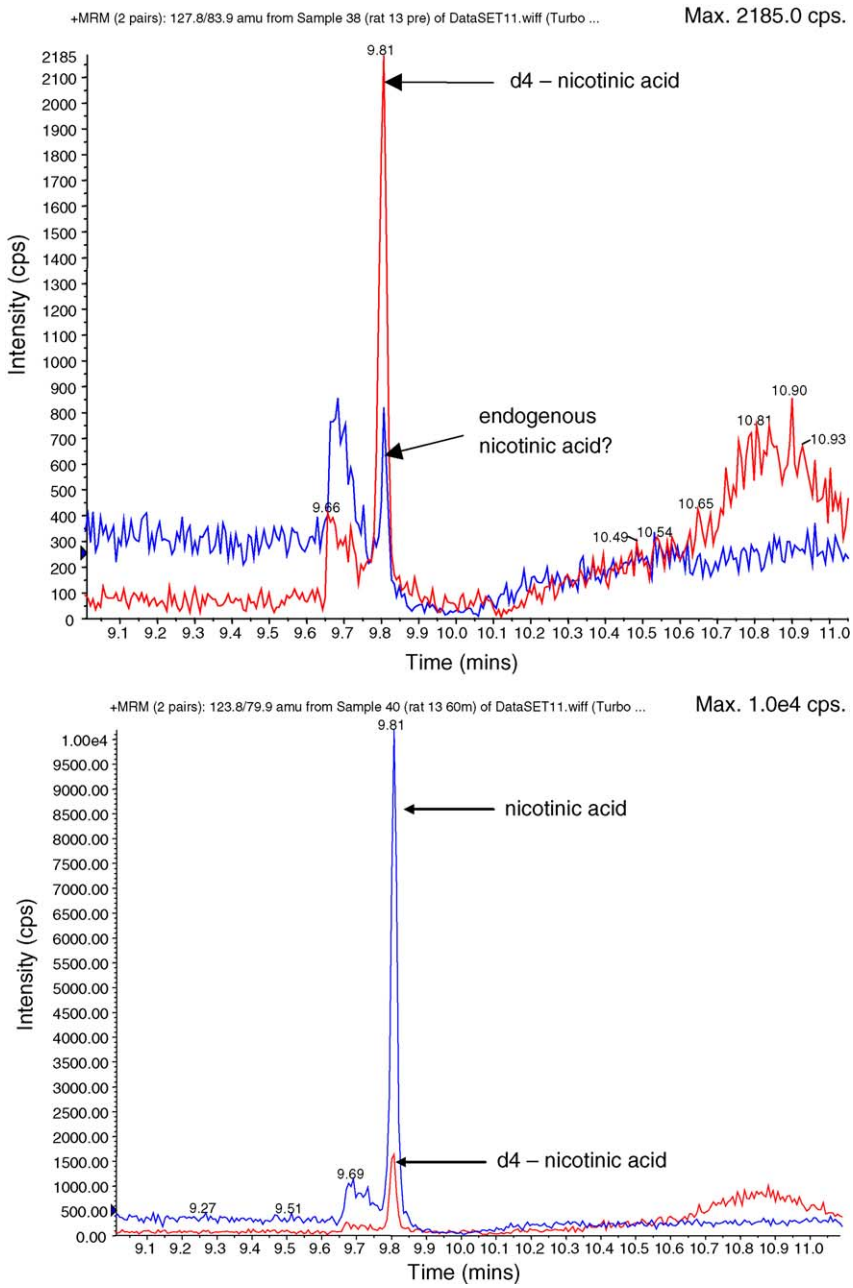

Fig. 5. (a) Cation exchange SPE/mixed-mode ion exchange/reversed-phase LC–MSMS analysis of an extract of a pre-dose plasma sample in a single oral dose, 100 mg/kg, fed vs. fasted rat study, spiked also with d4-nicotinic acid as internal standard. (b) Cation exchange SPE/mixed-mode ion exchange/reversed-phase LC–MSMS analysis of an extract of a plasma sample 1 h post a single oral dose, 100 mg/kg to a fed rat, spiked also with d4-nicotinic acid as internal standard.

cient of variation (CV) <18% and %bias <13%). It is also fair to suggest that because the new method uses MRM rather than selected ion monitoring (SIM) detection, it may offer selectivity benefits compared to the assay reported by Pfuhl, particularly due to possibility of interference in SIM chromatograms from the many compounds endogenous to plasma with similar low molecular mass as the analyte.

3.4. Throughput

It is obvious that, while a typical drug bioanalysis can be achieved within 2–3 min, this assay requires a commitment to a 25 min per sample run time. A large proportion of this time is made up of the long (∼14 min) re-equilibration time which might seem excessive as 14 min at 0.5 mL = 7 mL and is equivalent to approximately 40 column volumes (assuming the stationary phase occupies approximately 50% of the column volume). However, we found that reduction of the cycle time to 20 min with the same gradient profile resulted in a drift in the retention time for nicotinic acid. Likewise, attempts to reduce the gradient time led to instability in the retention time. Under the 25 min per sample regime, we found that the retention time for the analyte was very stable. The relatively long per sample analysis time is overcome in our laboratory by scheduling large

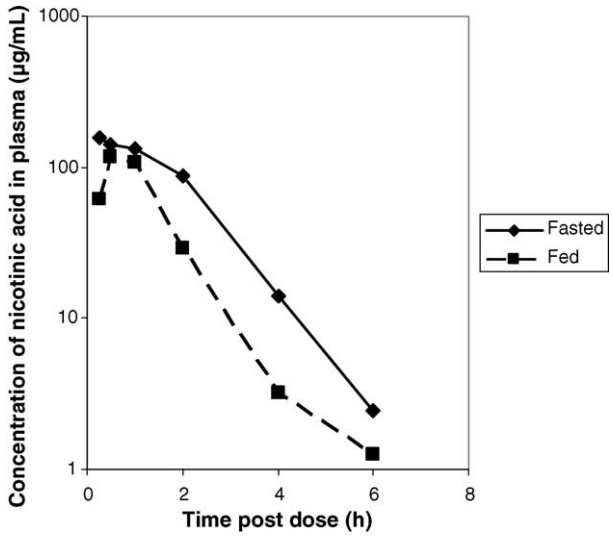

Fig. 6. Concentrations of nicotinic acid in plasma in rats following single oral dosing at 100 mg/kg.

assay batches to run unattended on the LC–MSMS system over the weekend. Injection of calibration standards at the beginning and end of these batches showed no significant deviation in the slopes of the resulting initial and final calibration lines indicating that any instability over this assay duration was effectively compensated by the use of the stable-isotope labeled internal standard.

Another concern in bioanalysis is the gradual build-up of micro-particulate matter derived from the plasma sample being deposited at the head of the chromatographic column and leading to a steady rise in the back-pressure observed in running the assay for large batches. Our experience was that the solid phase extraction procedure detailed here led to extremely clean extracts and no problems with increasing back-pressure. Indeed we observed that an initial back-pressure in the system of 177 bar rose to no more than 185 bar after at least 500 samples had passed through the column.

4. Conclusions

An assay for nicotinic acid in plasma samples has been developed based on ion exchange solid phase extraction followed by mixed-mode ion exchange/reversed-phase chromatography and positive ion tandem mass spectrometry. The assay is linear, accurate and precise over a wide calibration range (0.05–20 μg/mL) with a limit of quantification of 0.05 μg/mL. The assay is based on a small, 100 μL sample volume making it useful for the support of samples from animal studies, and is in regular use in our laboratory for the determination of nicotinic acid in plasma samples from many drug discovery support studies in several animal species. The assay avoids complicated, time-consuming derivatisation procedures or ion-pair reagents that are incompatible with LC–MSMS.

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