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LC-MS/MS analytical method for the estimation & validation of nutraceutical: Niacin in human plasma

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ABSTACT

Over the last 20 years the number of nutraceuticals available for self medication in pharmacies or and health food shops has grown enormously. Nutraceuticals usually appear to be packed and labeled as if they were medicine. Analytical method development is the process of creating a procedure to enable a new chemical entity of potential therapeutic value to be quantified in a biological matrix. A sensitive, specific, accurate, and reproducible HPLC/MS-method for the quantitative determination of Nutraceutical: Niacin in human plasma using Nevirapine as an internal standard for the hyperlipidimia conditions was developed and validated according to USFDA guidelines. Analyte and the internal standard were separated by Liquid-Liquid extraction. In the present study LC MS/MS (API 3000) Method of extraction procedure is short and simple, it consumes small amount of solvent and biological fluid for extraction and short turnaround time when compared to earliest methods. The chromatographic separation was achieved within 3 min by an isocratic mobile phase containing 0.1% Formic acid in water and Acetonitrile (20:80 v/v), flowing through HYPERSIL BDS,150×4.6mm, 5.0µm analytical column, at a flow rate of 1.0mL mil^{-1} . The calibration curves were linear in the measured range between 100.1 and 20009.7 ng/mL plasma. No indications were found for possible instabilities of niacin in plasma at −20 °C, in the extraction solvent or after repeated thawing/freezing cycles.

Key words: Nutraceuticals, niacin, human plasma, HPLC/MS.

INTRODUCTION

Niacin or nicotinic acid is a water-soluble B-complex vitamin and antihyperlipidemic agent. Niacin is also known as vitamin B_3 that prevents the deficiency disease pellagra. It is an organic

compound with the molecular formula $C_6H_5NO_2$. It is a derivative of pyridine, with a carboxyl group (COOH) at the 3-position. Niacin is essential for the metabolism of carbohydrates, fats, and many other substances in the body. Niacin shows hypolipemic activity at high doses [1, 2]. Niacin belongs to the hydrophilic vitamin B complex and is a component of nicotinamide adenine dinucleotide, which is biosynthesized through nicotinamide [3]. Niacin and nicotinamide are similar in their function as vitamins. The pharmacological effect of niacin differs from that of nicotinamide. Niacin also possesses vasodilating and fabrinolytic properties [4, 5].

Niacin is a well-accepted treatment for high cholesterol. Multiple studies show that niacin has significant benefits on levels of high-density cholesterol with better results than prescription drugs such as "statins" like atorvastatin. Niacin is approved for the treatment of niacin deficiency disease pellagra. Observational epidemiological studies have clearly established that the Low-Density Lipoprotein (LDL) cholesterol and High-Density Lipoprotein (HDL) cholesterol are risk factors for coronary heart disease. The Coronary drug project [6], completed in 1975, was designed to assess the safety and efficacy of nicotinic acid and other lipid-altering drugs in men 30 to 64 years old with a history of Myocardial Infarction (MI). Over an observation period of five years, nicotinic acid showed a statistically significant benefit in decreasing nonfatal, recurrent myocardial infarctions [7]. The Cholesterol-Lowering Atherosclerosis Study (CLAS) was a randomized, placebo-controlled, angiographic trial testing combined colestipol and nicotinic acid therapy in 162 non-smoking males with previous coronary bypass surgery [8]. The primary, per subject cardiac endpoint was global coronary artery change score. After two years, 61% of patients in the placebo cohort showed disease progression by global change score (N=82), compared with only 38.8% of drug-treated subjects (N=80), when both native arteries and grafts were considered. In a follow-up to this trial in a subgroup of 103 patients treated for four years, again, significantly fewer patients in the drug-treated group demonstrated progression than in the placebo cohort [9]. Literature review carried out for niacin, reveals there is no data were found on analytical methods to estimate its bulk form, formulation and in biological matrix[10].

MATERIALS AND METHODS

Niacin (Purity 99.92 %) obtained from Dr.Reddy's Laboratories, India. Nicotinamide (Purity 98.92 %), nicotinuric acid (Purity 98.50 %) and 2-Pyr (Purity 99.20%) were procured from Varda Biotech (P) Ltd., India. The internal standard nevirapine (Purity 99.48%) procured from IFPRESS (Indian Foundation for Pharmaceutical Reference Standard Substances). HPLC grade methanol and acetonitrile manufactured by J.T baker, ethyl acetate HPLC grade, manufactured by Merck Ltd. and formic acid GR grade, manufactured by Merck Ltd., were procured from Jignesh agency (Mumbai, India). High purity water was prepared in-house using a Milli-Q water purification system obtained from Millipore Pvt Limited. (Bangalore, India). Drug free (blank) human plasma was obtained from Cauvery laboratory (Hyderabad, India) and stored at −20 °C prior to use.

The liquid chromatography tandem mass spectrometry (LC-MS/MS) system consisted with a Shimadzu LC-20 AD series HPLC system (Shimadzu Corporation, Kyoto, Japan) coupled to an Applied Biosystems MDS Sciex API 3000 mass spectrometer (Sciex division of MDS health

group, Toronto, Canada) equipped with a TurboIon spray source for ion production. Data acquisition and integration were controlled by Applied Biosystems Analyst^{TM} Software.

Stock solutions preparation

About 10 mg of niacin working standards were transferred to a 10 mL volumetric flask and dissolve in small amount of methanol and made up the volume with methanol to give 1mg/mL stock solutions. The stock solutions were stored in refrigerator at 2-8°C. For the preparation of the IS stock solution, weighed accurately about 10 mg of nevirapine standard and dissolved in 10 mL methanol to give a 1mg/mL stock solution. The stock solution was stored in refrigerator at 2-8°C.

The stock solutions of niacin were diluted to suitable concentrations using methanol: water (60:40%, *v/v*) for spiking in to plasma to obtain Calibration Curve (CC) standards and Quality Control (QC) samples. All other final dilutions (system suitability dilutions, aqueous mixture, recovery etc.) of niacin were prepared in mobile phase. The stock solution of nevirapine was diluted to suitable concentration using methanol: water (60:40%, *v/v*). Other all final nevirapine dilutions (aqueous mixture, recovery etc.) were prepared in mobile phase.

Calibration curve standards and quality control samples

Different working solutions containing niacin was obtained by diluting the stock solutions with suitable concentrations using methanol: water (60:40 %, *v/v*) for spiking in to plasma to obtain calibration curve standards, quality control samples. Calibration curve standard consisting of a set of eight non-zero concentrations ranging from: 100.1 ng/mL to 20009.7 ng/mL for niacin; 10.19 ng/mL to 1602.58 ng/mL for nicotinamide, 10.19 ng/mL to 1602.53 ng/mL for nicotinuric acid and for 2-Pyr from 49.63 ng/mL 4963.37 ng/mL were prepared. Prepared quality control samples consisted of concentrations: for niacin 100.5 ng/mL (LLOQ QC), 300.9 ng/mL (LQC), 9999.3 ng/mL (MQC) and 18000.5 ng/mL (HQC). These samples were stored below -50°C until use. Twelve sets of LQC and HQC were transferred to the -20°C deep freezer to check the stability at -20°C.

Optimization of tandem mass spectrometry conditions

The tuning parameters were optimized by infusion of a standard solution (1000 ng/mL) of the niacin and nevirapine (IS) at a flow rate of 10 µL/min, using a syringe pump into the API 3000 mass spectrometer with ESI (TurboIon Spray) ion source probe. To provide optimum sensitivity and selectivity, electrospray ionization technique operated in the positive ion mode with multiple reaction monitoring analysis. The optimized mass parameters DP (Declustering Potential), CE (Collision Energy) and CXP (Collision cell Exit Potential).

The product ion mass spectrum of positively charged niacin shows the formation of characteristic product ions at m/Z 57.1, 70.9, 78.0, 80.2, 83.2, 96.1, 101.0 and 119.1. The most sensitive mass transition was monitored from m/Z 124.4 to m/Z 80.2.

The product ion mass spectrum of internal standard, Nevirapine shows the formation of characteristic product ions at m/Z 43.3, 57.1, 67.2, 71.5, 81.4, 95.2, 106.9, 125.4, 159.3, 226.3 and 249.5. The most sensitive mass transition was from m/Z 267.0 to 226.3.

Then using HPLC injection optimized the source parameters. The nitrogen curtain gas was optimized to a constant value of 7 psi; nebulizer gas optimized to 9 psi and the source temperature was optimized to 450^0C. The mass spectrometric parameters were optimized to obtain maximum sensitivity at unit resolution. For product ion scan, CAD gas was optimized to 4 psi to get the maximum sensitivity. Ion spray voltage was set at 4500V for positive ionization. The dwell time was fixed at 200 msec.

Procedure for Sample Preparation

Take out the required number of spiked plasma samples (STD Blank, CC, QC samples) from the deep freezer, thaw them at room temperature or in a water bath maintained at room temperature and vortex the tubes to mix. Aliquot 250ml of Plasma into pre-labeled Ependroff vials. Add 50µL of ISTD dilution (100ng/mL) to all the samples except STD Blank and vortex for about 10 seconds. Add 50µL of formic acid and vertex for 30 seconds. Add extraction solvent (Ethyl acetate) 4mL and shake for 20min at 150 rpm. Centrifuge the polypropylene tubes at 4,000 rpm and 10° C for 10 min, transfer approximately 3.0 mL of supernatant to Pre labeled glass tubes and evaporate to dryness. After completion of evaporation, reconstitute by using 1.0mL of Reconstitution solution, vortex for 30 seconds and transfer to pre labeled HPLC vials, then to the auto sampler.

Optimization of chromatographic conditions

Experiment 1: Mobile phase with composition of 0.1% formic acid: methanol (20:80%, *v/v*) and Kromosil C_{18} 250 × 4.6 mm, 5µm particle size column were used as chromatographic conditions to get the desirable peaks. But the higher retention times and low peak responses were observed.

Experiment 2: Mobile phase with composition of 5mM ammonium formate: methanol (25:75%, *v/v*) and Inertsil ODS 3V, 250 × 4.6 mm, 5µm particle size column was used as chromatographic conditions to get the reproducible peaks. But the response was not sufficient.

Experiment 3: Mobile phase with composition of 10mM ammonium acetate: acetonitrile (10:90%, *v/v*) and Zorbax eclipse XDB C_{18} 150 × 4.6 mm, 5µm particle size column was used as chromatographic conditions to get the satisfactory peaks. But the broad peaks with less peak response observed.

Experiment 4: A Thermo Scientific Hypersil BDS C_{18} column with 150 × 4.6 mm, 5 µm particle size, gave good retentions and baseline separation of the four analytes within a 2.0 min run time. The optimum LC-MS/MS mobile phase was determined to achieve good resolution and symmetric peak shapes for the analytes and IS, as well as short run time. It was found that an isocratic mobile phase system consisting of acetonitrile and 0.1% formic acid (80:20%, *v/v*) could achieve this purpose and was finally adopted as mobile phase. The high proportion of organic solvent (80% of acetonitrile) eluted niacin and IS at retention times of about 1.37. A flow rate of 1.0 mL/min (with splitter 60:40) produced good peak shapes and permitted a run time of 2.0 min only. The auto sampler cooler temperature and the injection volumes were optimized as 10°C and 10 µL respectively.

Several compounds were investigated to find a suitable internal standard. Choosing the appropriate internal standard is an important aspect to achieve acceptable method performance, especially with LC-MS/MS, where matrix effects can lead to poor analytical results. The reproducibility along with analytes was observed for nevirapine. Therefore, finally nevirapine was used as IS for the present method. Good chromatograms were obtained and no interferences at the retention times of all the analytes were observed.

RESULTS AND DISCUSSION

Plasma samples Stability at -20°C

Plasma samples stability at –20°C, using six sets each of LQC and HQC, was determined for 2 days. The quality control samples were quantified against the freshly spiked calibration curve standards of concentration range equivalent to that used for calculation of precision and accuracy, refer, Table 13e. The mean concentration of stability QCs were compared against the mean of the of the Ist day QCs when injected for first time after the bulk spiking. Niacin was found to be stable upto 2 days at –20°C as per the acceptance criteria. The percent mean nominal ranged from 88.05% to 97.56% and the precision ranged from 1.10% to 2.48%.

Freeze-thaw Stability

The stability in human plasma was determined during three freeze-thaw cycles. Six replicates of LQC and HQC were analysed after three freeze-thaw cycles. The freeze-thaw quality control samples were quantified against the freshly spiked calibration curve standards of concentration range equivalent to that used for the calculation of precision and accuracy, refer, Table 13a 13d. The percent nominal ranged from 95.70% to 102.16% for three freeze-thaw cycles and the precision ranged from 0.51% to 1.58%. Table 1

Table 1: Freeze Thaw Stability (FT– III Cycle)

FT- QC	Concentration (ng/mL)	
	LQC	**HQC**
	300.9	**18000.5**
97	290.7	18346.4
98	286.5	18372.1
99	292.5	18259.9
100	283.3	18360.0
101	282.3	18514.2
102	292.4	18479.8
Mean	287.95	18388.73
S.D.	4.554	93.303
C.V.(%)	1.58	0.51
% Nominal	95.70	102.16
N	6	6

Table 2: Stability in Plasma at –20°C (2 days)

-20°C QC	Concentration (ng/mL)	
	LQC	**HQC**
	300.9	**18000.5**
274	286.6	18797.7
275	294.0	18430.0
276	300.3	18668.3
277	281.0	19044.7
278	287.3	18665.2
279	283.4	18842.1
Mean	288.77	18741.33
S.D.	7.161	206.602
C.V.(%)	2.48	1.10
% Nominal	95.97	104.12
N	6	6
Mean % Nominal	**88.05**	**97.56**

Mean % Nominal Concentration = $\frac{\text{Mean concentration of stability LQC or HQC samples}}{\text{Mean concentration of LQC or HQC samples of first day of stability testing}} \times 100$

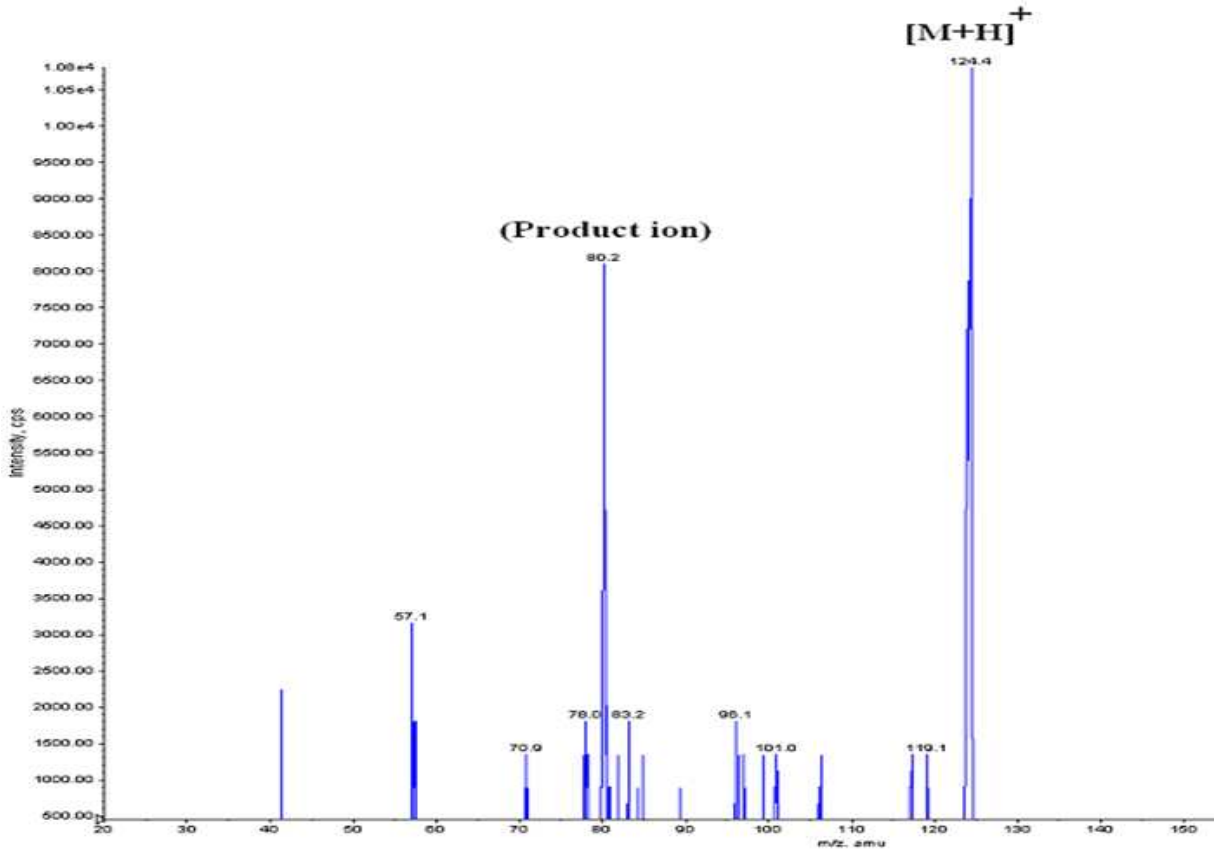
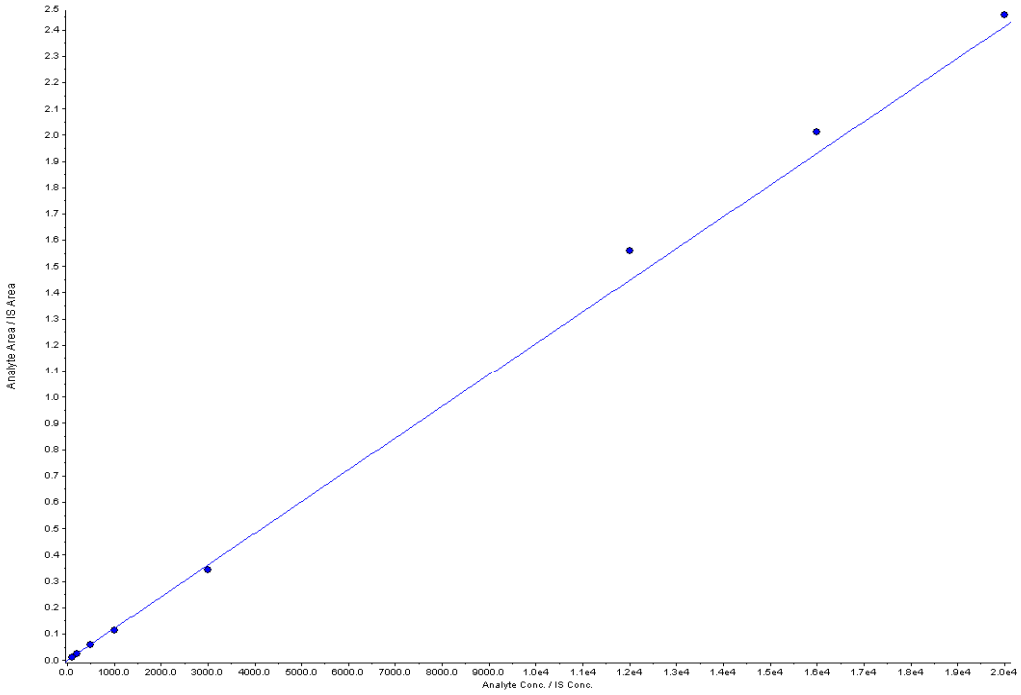


Figure 1: Niacin product ion scan



A representative calibration curve for regression analysis for niacin

CONCLUSION

A simple, sensitive, rugged, high throughput and effective method for determination of niacin in human plasma by LC-MS/MS in positive ion mode using multiple reaction monitoring was developed and fully validated according to International regulatory guidelines. The greater advantages of the current method are, short run time, simple method and very good sample processing technique (liquid-liquid extraction with ethyl acetate). The total chromatographic run time of 2.0 min for each sample made it possible to analyze more than 400 human plasma samples per day. This method was successfully adopted for the analysis of the samples received from a comparative bioavailability study, which was conducted on ten healthy human subjects.

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