Available online at www.ijpcr.com
International Journal of Pharmaceutical and Clinical Research 2014; 6(3): 206-213

ISSN- 0975 1556

Research Article

Bioanalytical Method Development and Validation of Niacin and Nicotinuric Acid in Human Plasma by LC–MS/MS

*Bratati Roy, Bhupinder Singh, Anjana Rizal, C.P. Malik

Jaipur National University, Jaipur, India

Available Online: 1st July 2014

ABSTRACT

An LC–MS method for the determination of Niacin and its metabolite Nicotinuric acid in human plasma was developed and validated. Sample preparation involved the solid phase extraction method. Chromatographic separation was performed on Phenomenex Gemini NX,5µm 4.6 mm x 100 mm column with the mobile phase consisting of Acetonitrile : (5 mM ammonium acetate buffer : Formic Acid::99.9:00.2 v/v) 70:30 v/v. The interface used with the API 3000 LC-MS/MS was a turbo ion spray in which positive ions were measured in MRM mode. The method was validated over the concentration range of 10.068-5002.086 ng/mL (NIC) and 10.157-5000.450 ng/mL (NIA). The recovery was 77.771% (NIC), 74.014 % (NIA) and the Lower imit of quantitation (LLOQ) was 10.068 ng/mL(NIC) and 10.157 ng/mL (NIA) . The intra- and inter-day precision of the method at four concentrations was 1.67-10.42% and 2.37-9.76% for NIC and 1.79-6.29% and 4.62-6.44% for NIA .Stability of compounds was established in a series of stability experiments. The method can be used for the simultaneous determination of Niacin and Nicotinuric acid in human plasma.

Key words: Niacin, Nicotinuric acid, LC-MS/MS, Bioanalysis, Bioequivalence, NIC, NIA

INTRODUCTION

Niacin also known as vitamine B3 is a endogenous molecule having medicinal role. This well known nutraceutical belongs to the water soluble Vitamin B complex family thatprevent pellagra and a antihyperlipidemic agent. Niacin is a component of nicotinamide adenine dinucleotide which is biosynthesized through nicotinamide (Iwaki *et al.,* 2000). It is a precursor of coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). These coenzymes triggers dehydrogenesis reactions and have catalytic impact of many biomolecules (Wan *et al.,* 2011). Niacin is basically reduces low density lipoprotein cholesterol (LDL), very low-density lipoprotein cholesterol (VLDL-C), and triglycerides (TG), but effectively increases high density lipoprotein cholesterol (HDL) (Villines *et al.,* 2012). Niacin alone (Bruckert *et al.,* 2010) or in combination with other lipid lowering agents such as statin (Taylor *et al.,* 2006) or ezetimibe (Taylor *et al.,* 2009) significantly reduces risk of cardiovascular disease and arthrosclerosis progression (Lukasova *et al.,* 2011) or hardening of the arteries. It lowers the risk of heart attacks. There is also some evidence that it might help in lowering the risk of Alzheimer's disease, cataracts, osteoarthritis, and type 1 diabetes. However, more research needs to be done. The present study is concerned with the development and validation of Niacin and its metabolite Nicotinuric acid in human plasma by HPLC–MS/MS and its application in bioequivalence studies.

EXPERIMENTAL

Chemicals and materials: NIC (mol.wt. 123.11; 99.95% w/w), NIA (mol.wt. 180.16; 98.75% w/w), and Niacin D4 (mol.wt. 127.10, 99.63 %) were obtained from Clearsynth Labs (P) Ltd. Control buffered (K_2EDTA) human plasma was procured from Laxmi Sai Clinical lab., India. All other reagents/chemicals were of AR grade.

LC–MS/MS instrumentation and settings: A high performance liquid chromatography system (Shimadzu Co., Kyoto, Japan) with Phenomenex Gemini NX ,5µm 4.6 mm x 100 mm column was used in this project. The column oven and autosampler temperature were maintained at 35± 2 °C and 5±1ºC respectively. The flow rate was set at 1.000 mL/min with split ratio of 70% flow to waste. Ionization and detection of analyte and IS was performed on a triple quadrupole mass spectrometer, API 3000 LC-MS/MS equipped with Turbo Ion spray®, from MDS SCIEX (Toronto, Canada) operated in the positive ion mode. Quantitation was done using MRM mode to monitor protonated precursor product ion transition of m/z 123.90 →80.10 amu, 181.00 →134.9 amu and 128.00 →84.10 for Niacin (NIC), Nicotinuric acid (NIA)and Niacin D4(IS) respectively. All the parameters of HPLC and MS were controlled by Analyst software version 1.4.2. The source dependent parameters maintained for analyte and IS were Nebulizer Gas (NEB): 10 psi, Curtain Gas (CUR): 9.00 psi, Collision Activation Dissociation (CAD): 4 psi, Turbo Heater Temperature (TEM): 450.00 ºC, Ion Spray Voltage (IS): 3500 V. The compound dependent parameters for analyte and IS were Declustering Potential: 52 V (NIC),42 V (NIA),43 V

*Author for correspondence: Email: bratatiroy@rediffmail.com

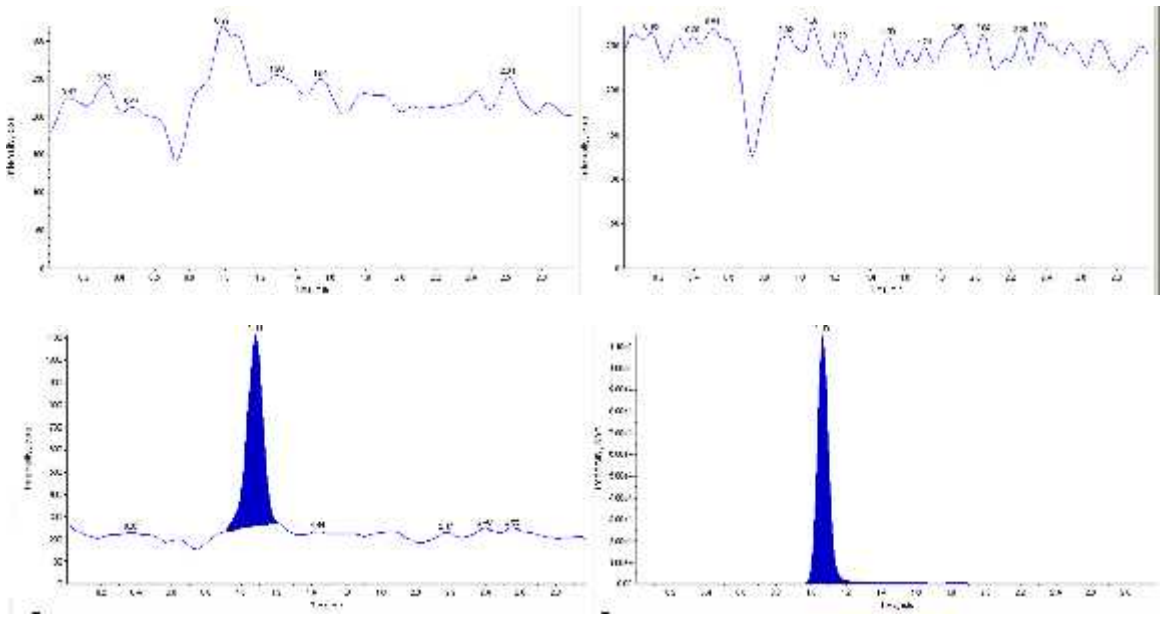

Fig.1A: Plasma blank LLOQ of NIC and NICD6

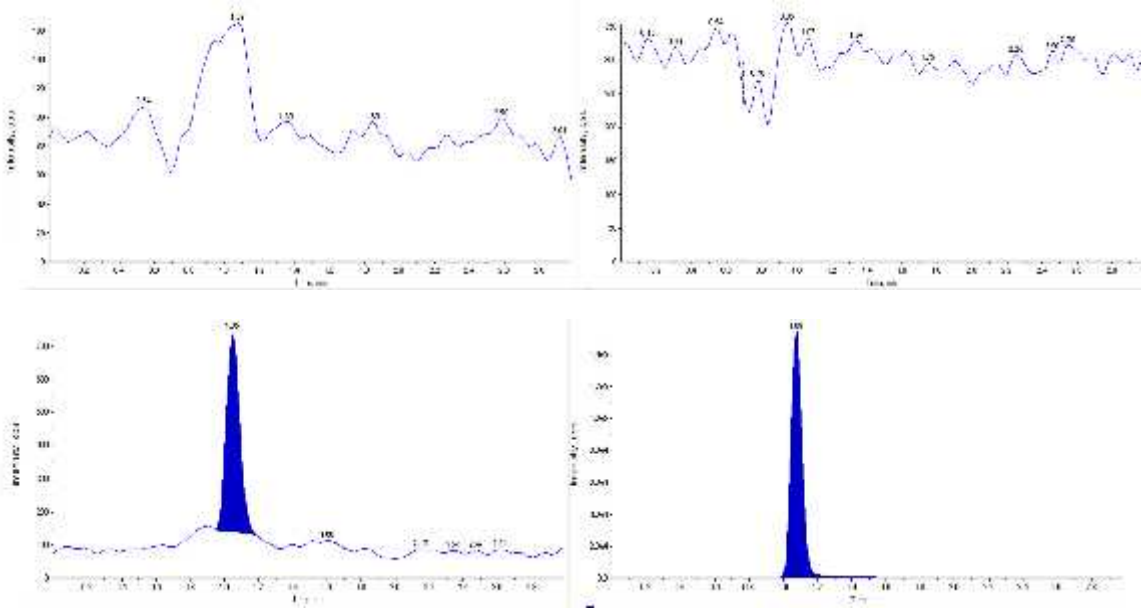

Fig.1B: Plasma blank and LLOQ of NIA and NICD6

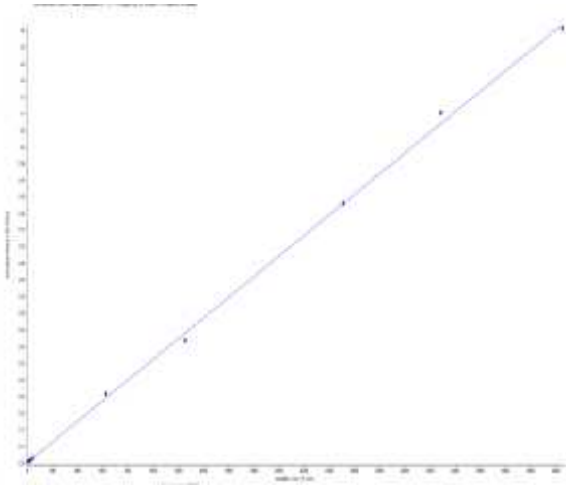

Fig. 2A: Calibration Curve of NIC

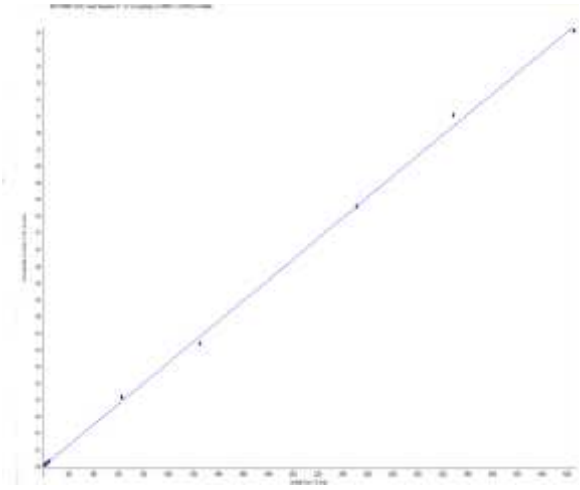

Fig. 2B: Calibration Curve of NIA

(IS),Focusing Potential (FP): 150V (NIC),135V(NIA),137V (IS) ,collision energy(CE):22V (NIC),15V(NIA),31V (IS), Collision Cell Exit Potential(CXP): 15V (NIC),9V(NIA),15 V(IS), entrance potential(EP): 10 V (NIC),10V(NIA),9V (IS).

Preparation of standard stock and plasma samples: The standard stock solution of Niacin and Nicotinuric acid (1mg/mL), and Niacin D4 (1mg/10mL) were prepared by dissolving in diluent solution (Methanol: Milli-Q Water: 50:50, v/v). Further dilutions from the stock solutions were prepared using diluent solution (Methanol: Milli-Q Water:: 50:50, v/v) for spiking in plasma to obtain calibration curve (CC) standards and quality control (QC) samples. CC standards consisting of a set of eight non-zero concentrations were prepared by spiking 1% of NIA and NIC aqous mixture to the plasma to obtain calibration standards and quality control samples. Calibration standards ranges 10.068 ng/mL , 20.136 ng/mL , 627.284 ng/mL , 1254.569 ng/mL , 2509.137 ng/mL , 3257.831 ng/mL , 4289.815 ng/mL and 5000.086 ng/mL for Niacin and 10.157 ng/mL , 20.314 ng/mL , 625.040 ng/mL , 1254.094 ng/mL , 2508.188 ng/mL , 3284.532 ng/mL , 4251.975 ng/mL and 5000.450 ng/mL for Nicotinuric acid.

The quality control samples were prepared worth concentration of Niacin at LLOQ QC 10.202 ng/mL, LQC 30.580 ng/mL, MQC 2590.836 ng/mL and HQC 4343.210 ng/mL and Nicotinuric acid at LLOQ QC 10.164 ng/mL, LQC 27.590 ng/mL, MQC 2508.188 ng/mL and HQC 3981.250 ng/mL respectively.

After bulk spiking, 500 µL of spiked plasma samples were pipetted out in pre-labeled polypropylene tubes. The calibration curve standards and quality control samples were logged in Ultra Low temperature deep freezer (Temp range: -55ºC to -75°C) except 30 samples each of LQC and HQC which were transferred for storage in Cell

Table 1: Back calculated concentration of calibration curve standards for NIC (n = 3).

		STD A	STD B	STD C	STD D	STD E	STD F	STD G	STD H
	Nominal con. (ng/mL)	10.068	20.136	627.284	1254.569	2509.137	3257.831	4289.815	5002.086
	CC ID	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml
PA BATCH 1	CC 1	10.416	18.324	619.951	1286.265	2523.909	3239.723	4288.825	4964.083
PA BATCH 2	CC 2	10.322	19.250	662.713	1255.311	2513.589	3267.720	4174.388	4918.228
PA BATCH 3	CC 3	10.059	20.058	665.643	1238.390	2433.450	3200.941	4271.463	4820.081
	N	3	3	3	3	3	3	3	3
	Mean	10.266	19.211	649.436	1259.989	2490.316	3236.128	4244.892	4900.797
	SD	0.1850	0.8677	25.5765	24.2779	49.5170	33.5343	61.6723	73.5664
	% CV	1.80	4.52	3.94	1.93	1.99	1.04	1.45	1.50
	Mean % Nominal	101.96	95.40	103.53	100.43	99.25	99.33	98.95	97.98

Table 2: Back calculated concentration of calibration curve standards for NIA (n = 3).

		STD A	STD B	STD C	STD D	STD E	STD F	STD G	STD H
	Nominal con. (ng/mL)	10.157	20.314	625.040	1254.094	2508.188	3284.532	4251.974	5000.450
	CC ID	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml
PA BATCH 1	CC 1	10.556	19.084	593.83	1251.902	2599.728	3416.332	4381.425	5375.586
PA BATCH 2	CC 2	10.437	19.178	665.90	1182.12	2509.111	3388.834	4207.319	5040.809
PA BATCH 3	CC 3	10.509	19.494	665.06	1186.501	2504.874	3413.269	4331.308	5042.465
	N	3	3	3	3	3	3	3	3
	Mean	10.501	19.252	641.594	1206.841	2537.904	3406.145	4306.684	5152.953
	SD	0.0599	0.2148	41.3713	39.0854	53.5828	15.0698	89.6269	192.8073
	% CV	0.57	1.12	6.45	3.24	2.11	0.44	2.08	3.74
	Mean % Nominal	103.38	94.77	102.65	96.23	101.18	103.70	101.29	103.05

Table 3: Inter day and intraday precision and accuracy of the method for NIC

PA BATCH 1	LLOQ QC		LQC		MQC		HQC	
NOMINAL CONC.	10.202	ng/mL	30.58	ng/mL	2590.836	ng/mL	4343.21	ng/mL
QC ID	Observed Conc	% Nominal	Observed Conc	% Nominal	Observed Conc	% Nominal	Observed Conc	% Nominal
1	11.916	116.80	31.212	102.07	2595.001	100.16	4163.677	95.87
2	11.443	112.16	29.766	97.34	2723.408	105.12	4150.609	95.57
3	11.562	113.33	28.866	94.40	2526.861	97.53	4235.352	97.52
4	10.408	102.02	29.422	96.21	2620.833	101.16	4194.269	96.57
5	8.824	86.49	30.383	99.36	2587.473	99.87	4171.936	96.06
6	10.619	104.09	31.931	104.42	2570.25	99.21	4339.259	99.91
N	6	6	6	6	6	6	6	6
Mean	10.795	105.82	30.263	98.96	2603.971	100.51	4209.184	96.91
SD	1.1252		1.1492		66.3102		70.308	
% CV	10.42		3.80		2.55		1.67	
Mean % Nominal	105.82		98.96		100.51		96.91	
PA BATCH 2	LLOQ QC		LQC		MQC		HQC	
7	10.585	103.75	29.599	96.79	2639.366	101.87	4298.795	98.98
8	10.479	102.72	29.142	95.30	2705.684	104.43	4431.198	102.03
9	11.627	113.97	30.304	99.10	2688.498	103.77	4404.81	101.42
10	12.956	126.99	31.676	103.58	2674.975	103.25	4425.303	101.89
11	10.161	99.60	30.654	100.24	2655.892	102.51	4434.144	102.09
12	10.231	100.28	30.734	100.50	2487.624	96.02	4921.487	113.31
N	6	6	6	6	6	6	6	6
Mean	11.007	107.89	30.352	99.25	2642.007	101.98	4485.956	103.29
SD	1.0916		0.8971		79.1668		219.3914	
% CV	9.92		2.96		3.00		4.89	
Mean % Nominal	107.89		99.25		101.98		103.29	
PA BATCH 3	LLOQ QC		LQC		MQC		HQC	
13	8.488	83.20	32.257	105.48	2625.83	101.35	4141.201	95.35
14	10.202	100.00	30.58	100.00	2590.836	100.00	4343.21	100.00
15	10.923	107.07	30.071	98.34	2611.352	100.79	4313.62	99.32
16	10.922	107.06	29.618	96.85	2695.754	104.05	4386.284	100.99
17	10.414	102.08	33.039	108.04	2667.183	102.95	4271.387	98.35
18	10.232	100.29	29.539	96.60	2625.32	101.33	4459.923	102.69
N	6	6	6	6	6	6	6	6
Mean	10.197	99.95	30.851	100.89	2636.046	101.74	4319.271	99.45
SD	0.8969		1.462		38.4908		108.5178	
% CV	8.8		4.74		1.46		2.51	
Mean % Nominal	99.95		100.89		101.74		99.45	
Global calculation								
	LLOQ QC		LQC		MQC		HQC	
N	18	18	18	18	18	18	18	18
Mean	10.666	104.55	30.489	99.7	2627.341	101.41	4338.137	99.88
SD	1.0411		1.1509		62.1913		181.0544	
% CV	9.76		3.77		2.37		4.17	
Mean % Nominal	104.55		99.70		101.41		99.88	

Frost deep freezer (Temp range: -17ºC to -27ºC) for the generation of Long Term Stability at – 22ºC ± 5ºC. These samples were used for performing the method validation.

Preparation of Mobile phase and Solid Phase Extraction method method: A buffer solution was prepared by dissolving approximately 385.4 mg of ammonium acetate in 1000 mL of milli-Q water and 2mL of formic acid in

the buffer solution. Mobile phase was prepared as the mixture of acetonitrile: buffer solution in the ratio 70:30, v/v. For bioanalysis a set of calibration curve standards and/or quality control samples were withdrawn from the deep freezer and allowed to thaw at room temperature. Solid Phase extraction was performed using Strata TM X 33 µm, Polymeric Sorbent cartridges. 50 µL of Niacin D4 as an internal standard (approximately 500.000 ng/mL)

Table 4: Inter day and intraday precision and accuracy of the method for NIA

PA BATCH 1	LLOQ QC		LQC		MQC		HQC	
NOMINAL CONC.	10.164	ng/mL	27.590	ng/mL	2508.188	ng/mL	3981.250	ng/mL
QC ID	Observed Conc	% Nominal	Observed Conc	% Nominal	Observed Conc	% Nominal	Observed Conc	% Nominal
1	11.496	113.11	29.988	108.69	2612.001	104.14	4307.045	108.18
2	11.554	113.68	27.534	99.80	2739.335	109.22	4188.090	105.20
3	11.769	115.79	28.905	104.77	2589.128	103.23	4087.946	102.68
4	10.733	105.60	29.114	105.52	2534.65	101.06	4262.472	107.06
5	10.578	104.07	28.301	102.58	2596.399	103.52	4189.026	105.22
6	10.469	103.00	24.927	90.35	2494.458	99.45	4188.295	105.20
N	6	6	6	6	6	6	6	6
Mean	11.100	109.21	28.128	101.95	2594.329	103.43	4203.812	105.59
SD	0.5685		1.7700		83.5538		75.1393	
% CV	5.12		6.29		3.22		1.79	
Mean % Nominal	109.21		101.95		103.43		105.59	
PA BATCH 2	LLOQ QC		LQC		MQC		HQC	
7	11.874	116.82	25.634	92.91	2381.613	94.95	3822.45	96.01
8	10.177	100.13	25.051	90.80	2552.537	101.77	3869.027	97.18
9	10.856	106.81	30.821	111.71	2449.402	97.66	3807.376	95.63
10	10.976	107.99	29.643	107.44	2509.956	100.07	3927.207	98.64
11	11.663	114.75	24.168	87.60	2438.556	97.22	4012.47	100.78
12	11.551	113.65	29.564	107.15	2324.221	92.67	4042.628	101.54
N	6	6	6	6	6	6	6	6
Mean	11.183	110.02	27.480	99.60	2442.714	97.39	3913.526	98.30
SD	0.6333		2.8447		82.9681		98.1705	
% CV	5.66		10.35		3.40		2.51	
Mean % Nominal	110.02		99.60		97.39		98.30	
PA BATCH 3	LLOQ QC		LQC		MQC		HQC	
13	10.197	100.32	29.633	107.40	2450.365	97.69	3818.222	95.91
14	9.749	95.92	29.151	105.66	2318.211	92.43	3919.981	98.46
15	10.862	106.87	29.837	108.14	2411.150	96.13	3734.461	93.80
16	9.566	94.12	30.248	109.63	2306.290	91.95	3874.39	97.32
17	10.535	103.65	30.863	111.86	2416.061	96.33	3856.189	96.86
18	11.450	112.65	29.457	106.77	2419.546	96.47	3746.521	94.10
N	6	6	6	6	6	6	6	6
Mean	10.393	102.25	29.865	108.25	2386.937	95.17	3824.961	96.07
SD	0.7063		0.6121		59.5787		73.2608	
% CV	6.80		2.05		2.50		1.92	
Mean % Nominal	102.25		108.25		95.17		96.07	
Global calculation								
	LLOQ QC		LQC		MQC		HQC	
N	18	18	18	18	18	18	18	18
Mean	10.892	107.16	28.491	103.27	2474.660	98.66	3980.766	99.99
SD	0.7019		2.1178		115.1192		183.8365	
% CV	6.44		7.43		4.65		4.62	
Mean % Nominal	107.16		103.27		98.66		99.99	

was added into ria vials and 400 µL of plasma samples were aliquoted from the pre-labeled polypropylene tubes into ria vials and vortexed. Additionally 100 µL of 50mM ammonium acetate buffer solution was added to the sample and vortexed. Cartridges were conditioned with 1mL of methanol followed by 1mL of 50mM ammonium acetate buffer solution. Samples were loaded and eluted gently. Washing of cartridges were done using 1 mL of washing solution (5% methanol in milli Q water). Cartridges were further kept for drying for 2 to 3 min at constant gas flow. Elution of samples were done with 1 mL of elution solution (0.5%formic acid in milliQ water solution and acetonitrile solution:: 10:90 v/v). Eluents were evaporated to dryness at 40 °C (at constant pressure) in nitrogen evaporator. Residue was reconstituted in 200 µL of mobile phase (5mM ammonium acetate and formic acid 99.80:0.200 solution and acetonitrile at 70:30 v/v) and analysed.

Method validation**:** The method has been validated for selectivity, sensitivity, linearity, matrix effect, calibration curve standards and quality control samples, precision and accuracy batches. The results of various stabilities i.e. (stock dilution stability at refrigerator temperature and room temperature, standard stock solution stability in refrigerator temperature and room temperature and photo degradation test in dark, auto sampler stability, re-injection reproducibility, freeze thaw stability, long term stability at - 65ºC ± 10°C and at - 22ºC ± 5ºC, reagent stability, bench top stability, dry ice stability, dry extract stability, extended bench top stability, wet extract stability in refrigerator, Lipemic and Haemolysed plasma stability), blood stability, effect of potentially interfering drugs, dilution integrity, recovery, ion suppression through infusion, ruggedness, robustness and extended batch verification meeting the acceptance criteria as per the USFDA guidelines (FDA, 2001). Selectivity was performed in six lots of Normal, three lots of Lipemic and three lots of Haemolysed Plasma containing K_2EDTA (Potassium salt of Ethylene di-amine Tetra Acetic Acid) as an Anticoagulant. Sensitivity of the method was determined on six LLOQ samples. For matrix effect, 12 blank samples were processed from 6 normal plasma lots (Two aliquots prepared from each plasma lot) and six blank samples were processed from 3 Lipemic plasma lots and six blank samples were processed from 3 Haemolysed plasma lots respectively. After drying these processed blank samples from each plasma lot were reconstituted with aqueous LQC and aqueous HQC dilution respectively. For comparison of matrix effect same prepared aqueous LQC and HQC dilution were used and six replicates were injected from each prepared aqueous LQC and HQC. Matrix effect was calculated as per the following formula:

Matrix factor = (Peak response in the presence of matrix ions)/ (Peak response in the absence of matrix ions)

%Matrix effect = (1-Mean of matrix factor) X 100

The precision of the assay was calculated as percent coefficient of variation over the concentration range of LLOQ QC, LQC, MQC and HQC samples, respectively. The accuracy of the assay was calculated as the ratio of the calculated mean values of the LLOQ QC, LQC, MQC and HQC samples to their respective nominal values. The data of three precision & accuracy batches were subjected for goodness of fit analysis. The back-calculated concentrations of Calibration Curve standards using 1/x and $1/x^2$ weighing were considered for finding the best fit for regression. Linearity was calculated using a regression equation with a weighting factor of $1/x^2$ for drug to IS concentration to produce the best fit for the concentration-detector response relationship for Niacin and Nicotinuric acid. Stock solution and stock dilution stability in refrigerator for Niacin and Nicotinuric acid and Niacin D4 (IS) was carried out for 11 days while Stock solution and stock dilution stability at room temperature was carried out for 72 hours. For all the aqueous related stability studies two aqueous mixtures were prepared, one from the stability standard stock solution and the other from fresh standard stock solution (comparison stock). Six replicates of aqueous mixture from each, stability stock and comparison stock were injected. The response of stability sample was corrected using a correction factor.

Correction factor= (Conc.of fresh standard sol.)/(conc.of stability standard sol.)

Corrected response=Stability Stock Response X Correction Factor

% Change= ((Mean Response of Comparison Samples - Mean Corrected Response of Stability Samples))/(Mean response of comparison samples) X 100

Aqueous recovery comparison samples (LQC, MQC and HQC) were prepared by adding 4 µL each of aqueous dilution of Niacin and Nicotinuric acid from respective quality control samples, 50 µL of internal standard dilution (~500.000 ng/mL) and 146 µL of mobile phase (representing 100 % extraction). The aqueous samples (LQC, MQC and HQC) of Niacin and Nicotinuric acid were compared against 6 sets of processed LQC, MQC and HQC samples. Recovery of internal standard was compared at LQC, MQC and HQC level.

% Recovery = (Mean Peak Area Response of Extracted sample) / (Corrected Mean Peak Area Response of Unextracted sample) X 100

The effect of Potentially interfering drugs (PIDs) i.e. Ibuprofen, Caffeine, Acetaminophen and Acetyl salicylic acid on NIC and NIA analysis was performed by spiking PID's at their approximately C_{max} concentration in the LLOQ sample in triplicate.

Bench top stability was determined for 12 hours using six sets each of LQC and HQC samples while Extended Bench Top Stability was determined in spiked samples to assess the stability of NIC and NIA at each step of extraction. The freeze and thaw stability was determined for five freeze thaw cycles. Six sets of LQC and HOC samples were analyzed after five freeze-thaw cycles. Long Term Stability (at - 65 ºC ± 10ºC and -22 ºC±5ºC) was carried out in plasma for 32 days by using six sets of LQC and HQC. Dry Extract stability was carried out by processing six sets of LQC and HQC, stored at -22 ºC±5ºC without reconstitution while Wet extract stability was carried out by processing the six sets of LQC and

HQC, stored at 2-8 ºC after reconstitution. The samples of wet extract and dry extract stabilities were analyzed after 75 hrs storage. All stability QC's were analyzed against the freshly spiked calibration curve standards and six sets of freshly spiked LQC and HQC (prepared from the fresh stock solution) to calculate the % change between the stability QC's and Comparison QC's.

For Robustness six sets of LQC and HQC were analysed against a calibration curve standards at different chromatographic conditions i.e. Robustness experiment was performed at different column temperatures (38ºC and 42ºC), at different flow rates (0.950 mL/min and 1.050 mL/min) and at different mobile phase compositions Acetonitrile: (5mm ammonium formate buffer : Formic Acid::99.80:00.20 v/v) 48:52 v/v and 52:48 v/v. To evaluate ruggedness, precision and accuracy batch was processed against calibration curve standards and analysed by a different analyst using different column and different sets of solutions.

RESULTS AND DISCUSSION

LC–MS/MS settings: Internal standards Niacin D4 was expected show nearly similar chromatographic behavior as of analyte because they are differing only in terms of possessing different isotopic atoms. Retention time (RT) of Niacin , Nicotinuric acid and Niacin D4 were found to be 1.08 ± 0.3 min ,1.05 ± 0.3 and 1.08 ± 0.3 min respectively. Furthermore, as expected IS produces similar recovery as of analyte in the positive ion mode. Electro Spray Ionization (ESI) provided high ionization efficiencies for both analyte and IS in positive ion mode which resulted in admirable sensitivity of the method.

Sample preparation: Solid Phase extraction method was used for sample preparation because of in liquid liquid extraction significant interferences were observed. Various extraction solvents were tried but for liquid liquid extraction but still the interferences were prudent. Solid phase extraction procedure were found to be most effective for extraction of Niacin, Nicotinuric acid and Niacin D4 with minimal interference.

Method validation: *Selectivity and matrix effect*: Figure 1(A and B) shows typical MRM chromatograms of a blank plasma sample, a plasma sample spiked with NIC and NIA at the LLOQ (10.068 (NIC) 10.157 (NIA)ng/mL) . No significant interference was observed from endogenous substances at the retention times of the analyte and internal standard in normal, haemolysed or lipemic Plasma. The variability of matrix factor (reported as %CV of matrix factor) was 3.54 % (HQC) and 9.16 % (LQC) for NIC, and 1.92 % (LQC) and 3.55 % (HQC) for NIA and 2.81 % (HQC) and 3.85 % (LQC) for NIC D6 and the variability of IS-Normalized Matrix Factor on normal plasma (reported as %CV of matrix factor) was -0.46 % (LQC) and 0.37 % (HQC) for NIC, 1.78 % (HQC) and 4.76 % (LQC) for NIA and -1.21 % (HQC) and 3.69 % (LQC) for NIA D6 . The results were within the acceptance criteria and indicate no ion suppression or enhancement due to the plasma matrix was consistent and would not interfere with the quantitation of analytes.

Linearity and sensitivity: The correlation coefficients (R^2) were greater than 0.99 over the range of 10.068 ng/mL and 5002.086 ng/mL (NIC) and 10.157 ng/mL and 5000.450 ng/mL (NIA) . Typical equations of calibration curves are as follows:

$y = 0.000517 x + (0.00335)$, $R^2=0.9992$ (for NIC)

$y = 0.000374 x + (0.00142)$, R2=0.9978 (for NIA)

Where y represents the analyte\ I.S. peak area ratio and x represents the plasma concentration of the analyte. The precision and accuracy for NIC at LLOQ was 6.84 % and 102.49 % and NIA at LLOQ was 7.83 % and 104.01 % respectively revealing a prodigious sensitivity of the method.

Precision and accuracy: Table 1 and 2 summarizes back calculated concentrations of calibration curve standards for NIC and NIA whereas Table 3 and 4 represents the intraday and inter days precision and accuracy data. The inter-day accuracy was 99.70%-104.55% (NIC) and 98.66% -107.16% (NIA). The inter-day precision was 2.37%-9.76% (NIC) and 4.62% -7.43% (NIA).The results showed that method is fairly precise and accurate within the acceptable limits.

Recovery: The mean % recovery of NIC was 77.771 % with a precision of 7.71 % and NIA was 74.014 % with a precision of 8.32 % while NIC D6 showed mean % recovery of 76.228 % with a precision of 1.13 %. The data show that the SPE procedure efficiently extracts analyte as well as IS from human plasma.

Stability and Other parameters: All the stabilities are been successfully established as per future PK study requirements. The outcomes of other parameters like Ruggedness, Reinjection reproducibility, Effect of Potentially Interfering Drugs (PID), Dilution Integrity, Extended Batch Verification and Robustness were found to be within the acceptance criteria as per USFDA guidelines (Anonymous, 2001).

CONCLUSION

A highly sensitive and selective method for the quantitative determination of NIC and NIA in human plasma was developed using HPLC–MS/MS with turbo-ion spray in positive ion mode. The method was validated as per USFDA guidelines and found to be well suited for the PK study. This method is more sensitive in comparison to all published literature till now This method allows for a much higher sample throughput due to the shorter chromatographic run time (3.0 min) .The method has been successfully used in a PK study of orally administered NIC extended release tablets.

ACKNOWLEDGMENT

We are thankful to Dr. Ritesh Kaur for her valuable suggestions.

REFERENCES

1. Anonymous. Guidance for Industry, Bionanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration Centre for Drug Evaluation and Research (CDER), Centre for Veterinary Medicine (CVM), May 2001.

2. Bruckert E, Labreuche J, Amarenco P (2010). "Meta-analysis of the effect of nicotinic acid alone or in combination on cardiovascular events and atherosclerosis". *Atherosclerosis* 210 (2): 353–61.
3. Iwaki M, E.Murakami and K.Kakehi (2000). *J. Of. Chrom*,747-229.
4. Nimra College of Pharmacy, Ibrahimpatnam, *International Journal of Pharmaceutical Invention* , September 2012, Volume 2(8)
5. Lukasova M, Hanson J, Tunaru S, Offermanns S (2011). "Nicotinic acid (niacin): New lipid-independent mechanisms of action and therapeutic potentials". *Trends in pharmacological sciences.* 32 (12): 700–7.
6. Villines TC, Kim AS, Gore RS, Taylor AJ (2012). "Niacin: The evidence, clinical use, and future directions". *Current atherosclerosis reports.* 14 (1): 49–59.
7. Wan P, Moat S, Anstey A (2011). "Pellagra: A review with emphasis on photosensitivity". *The British journal of dermatology.* 164 (6): 1188–200.