METHOD DEVELOPMENT AND VALIDATION OF RP-HPLC FOR THE SIMULTANEOUS ESTIMATION OF NIACIN AND *TRANS*-RESVERATROL IN BULK DRUG AND DOSAGE FORMS BY USING HUMAN PLASMA

A Dissertation submitted to

THE TAMILNADU DR.M.G.R. MEDICAL UNIVERSITY,

CHENNAI -600 032

In partial fulfilment of requirements for the award of the Degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

Submitted by

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CERTIFICATE

This is to certify that the dissertation work entitled "**Method Development and Validation of RP-HPLC for the Simultaneous Estimation of Niacin and** *Trans*-**Resveratrol in Bulk Drug and Dosage Forms by Using Human Plasma"** submitted by **university Reg. No 261730903 is** a bonafide work carried out by the candidate under the guidance of **Dr. S. Ravichandran, M. Pharm. PhD.,** and submitted to The Tamil Nadu Dr. M.G.R Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmaceutical Analysis** at the Department of Pharmaceutical Analysis, PSG College of Pharmacy, Coimbatore, during the academic year 2018 - 2019.

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DECLARATION

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EVALUATION CERTIFICATE

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Date:

Internal examiner **External examiner**

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INTRODUCTION

BIOANALYSIS

Bioanalysis is the method used to determine the concentration of drugs, their metabolites and/or endogenous substances in the biological matrices such as blood plasma, serum, cerebrospinal fluid, urine and saliva. Bioanalytical methods are widely used for quantitative estimation of drugs and their metabolites in the physiological matrices and the methods could be applied for studies in areas of human clinical pharmacology and non-human pharmacology toxicology. Bioanalytical method employed for the quantitative determination of drugs and their metabolites in biological fluids plays a significant role in the evaluation and interpretation of bioequivalence, pharmacokinetics and toxic kinetic studies. In initial stages, these studies are done only to find out over dosage conditions and in toxicological studies. When concentration of drug in biological matrix was known, then pharmacokinetic parameters are calculated from that. Bioanalytical studies are important in drug discovery and development. So, these studies are performed carefully.

Therapeutic efficacy of the particular drug can be known by bioanalysis. In pharma field bioanalysis plays a significant role. Bioanalysis involves the following steps.

- Selection and collection of biological fluid.
- Preparation of sample - Analyte extraction from biological matrix.
- Analyte detection done by various methods.

NEED FOR BIOANALYTICAL METHOD:

The reason for validating a bioanalytical procedure is to demonstrate the performance and reliability of a method and hence the confidence that can be placed on the results. It is mandatory that all bioanalytical methods must be validated if the results are used to support registration of a new drug or the reformulation of an existing one. Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of the method are suitable and reliable for the intended analytical applications.

1. It is essential to used well-characterized and fully validated bioanalytical methods to yield reliable results that can be satisfactorily interpreted.
2. It is recognized that bioanalytical methods and techniques are constantly undergoing changes and improvements; they are at the cutting edge of the technology.
3. It is also important to emphasize that each bioanalytical technique has its own characteristics, which will vary from analyte to analyte, specific validation criteria may

need to be developed for each analyte.

4. Moreover, the appropriateness of the technique may also be influenced by the ultimate objective of the study. When sample analysis for a given study is conducted at more than one site, it is necessary to validate the bioanalytical method(s) at each site and provide appropriate validation information for different sites to establish inter-laboratory reliability.

PREPARATION AND PRESERVATION OF BIOLOGICAL SAMPLES

Sample preparation was important step for analysis of drugs and metabolites in bioanalytical study. Biological samples contain proteins, various endogenous and exogenous substances that may interfere with analyte. The objective of sample preparation is to free analyte of interest from all possible unwanted substances without significant loss of analyte. When drugs are susceptible to plasma esterase, the addition of esterase inhibitors, such as sodium fluoride was immediately added after collection helps to prevent decomposition.

When collecting and storing biological samples, there are chances for the analyte to get contaminated with storage vessels. For example, plastic containers contains high boiling liquid bis (2-ethylhexyl) phthalate. similarly, the plunger-plugs may contain tri-butoxyethyl phosphate which can be interfere with certain drug analysis. Hence, care should be taken in selecting the material of containers for sample storage and preservation.

ANALYSIS OF DRUG IN VARIOUS BIOLOGICAL MEDIA

The most common samples obtained for biopharmaceutical analysis are blood, urine, and faeces, especially if the drug or metabolite is poorly absorbed or extensively excreted in the bile. Other media that can be utilized includes saliva, breath and tissue.

The choice of sampling media was determined largely by the nature of the study. For example, drug levels in a clinical pharmacokinetic study demand the use of blood, urine, and saliva. A bioavailability study may require drug level data in blood and / or urine where as a drug identification or drug abuse problem may be solved with only one type of biological sample.

Detection of drug or its metabolite in biological media is usually complicated by the matrix. Because of this, various types of cleanup procedures involved i.e. solvent extraction and chromatography are employed to effectively separate drug components from endogenous biological materials. The sensitivity and selectivity of the assay method was limited by the efficiency of the clean up methodology.

If the blood was allowed to clot and then centrifuged, about 30 to 50% of the original volume was collected as serum (upper level). Plasma generally was preferred because of its greater yield from blood. Blood, serum or plasma samples can be utilized for bioanalytical studies and may require protein denaturation steps before further processes.

If plasma or serum was used for the analytical procedure, the fresh whole blood should be centrifuged immediately at 5000 rpm for approximately 5 to 10 min, and the supernatant should be transferred by means of a suitable device, such as pasture pipette, to a clean container of appropriate size of storage.

Urine was the easiest one to obtain from the patient and also permits collection of large and frequently more concentrated samples. The lack of protein in a healthy individual's urine obviates the need for denaturation steps, because urine samples are readily obtained and often provide the greatest source of metabolites, they are frequently analszed in drug metabolism studies.

EXTRACTION PROCEDURE FOR BIOLOGICAL SAMPLE

Sample preparation was a technique used to clean up a sample before analysis and /or to concentrate a sample to improve its detection. When samples are biological fluids such as plasma, serum or urine, this technique is described as bioanalytical sample preparation.

Objectives of Bio-analytical sample preparation:-

1. Removal of unwanted matrix components (primarily protein) that would interfere with analyte determination.
2. Concentration of analyte to meet the detection limits of the analytical instrument.
3. Exchange of the solvent or solution in which the analyte resides so that it was compatible with mobile phase for injection into a chromatographic system.
4. Dilution to reduce solvent strength or avoid solvent incompatibility.
5. Stabilization of analyte to avoid hydrolytic or enzymatic degradation.

After selection of biological fluid the required analyte should be extracted from it. This step in bioanalytical method was more important because sample preparation can be done by different methods of extraction. The sample preparation is a time taking process and it should be done carefully because of its importance. If biological matrix was in liquid state like blood, plasma and urine then liquid-liquid extraction is used or it is solid then liquid-solid extraction can be done.

Different types of extracting methods are

- Dilution followed by injection
- Solid Phase extraction (off line/online)
- Protein precipitation
- Filtration
- Liquid-liquid extraction
- Protein removal by equilibrium dialysis or ultrafiltration
- Restricted access media
- Solid-supported liquid-liquid extraction
- Monolithic columns
- Immunoaffinity extraction

Out of all these methods the most prominent and widely used techniques for extraction are:

- Protein precipitation method.
- Liquid-liquid extraction method.(LLE)
- Solid-phase extraction method.(SPE)

Protein precipitation

Protein precipitation was based on the interaction between the precipitation reagent and protein groups. Soluble proteins generally have a hydrophobic core surrounded by a hydrophilic surface including ionic groups that are not involved in intra-molecular binding. Organic solvents interfere with the intra-molecular hydrophobic interactions of proteins.

The addition of a volume of solvent (frequently acetonitrile) to the serum causes the proteins of the serum to precipitate and leaves the analyte of interest in the solvent, which can either be injected directly or dried down and reconstituted in a smaller volume to concentration before injection. While this is the fastest and simplest method for sample preparation, it is the most likely to cause ion suppression issues, especially in ESI, where the coelution of endogenous compounds such as lipids, phospholipids and fatty acids affect the ESI droplet de-solvation process.

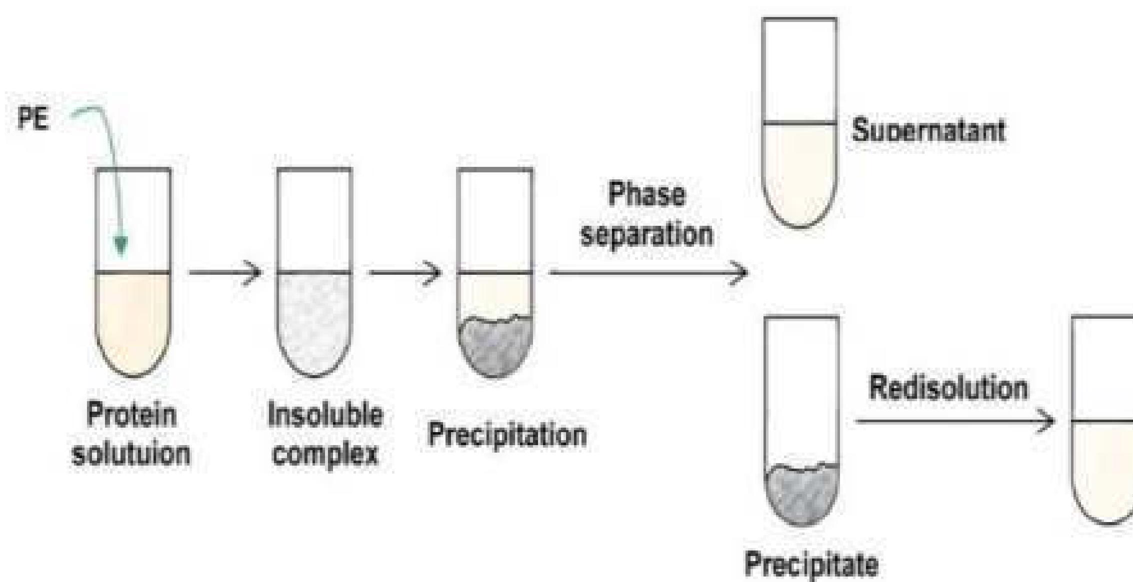


Fig 1: Schematic diagram of protein precipitation technique.

Procedure

a) **In case of acids:** Take 0.5 to 1.0 ml of plasma/serum/urine and add 100 to 200µl of 10 to 20 % perchloric acid or trichloroacetic acid or sometimes decreasing the volume and increasing the percentage of acid are also recommended.

b) **In case of organic solvents:** Take 0.5 to 1.0 ml of plasma and add 0.5 to 2.0ml of solvent methanol or acetonitrile.

After adding the acid or solvent vortex the vial for complete precipitation of protein then centrifuge and inject the supernatant. It is recommended to filter the sample whenever the technique is used to avoid clogging of the column.

ESTIMATION OF DRUGS IN BIOLOGICAL SAMPLES BY HPLC:

High performance liquid chromatography is one of important techniques in chromatography. HPLC is an advanced form of liquid chromatography used in separating the complex mixture of molecules encountered in chemical and biological systems. HPLC is really the automation of traditional liquid chromatography under conditions which provides enhanced separations during shorter periods of time. It is ideally suited for separation and identification of amino acids, proteins, nucleic acids, hydrocarbons, carbohydrates, pharmaceuticals, pesticides, antibiotics, steroids etc. Its simplicity high specificity and wide range of sensitivity make it ideal for the analysis of many drugs in both dosage forms and biological fluids. HPLC is a versatile analytical tool useful in identification and quantitative

estimation of low concentration of drugs and metabolites in biological matrices. HPLC is having many advantages most of drugs in biological matrix can be estimated by this. Some of its advantages than other methods are:

- Rapid speed.
- Improved resolution (wide variety of stationary phase).
- Precise and reproducible.
- Easy recovery of sample, handling and maintenance.
- Greater sensitivity.
- Calculations are done by integrator itself.
- Ideal for substances of low volatility.
- Reusable column

Hence HPLC can be applied to any sample, such as pharmaceuticals, nutraceuticals, cosmetics, environmental matrices, forensic samples, industrial chemicals.

PRINCIPLE:

The principle of separation is based on the typical modes of separation of that of a classical chromatography i.e., Adsorption, Partition, Ion exchange and Gel permeation.
The HPLC principle is based upon the Adsorption techniques. HPLC is historically divided into two different sub classes based on the polarity of the mobile and stationary phases.

- Normal phase high performance liquid chromatography
- Reverse phase high performance liquid chromatography

PHASES OF CHROMATOGRAPHY:

Normal phase mode

The stationary phase is polar and the mobile phase is nonpolar in nature. In this technique, nonpolar compounds travel faster and are eluted first. This is because of the lower affinity between the nonpolar compounds and the stationary phase. Polar compounds are retained for longer times because of their higher affinity with the stationary phase. These compounds, therefore take more times to elute. Normal phase mode of separation is therefore, not generally used for pharmaceutical applications because most of the drug molecules are polar in nature and hence take longer time to elute.

Reverse phase mode

It was the most popular mode for analytical and preparative separations of compound of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this mode, the stationary phase is nonpolar hydrophobic packing with octyl or octa decyl functional group bonded to silica gel and the mobile phase is polar solvent. An aqueous mobile phase allows the use of secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pairing and complexation) to control retention and selectivity. The polar compounds gets eluted first in this mode and nonpolar compounds are retained for longer time. The most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer times and hence elute faster. The different columns used are octa decyl silane (ODS) or C18, C8, C4, etc., (in the order of increasing polarity of the stationary phase).

QUANTITATIVE ANALYSIS BY HPLC:

For quantitative of analysis in HPLC generally three methods are used. They are

1) External standard method.

2) Internal standard method.

3) Standard addition method.

External standard method*:*

In this method standard and sample peak area or height are directly compared. In external standard method can be done by using single standard or up to three different standard solutions.

In this method standard and unknown sample are injected and then the concentration of unknown sample can be determined by plotting calibration curve graphically or by numerically using response factors. For good quantitation in this method constant chromatographic conditions should be maintained for both standard and sample separation.

Internal standard method:

In this method a known quantity of a compound is added to known amount of sample to give separate peaks, which compensates the loss of compound of interest during sample pre-treatment. In order to overcome various analytical errors addition of internal standard is commonly is used quantitation method. The compound selected for internal standard should be completely separate from the sample and should not interfere it. In chromatographic

analysis internal standard is added to the compound to be analyzed are desirable, if any loss of compound may occur during handling.

In this way both internal standard and concentration of sample ratio remains constant regardless of amount of solution lost. Any loss of compound of interest will be accompanied by the loss of an equivalent fraction of internal standard. The internal standard selected should have similar properties of that of the interested compound or any other compound with other properties also can be taken. In chromatographic analysis internal standards are frequently used. The internal standard used should be added to sample before sample preparation and mixed properly. By using response factor (Rf) concentration of sample can be known.

Necessary of internal standard

- It should elute closely to the interested compound.
- No interferences should present in a completely resolved peak.
- Stable one, unreactive with compound of interest, mobile phase and column packing.
- Behaved equally to the compound of interest for analysis like pre-treatment, derivative formation, etc.
- Not be present in the original ample.
- Available in high purity.
- It should be added at a concentration, which gives peak area or peak height equal or unity with the compound of interest.

BIOANALYTICAL METHOD VALIDATION (BMV):

A bioanalytical method is a set of procedures involved in the collection, processing, storage, and analysis of a biological matrix for a chemical compound. Bioanalytical method validation (BMV) is the process used to establish that a quantitative analytical method is suitable for biomedical applications. Method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum, or urine, is reliable and reproducible for the intended use.

Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of the method are suitable and reliable for the intended analytical applications.

Bioanalytical method validation is vital not only in terms of regulatory submission but also for ensuring generation of high-quality data during drug discovery and development. BMV assures that the quantification of analyte in biological fluids was reproducible, reliable and suitable for the application. Method validation is a process that demonstrates that the method will successfully meet or exceed the minimum standards recommended in the Food and Drug Administration (FDA) Guidance for accuracy, precision, selectivity, sensitivity, reproducibility, and stability of the developed method.

Types of Bioanalytical Method Validation:

- Full validation
- Partial validation
- Cross validation

Validation parameters:

The common parameters used in the bioanalytical validation is given as follows,

1. Accuracy
2. Precision
3. Linearity / Range
4. Specificity / Selectivity
5. Limit of Detection (LOD)
6. Limit of Quantification (LOQ)
7. Recovery
8. Robustness
9. Ruggedness.
10. System suitability

Accuracy:

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value (concentration)of the analyte. The two most commonly used ways to determine the accuracy bias of an analytical method, are

(i) Analyzing control samples spiked with analyte and

(ii) By comparison of the analytical method with a reference method.

Precision:

The precision of an analytical method is the degree of agreement between a serious of measurement obtained from multiple sampling a homogeneous sample of biological matrix. The Precision of an analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements.

The precision of an analytical procedure is usually expressed as the variance standard deviation or coefficient of variation of a series of measurements

- **Repeatability** – Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-day-assay precision
- **Intermediate precision** - Intermediate precision expresses within laboratory variation, as on different days, or with different analysts or equipment within the same laboratory.
- **Reproducibility** - Reproducibility refers to the use of the analytical procedure in different laboratories, as in a collaborative study.

Specificity:

Specificity is the ability to assess unequally the analyte in the presence of components which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure.

Linearity & Range:

The linearity of an analytical method is its ability to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample within a given range. The linearity of a method is measure of how well a calibration plot of response Vs concentration approximates a straight line. The linearity can be assessed by using a Linearity least – square regression.

The range of an analytical method is the interval between the upper and lower levels of analyte (including these levels) that has been demonstrated with a suitable level of precision, accuracy and linearity using the method as written. For studying linearity studies calibration curve was plotted by using concentration of drug solution on x-axis and peak area on y-axis. The resulting plot slop, intercept and cross correlation coefficient provides the

desired information on linearity. The range is normally expressed in the same unit as test results (e.g., percent, parts per million) obtained by the analytical method.

Limit of detection (LOD):

The detection limit is a characteristic of limit test. It is the lowest amount of analyte in a sample that can be detected, but not necessarily quantified as an exact value. The detection limit is usually expressed as the concentration of analyte (e.g., percent, parts per million) in the sample. The limit test merely substantiate that the amount of analyte is above or below a certain level. Detection limit corresponds to the concentration of related substance in the sample that will give a signal-to-noise ratio of 3:1.

Limit of quantification (LOQ):

The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be determined quantitatively with suitable precision and accuracy. Quantification limit is the concentration of related substance in the sample that will give a signal-to-noise ratio 10:1.

LOD and LOQ can determine by

- Based on visual evaluation
- Based on signal-to-noise
- Based on standard deviation of response and slope

It can be calculated as:

$$LOD = 3.3 \times SD/S$$

$$LOQ = 10 \times SD/S$$

Where, S.D = Standard deviation of calibration curves

S = Slope of intercepts of calibration curves

Robustness:

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. This is to verify that the method performance is affected by typical changes in normal experiments.

Ruggedness:

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the same samples under a variety of conditions, such as different laboratories, analysts, instruments, lots of reagents, elapsed assay times, assay temperature, or days.

Ruggedness is normally expressed as the lack of influence on test result of reproducibility of test results under the variation in condition normally expected from laboratory to laboratory and from analyst to analyst.

System suitability requirements

System suitability is a part of method validation, measure of the performance of a given day with in a particular sample analysis set. Before injecting a standard solution in creating standard plot, it is necessary to ensure the system is performing adequately. System suitability, an integral part of analytical procedures, based on concept that equipment, samples, electronics an integral system that can be evaluated.

The main objective is to recognize where or not system operation is adequate on a given day with a particular sample analysis set and checking variability. System suitability should be based on criteria and parameters collected as a group.

Some of common parameters include precision of repetitive injections (n=5 or 6), resolution(R), tailing factor (T), number of theoretical plates (N), HETP and capacity factor. (Satinder Ahuja)

Limits for system suitability

SST	**Limits**
Resolution (Rs)	>2.0
Injection repeatability (RSD)	<1.0% for five replicates
Plate count (N)	>2000
Tailing factor (T_f)	≤2.0
Separation factor	>1.0
Theoretical plates	Should not fall below 2000

Recovery:

It can be calculated by comparison of the analyte response after sample workup with the response of a solution containing the analyte at the theoretical maximum concentration. Therefore, absolute recoveries can usually not be determined if the sample workup includes a derivation step, as the derivates are usually not available as reference substances. However, during method development one should of course try to optimize recovery.

Matrix effect:

Matrix effect is the effect on bio analytical method caused by all other components of the sample except the specific compound to be quantified. It happens due to ion suppression/enhancement by the others ions present in the biological matrix which might get ionized during detection and will give false results.

Dilution integrity:

Dilution integrity is performed in order to check the validity of method incase the sample needs to be diluted during analysis. It is done by spiking analyte working standard in drug free and interference free plasma to get concentration of 2xULOQ. Two and four fold dilution made of the original concentration using screened and pooled plasma and analysed against a fresh calibration curve. The concentration will be calculated using the dilution factor.

Stability:

The chemical stability of an analyte in a given matrix specific conditions for given time intervals is assessed in several ways. Pre-study evaluations should cover the expected sample handling and storage condition during the conduct of the study, including conditions at the clinical site, during shipment and at all other secondary sites. Stability samples should be compared to freshly made calibrations or freshly made Quality control samples. At least three replicates at each of the low and high concentrations should be assessed. Assessments of analyte stability should be conducted in the same matrix as that of the study samples. All stability determinations should use samples prepared from a freshly made stock solution.

Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analyte (e.g., long-term, bench top and room temperature storage; and freeze-thaw cycle). If during sample analysis, storage conditions changed and/or exceed the sample storage conditions evaluated during method validation,

stability should be established under the new conditions. Stock solution stability also be assessed. Stability sample results should be within 15% of nominal concentration.

- **Stock solution stability:**

  The stability of the stock solution should be evaluated at room temperature for at least for 6 hours. When the stock solution exists in a different state (solutions vs solid) or in a different buffer composition (generally the case for macromolecules) from the certified reference standard, the stability data on this stock solution should be generated to justify the duration of stock solution storage stability.
- **Short-term temperature stability:**

  The stability of the analyte in biological matrix at ambient temperature should be evaluated. Three aliquots of low and high concentration should be kept for at least 24 hours and then analysed.
- **Long-term temperature stability:**

  The stability of the analyte in the matrix should exceed the time period from sample collection until the last day of analysis.
- **Freeze and thaw stability:**

  The stability of the analyst should be determined, after three freeze and thaw cycles. Three aliquots of low and high concentration should be frozen for 24 hours and then thawed at ambient temperature.
- **Post-preparative stability:**

  The stability of the analyte during stages of the analysis process should be evaluated.
- **Bench-top stability:**

  Bench top stability experiments should be designed and conducted to cover the laboratory handling conditions that are expected for study samples.
- **Processed sample stability:**

  The stability of processed samples, including the time until completion of analysis, should be determined.

FDA GUIDELINES ON BIOANALYTICAL METHOD VALIDATION:

For bioanalytical method validation FDA had given some guidelines. These guidelines are given the validation performed was to be accurate. They are:

- Analyte stability in biological matrix at intended storage and operating conditions should be kept.
- Standard curve for matrix-based one should contain minimum 5 standards without including blank and it should cover the entire range of concentrations expected.
- Essential parameters that are performed for acceptability of bioanalytical method are precision, accuracy, selectivity, sensitivity and reproducibility.
- Then the lower limit of quantitation should serve as lowest concentration on the standard curve and that is not confused with limit of detection.
- All these parameters are to be defined during the Full validation of a bioanalytical method.

LITERATURE REVIEW

Gurinder Singh and Roopa S. Pai *et al.,* [2014] "A Rapid Reversed-Phase HPLC Method for Analysis of *Trans*-Resveratrol in PLGA Nanoparticulate Formulation". The desired chromatographic separation was achieved on a Phenomenex C_{18} column under isocratic conditions using UV detection at 306 nm. The optimized mobile phase consisted of a mixture of methanol: 10mMpotassium dihydrogen phosphate buffer (pH 6.8): acetonitrile (63 : 30 : 7, v/v/v) at a flowrate of 1 mL/min. The linear regression analysis for the calibration curves showed a good linear correlation over the concentration range of 0.025–2.0 μg/ml, with determination coefficients, R2, exceeding 0.9997. The accuracy with bias not exceeding 15% and percentage recovery was found to be in the range between 94.5 and 101.2. The limits of detection and quantification were 0.002 and 0.007 μg/ml, respectively.

Robert Ohmacht *et al.,* [2005] "A Validated HPLC Method for the Quantitative Analysis of *Trans*-Resveratrol and *Trans*-Piceid in Hungarian Wines". The samples are injected without pretreatment and UV–vis and mass spectrometric (MS) detection has been applied. The detection limit for *trans*-resveratrol and for *trans*-piceid is found to be 0.9 and 0.6 p/mol for the UV–vis detection method and 0.3 and 0.2 p/mol for the MS detection method. *Trans*-resveratrol and *trans*-piceid are found in red wines from 0.1 to 14.3 mg/L and from 3.8 to 16.4 mg/L concentrations, respectively.

Zika S. Cvetkovic *et al.,* [2015] "Development and validation of an RP-HPLC method for quantification of *trans*-resveratrol in the plant extracts". Methanol was used as a mobile phase with a flow rate of 1.0 cm^3/min, while the quantification was effected at 306 nm. The separation was performed at 35 °C using a C_{18} column. The results showed that the peak area response was linear in the concentration range of 1–40 µg cm^{-3}. The values of LOD and LOQ were found to be 0.125 and 0.413 µg cm^{-3}, respectively. The antioxidant activity of the extracts was determined using DPPH assay. The ability of DPPH radicals inhibition decreases in the following order: the extract of grape exocarp > *trans*-resveratrol standard > the extract of grape seeds.

Manish Patidar *et al.,* [2013] "Development and Validation of RP-HPLC Method for Simultaneous Determination of Resveratrol and Curcumin in Pure Form". The retention time of Resveratrol and Curcumin were found to be 2.90 and 4.11 minutes. Detection was carried out at 424 and 306nm. The regression coefficient value of Resveratrol and Curcumin is 0.9904 and 0.9937 which was found to be linear in the detection range. Limit of detection and limit of quantification of Resveratrol was found to be 0.08µg/ml and 0.32 µg/ml and Curcumin is 0.05 µg/ml and 0.17 µg/ml. Analysis was performed using a C18 column (250 X 4.6 mm) at room temperature in isocratic mode. The mobile phase used was Citric acid (pH adjusted to 3.5): Acetonitrile (40:60) at flow rate of 1.0 ml/min.

Michael A. Koupparis *et al.,* [2007] "HPLC Method with UV Detection for the Determination of *trans*‐Resveratrol in Plasma". The proposed method for the determination of *trans*‐resveratrol in blood plasma is based on reversed phase HPLC utilizing UV detection (310 nm), under isocratic conditions (1.0 mL/min), with mobile phase consisting of acetonitrile‐phosphate buffer pH 4.8 (30 mM) 25∶75 v/v, a C_{18} Novapack 150×4.0 mm column (4 µm particle size), and carbamazepine as internal standard (5 µg/mL). Plasma samples (1 mL) are buffered with phosphate buffer (0.4 mL) pH 6.0 and extracted with 3×3 mL of ethyl acetate. *trans*‐resveratrol is eluted at around 4.6 min, whereas carbamazepine at approximately 9.6 min, yielding a resolution of 4.4. The method appears to be linear within a range of 0.15–4.0 µg/mL (r=0.9998), with good repeatability (%RSD=0.86) and reproducibility (%RSD=2.2). Detection and quantification limits were found equal to 0.10 and 0.33 µg/mL, respectively. Accuracy, expressed as recovery determined at two concentrations of 0.3 and 3.0 µg/mL (6‐replicates) were found to be 88.3±7.5% and 100.7±0.7%, respectively.

Gurinder Singh *et al.,* [2016] "Development and validation of a HPLC method for the determination of trans-resveratrol in spiked human plasma". Caffeine was employed as an internal standard (IS). The chromatographic separation was achieved on a Phenomenex C18 column (250 mm × 4.6 mm, 5 µm) at room temperature in isocratic mode, and the column effluent was monitored by UV detector at 306 nm. The mobile phase used was methanol: phosphate buffer (pH 6.8 adjusted with 0.5% (v/v) orthophosphoric acid solution in Milli-Q water) (63:37%, v/v) at a flow rate of 1.0 ml/min. Nominal retention times of trans-resveratrol and IS were 3.94 and 7.86 minutes, respectively. Limits of detection and Limits of quantification

of trans-resveratrol were 0.006 µg/ml and 0.008 µg/ml, respectively. This method was linear over the range of 0.010 to 6.4 µg/ml with regression coefficient greater than 0.9998. The inter- and intra-day precisions in the samples, 0.010, 3.2 and 6.4 µg/ml of trans-resveratrol was in the range 0.63 to 2.12% relative standard deviation (RSD) and 0.46 to 1.02% RSD, respectively. Resveratrol was found to be stable for a period of 15 days on storage at -20°C.

G.S. Devika *et al.,* [2012] "Development and Validation of RP- HPLC Method for Simultaneous Determination of Niacin (Extended Release) and Lovastatin in Oral Solid Dosage Form". Chromatographic separation of the two drugs was performed on a Purospher BDS C_8 column (150mmx4.6mmid, 5pm particle size). The mobile phase used was a mixture of 0.1% v/v triethylamine (pH 5.0), containing 20 mM of ammonium acetate buffer: Methanol (30:70% v/v).Detection was performed at 237nm and sharp peaks were obtained for niacin and Lovastatin at retention times of 3.2±0.01 min and.6.4±0.01 min respectively. The calibration curve was linear in the concentration range 100-700µg/ml for niacin 3-18µg/ml for Lovastatin; the correlation coefficients were 0.9991 and 0.9992, respectively.

Indrajeet Gonjari *et al.,*[2016] "Development and validation of RP-HPLC method for determination of Atorvastatin calcium and Nicotinic acid in combined tablet dosage form". The analysis has been performed by using Agilent ZORBAX SB-C18 (150 × 4.6 mm, 3.5 u) and mobile phase containing acetonitrile: distilled water (85:15) at pH 4.5 (adjusted with phosphoric acid). The detection was carried out at 261 nm with a flow rate of 1.0 ml/min. The retention times of Atorvastatin calcium and Nicotinic acid were 6.092 and 3.125 min, respectively. The linearity for Atorvastatin calcium and Nicotinic acid were in the range of 2–12 and 10–80 µg/ml respectively. The recoveries of Atorvastatin calcium and Nicotinic acid were found to be in the range of 99.031% and 99.744% respectively.

Akula Ganesh *et al.,* [2016] "Validated RP-HPLC Method for the Simultaneous Estimation of Simvastatin and Niacin". Inertsil ODS, RP-18 Column (250 x 4.6 mm ID, 5µ) was used with a mobile phase containing a mixture of Phosphate buffer pH 2.5, Methanol and Acetonitrile in the ratio of 45: 20: 35. The procedure was carried out at pH-3.5. The compounds were eluted at a flow rate of 1.0 ml/min. results were determined at 220 nm with fixed wavelength PDA detector. The linearity for Niacin was found between 75–175 µg/ml and between 3–7 µg/ml for

Simvastatin. The retention times were found as 4.747 min and 2.970min for Niacin and Simvastatin respectively.

N.Nagi Reddy *et al.,* [2011] “new validated RP-HPLC method for the simultaneous estimation of niacin and its metabolite 1-methylnicotinamide in rat plasma”. Separation was done by using 25×4.6 cm i.d, 5um particle, Hichrome KR 100 column with 70:30 (Acetonitrile & 10%KH2PO4) as mobile phase, at flow rate of 1ml/min and UV-detection at 275nm. Total run time was 20 mins: N & 1-MN were eluted with retention times of 13.9 min & 8.4mins respectively.

G.Tuljarani *et al.,*[2014] “simple and rapid isocratic RP-HPLC method to determine lovastatin and niacin in bulk and pharmaceutical formulation”. The determination was carried out by using Symmetry C8 (4.6×250 mm, 5μm) column with the mobile phase containing acetonitrile: phosphate buffer (pH4.0±0.5) in the ratio of 65:35 v/v. The optimized flow rate was 0.7 ml/min and the UV detection was carried out at 240 nm. The retention time of lovastatin and niacin were found to be 3.093 min and 6.196 min respectively. The method was found to be linear in the concentration range 2.0 -10 μg/ml and 25-125 μg/ml for lovastatin (LS) and niacin (NC) respectively.

Ripunjoy Bordoloi *et al.*, [2017] “Development and Validation of UV-Spectroscopic Method for Estimation of Niacin in Bulk and Pharmaceutical Dosage Form”. The λmax of niacin was found to be 262 nm. Linearity in the concentration range of 01-19μg/ml was found to be exhibiting good correlation coefficient (R2=0.9991). The validation parameters were selected as per the ICH [Q2 (R1)] guideline.

Ganesh Akula *et al.,* [2016] “Validated RP-HPLC Method for the Simultaneous Estimation of Simvastatin and Niacin”. Inertsil ODS, RP-18 Column (250 x 4.6 mm ID, 5μ) was used with a mobile phase containing a mixture of Phosphate buffer pH 2.5, Methanol and Acetonitrile in the ratio of 45:20:35. The procedure was carried out at pH-3.5. The compounds were eluted at a flow rate of 1.0 ml/min. results were determined at 220 nm with fixed wavelength PDA detector. The linearity for Niacin was found between 75-175 μg/ml and between 3-7 μg/ml for Simvastatin.

The retention times were found as 4.747 min and 2.970min for Niacin and Simvastatin respectively.

RS Mehta *et al.,* [2007]"RP-HPLC method for the determination of atorvastatin calcium and nicotinic acid in combined tablet dosage form" A phenomenex Luna C-18, 5 mm column having 250 × 4.6 mm i.d. in isocratic mode, with mobile phase containing 0.02 M potassium dihydrogen phosphate: methanol: acetonitrile (20:40:40, pH 4) was used. The flow rate was 1.0 ml/ min and effluents were monitored at 240 nm. The retention times of atorvastatin calcium and nicotinic acid were 3.6 min and 2.4 min, respectively. The linearity for atorvastatin calcium and nicotinic acid were in the range of 2-24 μg/ ml and 60-250 μg/ ml, respectively. The recoveries of atorvastatin calcium and nicotinic acid were found to be in the range of 97.93-101.16% and 98.82-101.30%, respectively.

Bratati Roy *et al.,*[2014] "Bioanalytical Method Development and Validation of Niacin and Nicotinuric Acid in Human Plasma by LC–MS/MS". 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 LCMS/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.

PingpingZhang *et al.,*[2015] "Quantification of Niacin and Its Metabolite Nicotinuric Acid in Human Plasma by LC-MS/MS: Application to a Clinical Trial of a Fixed Dose Combination Tablet of Niacin Extended-Release/Simvastatin (500mg/10mg) in Healthy Chinese Volunteers". Following protein precipitation with acetonitrile, theNA, NUA, and internal standard (5fluorouracil) were separated on a Zorbax300SB-C8 column (250mm × 4.6mm,5μm) with a mobilephase consisting of methanol 2mM ammoniumacetate (3:97,v/v) at a flowrate of 1mL/min(split1:1). The linear concentration ranges of the calibration curves were 5–800ng/mL

for NA and NUA. The intra-assay RSD for quality control (QC) samples were from 5.0% to 8.7% for NA, and 5.5% to 7.6% for NUA. The interassay RSD for QC samples were from 2.8% to 9.4% for NA, and 3.7% to 5.8% for NUA. The relative errors for QC samples were from −2.2% to 2.3% for NA, and −0.6% to 3.2% for NUA.

Indranil Chanda *et al.,* [2017] "Development and Validation of UV-Spectroscopic Method for Estimation of Niacin in Bulk and Pharmaceutical Dosage Form". The λmax of niacin was found to be 262 nm. Linearity in the concentration range of 01-19µg/ml was found to be exhibiting good correlation coefficient (R2=0.9991).

Rudra Pangeni *et al.,* [2017] "Design expert-supported development and validation of stability indicating high-performance liquid chromatography (HPLC) method for determination of resveratrol in bulk drug and pharmaceutical formulation". Optimum chromatographic separation was achieved by mobile phase consisting of methanol, water and acetic acid in 69:30:1 ratio respectively. The flow rate of 1 ml/ml with standard RT of 2.8 min was optimized in the present study. The method was linear in the concentration range of 7.5-60 µg/ml with a regression coefficient (R^2) of 0.999. The LOD and LOQ was found to be 1.463 and 4.737 µg/ml respectively.

LaurianVlase *et al.,* [2009] "A Rapid Method for Determination of Resveratrol in Wines by HPLC-MS". The optimum conditions were as follows: a mobile phase consisting of a mixture of 1 mM ammonium acetate:acetonitrile (73:27, v/v) with a flow rate of 1 mL/min, MS/MS detection with an APCI interface, operated in negative ionization mode. The monitored ion transition was m/z 227→184.7. Calibration curves were generated for both isomers of resveratrol in the range of 10.47–837.86 ng/mL (*trans* isomer) and 9.12 − 730.14 ng/mL (*cis* isomer), respectively.

AIM AND OBJECTIVE

According to the survey of the literature, there was no method found out so far for the simultaneous estimation of Niacin and Resveratrol. So, it has been planned to develop an accurate, precise, economical Bio-analytical method by RP-HPLC methods.

The main objective of the present work was to develop and validate the Bio-analytical method with the help of RP-HPLC which can separate and simultaneously quantify the drug components from the bulk and dosage forms.

This research proposal was based on feasibility and applicability for the method development of Nutraceuticals. The literature survey revealed that various analytical method has been reported for the estimation of Niacin and Resveratrol individually.

For our Research purposes, the quantitative estimation of Niacin and Resveratrol in its pharmaceutical dosage form was paramount. For our knowledge, we don't come across any Analytical (or) Bioanalytical method for simultaneous determination of Niacin and Resveratrol. In this method, effort was made to develop a simple, easy and economic RP-HPLC method for estimation of Niacin and Resveratrol in human plasma.

PLAN OF WORK

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD:

The present study, aims to develop the Bioanalytical method for the estimation of Niacin and Resveratrol in human plasma.

PHASE I

1. Optimization of chromatographic conditions

- Selection of wavelength
- Selection of initial separation conditions
- Selection of mobile phase (pH, peak modifier, solvent strength, ratio and flow rate)
- Selection of the stationary phase
- Selection of internal standard
- Sample preparation by solid phase extraction method
- Estimation of escitalopram oxalate in human plasma

PHASE II

2. Validation of the developed method

The developed method were proposed to be validated using various validation parameters such as,

- Accuracy
- Precision
- Linearity
- Limit of Detection (LOD)
- Lower Limit of Quantitation (LLOQ)
- Selectivity / Specificity
- System suitability.
- Ruggedness

The drugs present in the biological fluid were proposed to be estimated.

3. The Niacin and Resveratrol present in the biological fluid were proposed to be Estimated.

DRUG PROFILE

Nutraceutical

The term nutraceutical was coined by the combination of terms 'nutrition' and pharmaceutical in 1989 by Dr Stephen Defelice, chairman of the foundation of innovative medicine in 1989. About many years ago Hippocrates correctly emphasized that let food be our medicine and medicine be our food. This is very correct now a days because of the recognition that nutraceutical play a major role in health benefits. About 400 nutraceutical products are available with documented health benefits. Nutraceuticals are products which are derived from food sources that provide extra health benefits. In the us, "nutraceutical" do not exist as a regulatory category; they are regulated as dietary supplements, food additives by the FDA, drug and cosmetic act.

Niacin

Niacinamide is the active form of vitamin B_3 and a component of the coenzyme nicotinamide adenine dinucleotide (NAD). Niacinamide acts as a chemo- and radio-sensitizing agent by enhancing tumor blood flow, thereby reducing tumor hypoxia. This agent also inhibits poly (ADP-ribose) polymerases, enzymes involved in the rejoining of DNA strand breaks induced by radiation or chemotherapy.

Niacinamide or vitamin b_3 is an important compound functioning as a component of the coenzyme NAD. Its primary significance is in the prevention and/or cure of black tongue and pellagra. Most animals cannot manufacture this compound in amounts sufficient to prevent nutritional deficiency and it therefore must be supplemented through dietary intake. Niacinamide is used to increase the effect of radiation therapy on tumor cells. Niacin (nicotinic acid) and niacinamide, while both labelled as vitamin B_3 also have different applications. Niacinamide is useful in arthritis and early-onset type 1 diabetes while niacin is an effective reducer of high cholesterol levels.

Resveratrol

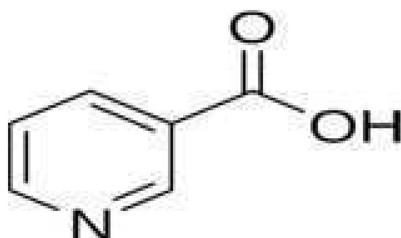
Resveratrol is natural occurring phytochemical obtained from the roots of polygonum Cuspidatum and leaves of veratrum grandiflorum belongs to the family polyganaceae. The Source of resveratrol in food include several species of plant including skin of grapes, red wine, peanuts, pine trees and berries. It belong to class of phytochemical known as stilbenes and subclass phytoalexin. Because it is produced in several plants in response to injury or when the plant is in attack by pathogens such as bacteria and fungi or under ultraviolet or when the plant is harmed such as cutting, crushing. Structurally resveratrol is 3,5,4'-trihydroxy-trans-stilbene.

Resveratrol has wide range of biological action with many targets with mechanism of action. These include inhibition of lipid peroxidation, inhibition of platlets, inflammatory activity and free radical scavenging. It is an effective antioxidant due to its protective effect on cellular membrane. Resveratrol is a fat soluble substance which can be in both *trans* and *cis* configuration.

NIACIN:

Cas register number	:	59-67-6
IUPAC name	:	Pyridine-3-carboxylic acid

Structure:

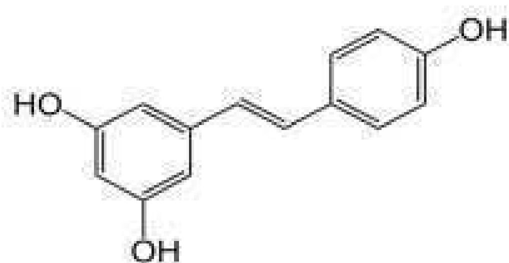


Molecular weight	:	123.111 g/mol
Molecular formula	:	$C_6H_5NO_2$
Melting point	:	236-239 °c
Pka value	:	2.0,4.85
Log p value	:	0.4
Physical form	:	white crystalline powder
Solubility	:	soluble in water, ethanol, dimethyl sulfoxide, acetone, acetonitrile and diethyl ether
Storage	:	store at room temperature.
Indications .	:	Niacin deficiency (Pellagra), cardiovascular disease, Anti-cholesterol levels, atherosclerotic disease, pancreatitis
Contra–indication	:	Malabsorption syndrome, diarrhoea, confusion (dementia), tongue redness/swelling, peeling red skin, hepatic impairment, Liver Dysfunction. Aspirin may decrease the metabolic clearance of nicotinic acid.

RESVERATROL:

Cas register number	:	501-36-0
IUPAC name	:	5,2(4-hydroxyphenyl)ethenyl-benzene 1,3 -diol

Structure:



Molecular weight	:	228.25 g/mol-1
Molecular formula	:	$C_{14}H_{12}O_3$
Melting point	:	261 to 263◦c
Pka value	:	8.49
Log p value	:	3.10
Stability	:	stable under normal storage condition
Physical form	:	off white powder
Solubility	:	soluble in water ethanol, methanol and acetone
Indications	:	Antioxidant, nutritional deficiencies, inflammation,
heart		attack, Anti-ageing and life longevity
Contra- indications	:	Blood thinners, bleeding, blood clotting. Sometimes it act like estrogen.
Drug interactions	:	Resveratrol decrease break down of some medicines in liver.

MATERIALS AND METHODS

DRUGS AND CHEMICAL REAGENTS USED:

The reference standard of Niacin and Resveratrol were purchased from Sami Labs Limited, Karnataka. The commercial product of Resveratrol (Gelnova) and Niacin (apex) was bought from market separately. HPLC grade Methanol were purchased from Sigma aldrich, India. Ortho Phosphoric acid was purchased from Thermo fischerpvt ltd. Ultra pure water was obtained from Mille Q- water purification system from millipore(milford) USA. Blank plasma were obtained from blood bank services at PSG Hospitals, Coimbatore, India.

INSTRUMENTATION

HPLC system	Shimadzu
HPLC software	LC lab solution
Pump	LC-20AR series
Instrument model number	CBM-20A
Photo diode array detector	Shimadzu-SPD-M20A diode array detector
Column	C_{18} Column Sun fire(250x4.6mm, 5µ)
Column manufacture injector	Rheodyne injector 20µl
UV double spectrophotometer	UV-1650PC, Shimadzu
pH meter	LAB INDIA. New Delhi.
Sonicator	Ultra sonicSonicator
Micro balance	AND HR 200

STANDARD AND SAMPLE PREPARATION:

a) Dilution preparation

99.7% methanol was used for preparation of stock solution and mobile phase was used for preparation of further stock solutions and dilutions.

b) Standard preparation

1. **Preparation of Niacin:**

18mg of Niacin was taken in a 10ml volumetric flask and make up to 10ml with 99.7% methanol. keep it for sonication upto 10 min. 2ml from stock solution were taken and transferred into 100ml volumetric flask and makeup to the volume with mobile phase to get a concentration 36µg/ml. From the above stock 1,2,3,4,5,6 and 7ml of stock solution was taken in 10ml standard flask and make up 10ml with mobile phase to get the concentrations of 3.6, 7.2, 10.8, 14.4, 18, 21.6 and 25.2µg/ml.

2. **Preparation of Resveratrol:**

5mg of Niacin was taken in a 10ml volumetric flask and make up to 10ml with 99.7% methanol. keep it for sonication upto 10 min. 2ml from stock solution were taken and transferred into 100ml volumetric flask and makeup to the volume with mobile phase to get a concentration 10µg/ml. From the above stock 1,2,3,4,5,6 and 7ml of stock solution was taken in 10ml standard flask and make up 10ml with mobile phase to get the concentrations of 1, 2, 3, 4, 5, 6 and 7µg/ml.

3. **Preparation of internal standard solution:**

10mg of Caffeine was taken and dissolved in 99.7% methanol. 1ml of caffeine was taken and dissolved in 10ml of methanol. 1ml of the above stock solution was taken in 100ml flask and makeup to the volume with the mobile phase (10µg/ml).

Analysis of marketed formulation

To determine the Niacin and Resveratrol content in conventional tablets and capsules respectively. 5 tablets and 5capusles was individually taken and weighed and Equivalent weight was taken and extracted. The extraction was done in most soluble organic compound (ie) Methanol. Drug was extracted countinously for 3 times and then filtered. Dilutions were followed as per standard preparations and injected. The wavelength in which the drug shows maximum absorbance is at 270nm which is confirmed in UV double beam spectroscopy.

Extraction Procedure

Transfer 50µl of the blank plasma, add 18µl and 5µlof drug mixture (NIA + RESV) from each level separately and vortex for 1 min, then add 20µl of the internal standard solution (caffeine) to a eppendorf vial and vortex for 1 min Then add 25µl as the extracting solvent and vortex for 3 mins. Kept at centrifuge at 4,000 rpm for 3 mins (at 40˚C). Separate the non -extracted samples were prepared and injected.

OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS:

Selection of wavelength

The selection of wavelength for the analysis was selected from UV Spectrum range at 200-400 nm for both Niacin and Resveratrol showed that 214nm and 306nm was the suitable wavelength for detection due to maximum absorbance. From the UV spectrum, the wavelength was selected as 270nm as it showed good absorbance.

Selection of mode of separation

The selection of method depends up on the nature of the sample, its molecular weight and solubility. The drug selected in the study is polar in nature and RP-HPLC method was prefer due to the suitability.

Effect of Mobile phase:

Methanol and Water of pH with 30:70, 40:60 and 50:50 ratios were used as mobile phase. At 30:70 Methanol and Water, the peak was eluted at 2.9min for Niacin and 6.2min for Resveratrol. At 40:60 ratio, the peak was eluted at 2.1 and 5.9mins. At 50:50 ratio, the peak was eluted at 1.8min and 5.1min with symmetric and well retained peak. For the present study 50:50 ratio was selected.

Initial chromatographic condition:

Trial 1: The chromatographic conditions used are

Stationary phase	Sunfire^{TM} C_{18} (250X4.6mm) 5µm column
Mobile phase	Methanol : Water
Solvent ratio	30 :70
Detection wavelength	270
Column temperature	Room temperature of about 20˚C
Flow rate	0.8ml/min
Injection volume	20µl
Photo diode array detector	Shimadzu-SPD-M20A diode array detector

From the isocratic run approximate percentage of Methanol, Triethylamine and Water (40:5:55) (pH 5.5) was adjusted by using Ortho phosphoric acid. The standard and sample

solution were injected. The retention time of Niacin and Resveratrol was found to be 2.5 and 7.9min respectively. The chromatogram was not good. To increase the retention and peak shape of the drugs Methanol and Water (30:70) was used and adjusted the pH with OPA. The retention time of Niacin was 2.9min and Resveratrol was 6.2min. The peak shape was not symmetrical. Instead of 30ml of Methanol 50ml of Methanol was used and the Mobile phase was Methanol:Water (50:50%v/v). Retention time of Niacin was 1.8min and Resveratrol was 5.1min and adjusted to the pH 3.5. The chromatogram was recorded and the peak was found to be symmetrical.

In this chromatographic condition, peak was well separated. So, column and mobile phase was fixed C_{18} 5µm (250X4.6mm) which showed a good resolution of peaks.

Effect of flow rate:

Flow rates of 0.8, 1 and 1.2 ml/min were used and chromatograms are recorded. When 1.2ml/min was used the elution time was found to be 2.9min for Niacin and 6.2min for Resveratrol. When 1ml/min was used the elution time was 2.1min and 5.9min. For 0.8ml/min the peak was found at 1.8min and 5.1min respectively. All these flow rate gave symmetric and well retained peak. For the present study, 0.8ml/min was selected.

Effect of pH

The standard solution was chromatographed for 20min using Methaol : water (50:50) in different pH ranging from 3.0 to 7.0 (ortho phosphoric acid) as the mobile phase at a flow rate of 0.8ml/min using Sunfire C_{18} column as the stationary phase. The retention time of Niacin and Resveratrol was found to be 1.8, 2.1, 2.6, 2.9 and 5.1, 5.9, 6.2, 6.9at the pH of 3.5, 4.5, 5.5, 6.2 respectively.It was observed that pH of the mobile phase did not alter the elution pattern of the drug. For, the present study pH of 3.5 was selected .

Effect of nature of stationary phase

The different selection of stationary phase

1. Phenomenex luna C_{18} (150×4.6mm), 5µm
2. Sunfire C_{18} column (250×4.6mm), 5 µm

With phenomenex luna C_{18} column the drug was not eluted. No peaks were obtained. The column was changed to Sunfire C_{18}, initialy methanol was used which gave high pressure which is beyond the limit. So mobile phase were changed and different mobile phase

was tried to improve the peak shape as well as peak symmetry. The result was good, with the ratio of Methanol and Water (50:50) in which Niacin peak showed at 1.8min and Resveratrol at 5.1 mins. The pressure was within limited range hence Sunfire C_{18} was selected.

Selection of Internal standard:

Based on the polarity and solubility, caffeine (10µg/ml) was selected and chromatographed along with the standard drug. The elution time of caffeine was 2.69 mins. the peak of IS was well resolved and symmetric. For the present study, caffeine was selected as an Internal standard.

Fixed chromatographic conditions

Stationary phase	Sunfire^{TM} C_{18} 5µm, (250X4.6mm) column
Mobile phase	Methanol : Water
Solvent ratio	50 :50
Detection wavelength	270
Flow rate	0.8ml/min
Injection volume	20µl
Column temperature	25˚C
Photo diode array detector	Shimadzu-SPD-M20A diode array detector

ICH Q2 (R1) VALIDATION OF ANALYTICAL PROCEDURE:

This guideline extends the guideline Q2A to include the validation parameters needed for a variety of analytical methods. It also discusses the characteristics that must be considered during the validation of the analytical procedures which are included as part of registration applications and describes the actual experimental data required,along with the statistical interpretation, for the validation of analytical procedures. The content of this guideline was previously included in two separate guidelines coded Q2A and Q2B ,which were unified to Q2(R1) guideline in November 2005.

RESULT AND DISCUSSION

OPTIMISATION OF CHROMATOGRAPHIC CONDITIONS FOR THE DETERMINATION OF NIACIN AND RESVERATROL:

Proper selection of the method depends upon the nature of the sample, its molecular weight and solubility. The drugs selected in the present study are polar in nature and hence RP-HPLC method was used because of its simplicity and suitability.

UV scanning at 200-400 nm for both Niacin and Resveratrol show that 214nm and 306nm is the suitable wavelength for detection due to maximum absorbance. From the UV spectrum, the wavelength was selected as 270nm as it showed good absorbance.

The Linearity of Niacin was 3.6-25.2µg/ml and Resveratrol was 1-7µg/ml. Different mobile phases were tried for selecting the ideal mobile phase. At 30:70 Methanol and Water, the peak was eluted at 2.9min for Niacin and 6.2min for Resveratrol. At 40:60 ratio, the peak was eluted at 2.1 and 5.9mins. At 50:50 ratio, the peak was eluted at 1.8min and 5.1min with symmetric and well retained peak. For the present study 50:50 ratio was selected.

With Phenomenex luna C_{18} column the drug was not eluted. No peaks were obtained. The column was changed to Sunfire C_{18}, initially methanol was used which gave high pressure which is beyond the limit. So mobile phase were changed and different mobile phase was tried to improve the peak shape as well as peak symmetry. The result was good, with the ratio of Methanol and Water (50:50) in which Niacin peak showed at 1.8min and Resveratrol at 5.1mins. The pressure was within limited range hence Sunfire C_{18} was selected.

Caffeine (10µg/ml) was selected as internal standard and chromatographed along with standard drug. The elution time of caffeine was 2.6mins. The peak of caffeine was well resolved and symmetric.

The retention time of Niacin and Resveratrol was found to be 1.8, 2.1, 2.6, 2.9 and 5.1, 5.9, 6.2, 6.9 at the pH of 3.5, 4.5, 5.5, 6.2 respectively. It was observed that pH of the mobile phase did not alter the elution pattern of the drug. For, the present study pH of 3.5 was selected. The correlation coefficient was found to be 0.999 and 0.998 for Niacin and Resveratrol Respectively.

The percentage recovery was good which indicates reproducibility and accuracy of the developed method. The percentage recovery of the drugs were found to be 76 - 78%. Similarly, the RSD value for Precision was also found to be within the acceptance limit and more fatal was that the estimation was performed in short time. The LOD and LOQ were found to be 0.05, 0.03 for Niacin and 0.15, 0.10 for Resveratrol.

Based on the above studies, the following optimized chromatographic condition was selected for the analysis of the drugs in standard solution.

Stationary phase	Sunfire^{TM} C_{18} 5µm, (250X4.6mm) column
Mobile phase	Methanol : Water
Solvent ratio	50 :50
Detection wavelength	270 nm
Flow rate	0.8ml/min
Injection volume	20µl
Internal standard	Caffeine
Temperature	25□

In optimised condition the blank plasma, standard solutions were injected and the chromatograms were recorded. The optimised conditions are used for the estimation have well defined separation between the drugs, internal standard and endogenous components. The blank plasma showed no interference at retention time of the drugs and their internal standard.

The standard solution and sample solution were injected with the above chromatographic conditions and the chromatogram were recorded. The retention time for Niacin, caffeine and Resveratrol was 1.8, 2.3 and 5.1mins respectively. The response factor (peak area of drug and IS) of the standard solution and the concentration of the Niacin and Resveratrol present in the plasma samples was calculated.

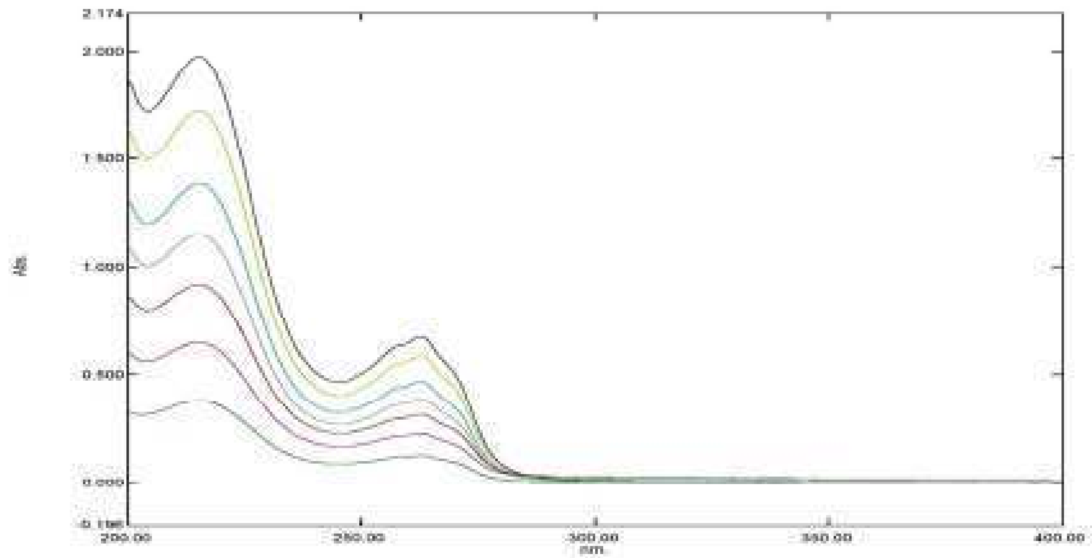


FIGURE:1 Overlain Spectrum of Niacin

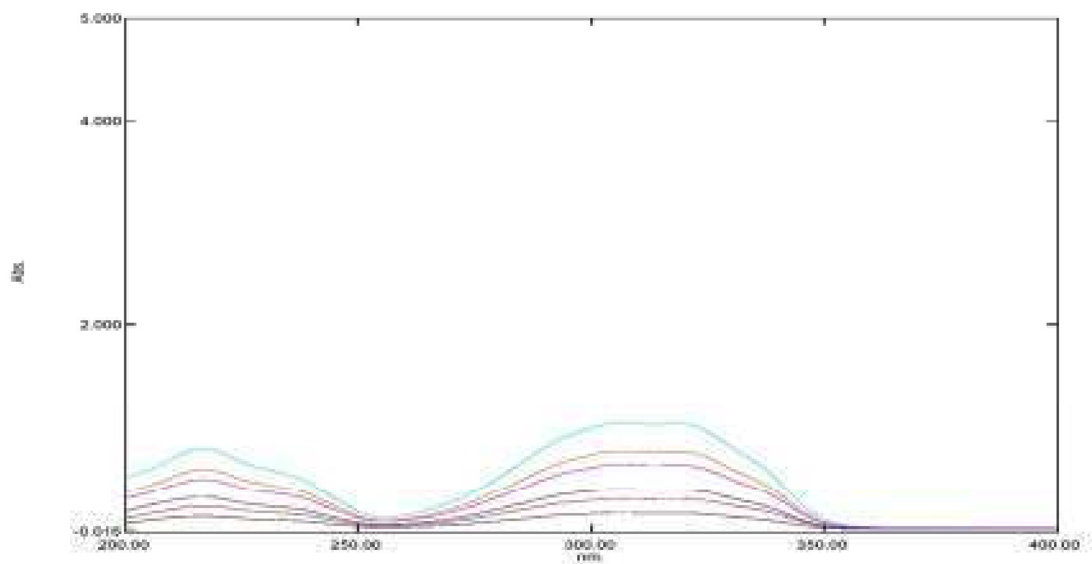


FIGURE:2 Overlain Spectrum of Resveratrol

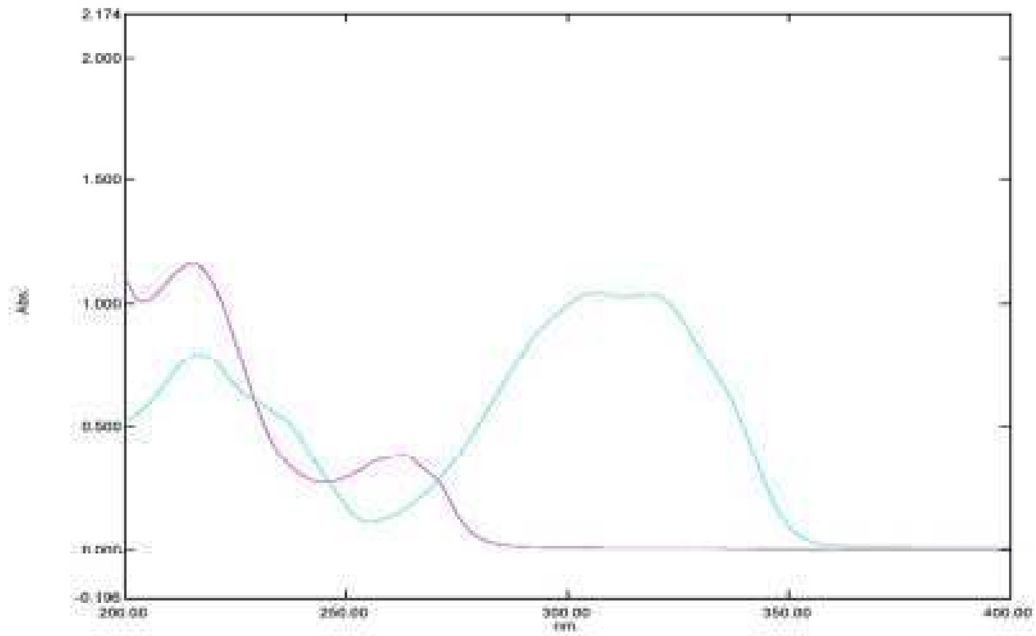


FIGURE:3 Overlay of UV Spectroscopy of Niacin and Resveratrol

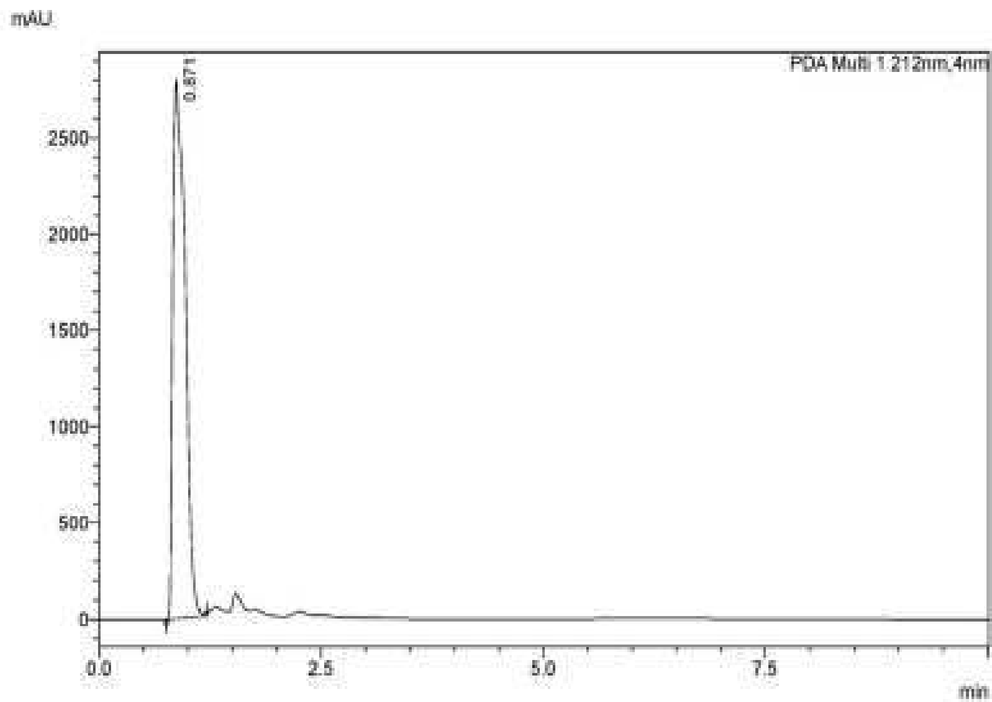


FIGURE:4 Representative chromatogram of Blank plasma

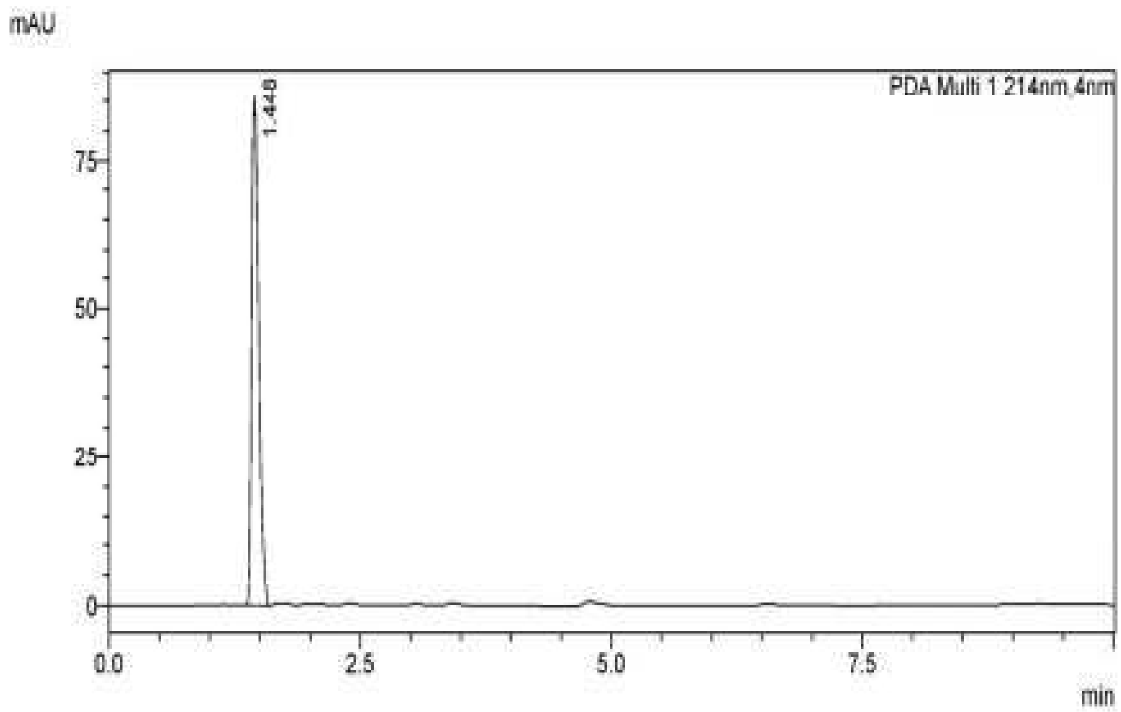


FIGURE:5 Representative chromatogram of Niacin

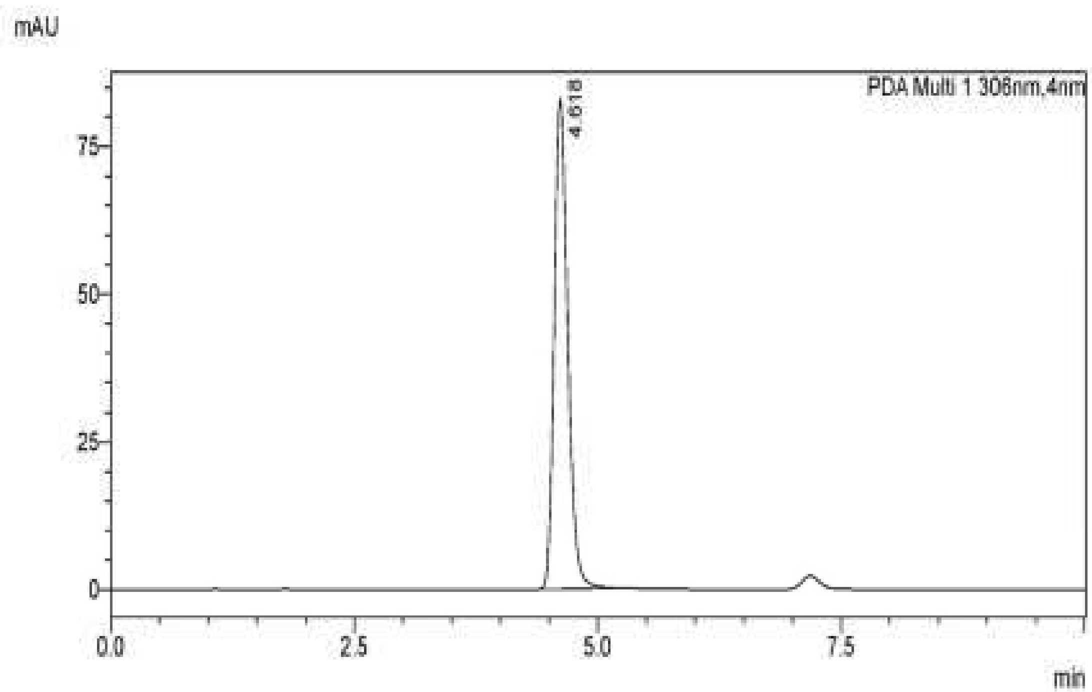


FIGURE:6 Representative chromatogram of Resveratrol

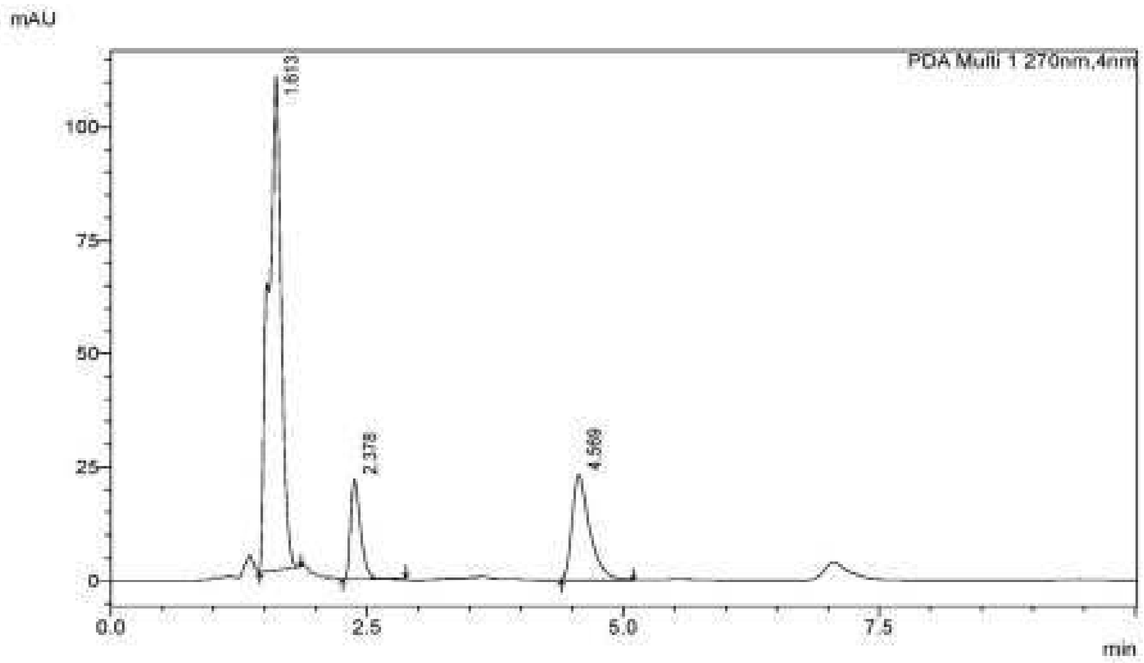


FIGURE:7 Representative chromatogram of Drugs and IS

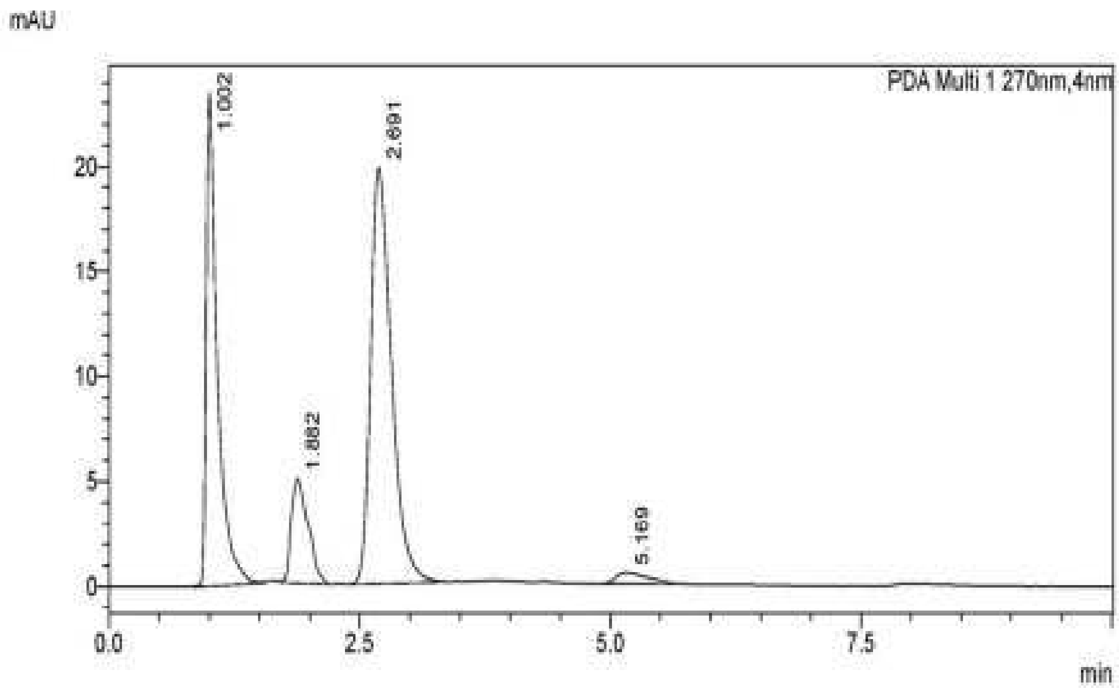


Figure 8: Representative chromatogram of Low quality control (LQC) sample

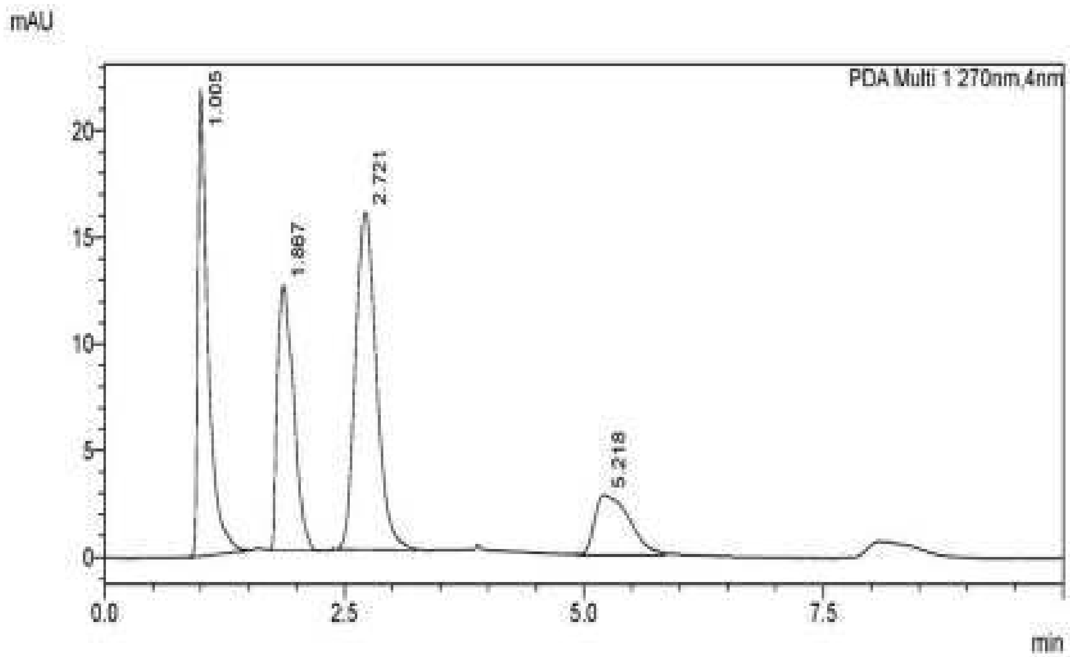


Figure 9: Representative chromatogram of Middle quality control (MQC) sample

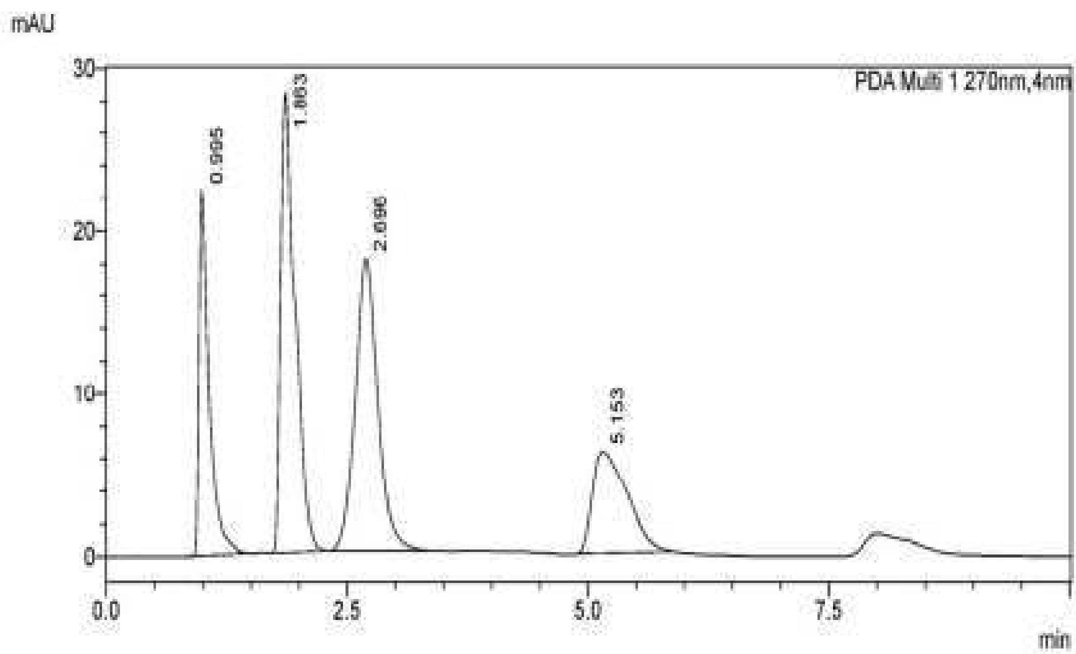


Figure 10: Representative chromatogram of High quality control (HQC) sample

VALIDATION OF THE DEVELOPED METHODS

Validation of bioanalytical methods include performing all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix (e.g., blood, plasma, serum or urine) reliable and reproducible for the intended use.

Accuracy and Precision:

The accuracy of the optimized methods was determined by relative and absolute recovery experiments. Accuracy and Precision was determined by replicate analysis of samples containing known amounts of the analyte. Precision was determined by their intra-day and inter-day responses. A minimum of three concentrations in the range of expected study sample concentrations was recommended. Based on the calibration curve of Niacin and Resveratrol of 10.8, 18, 25.2µg/ml and 3, 5, 7µg/ml repectively as LQC, MQC, and HQC. Each concentration range was injected 6 times. Based on the area and height of the peak, back calculated concentration (µg/ml) was obtained. The percentage accuracy was obtained by simplifying back calculated concentration with actual nominal concentration and % CV was calculated.

TABLE : 1

Back calculated concentration of Quality control samples of Niacin

Nominal concentration (µg/ml)	**LQC**		**MQC**		**HQC**	
	10.8		18		25.2	
S.No	Calculated concentration (µg/ml)	Accuracy (%)	Calculated concentration (µg/ml)	Accuracy (%)	Calculated concentration (µg/ml)	Accuracy (%)
1	10.62	98.33	17.96	99.77	25.06	99.44
2	10.75	99.54	18.4	100.22	25.16	99.84
3	10.89	100.84	17.83	99.05	24.93	98.93
4	10.67	98.79	17.94	99.66	25.26	100.23
5	10.78	99.82	17.79	98.83	24.82	98.49
6	10.69	98.99	17.73	98.50	25.12	99.68
Mean	10.73		17.94		25.05	
%CV	0.89		1.34		0.64	
%Nominal	99.35		99.66		99.40	

TABLE : 2

Back calculated concentration of Quality control samples of Resveratrol

Nominal concentration (µg/ml)	**LQC**		**MQC**		**HQC**	
	3		5		7	
S.No	Calculated concentration (µg/ml)	Accuracy (%)	Calculated concentration (µg/ml)	Accuracy (%)	Calculated concentration (µg/ml)	Accuracy (%)
1	2.96	98.67	5.01	100.25	6.95	99.28
2	3.02	100.66	4.98	99.50	6.98	99.71
3	2.98	99.34	4.89	97.25	6.87	98.14
4	2.95	98.33	4.93	98.60	6.79	98
5	3.01	101	4.92	98	6.93	99
6	2.94	98	4.95	98.75	7.03	100.42
Mean	2.98		4.95		6.92	
%CV	1.09		0.87		1.22	
%Nominal	99.33		99		98.85	

The mean value was within 15% of the nominal value. The coefficient of variation (%) of these values was less than 5%. The accuracy values ranged for Niacin was 98.33% to 100.84% and Resveratrol was 97.25% to 101%. The Precision values ranges from 0.64% to 1.34%for Niacin and 0.87% to 1.22% for Resveratrol. Thus, the derived developed method are accurate, precise and reliable.

Specificity and Selectivity:

Selectivity is the ability of an analytical method to differentiate and quality the analyte in the presence of other components in the sample. The six blank plasma samples obtained from six different volunteers were analysed and the chromatograms were recorded and compared with the chromatograms obtained from standard solutions. Each blank is tested for interference and selectivity is ensured at the lower limit of quantification. Selectivity was evaluated by injecting extracted blank plasma and comparing with the response of extracted LLOQ samples processed with internal standard endogenous interference were not detected at the retention time of selected drugs and internal standard. These observations show that the developed assay method is specific and selective.

Linearity:

A calibration curve is the relationship between the instrument response and known concentration of analyte. It was observed that the optimized methods were linear within a specific concentration range for individual drugs. The linearity concentration ranges from 3.6 – 25.2µg/ml for Niacin and 1 – 7 µg/ml.

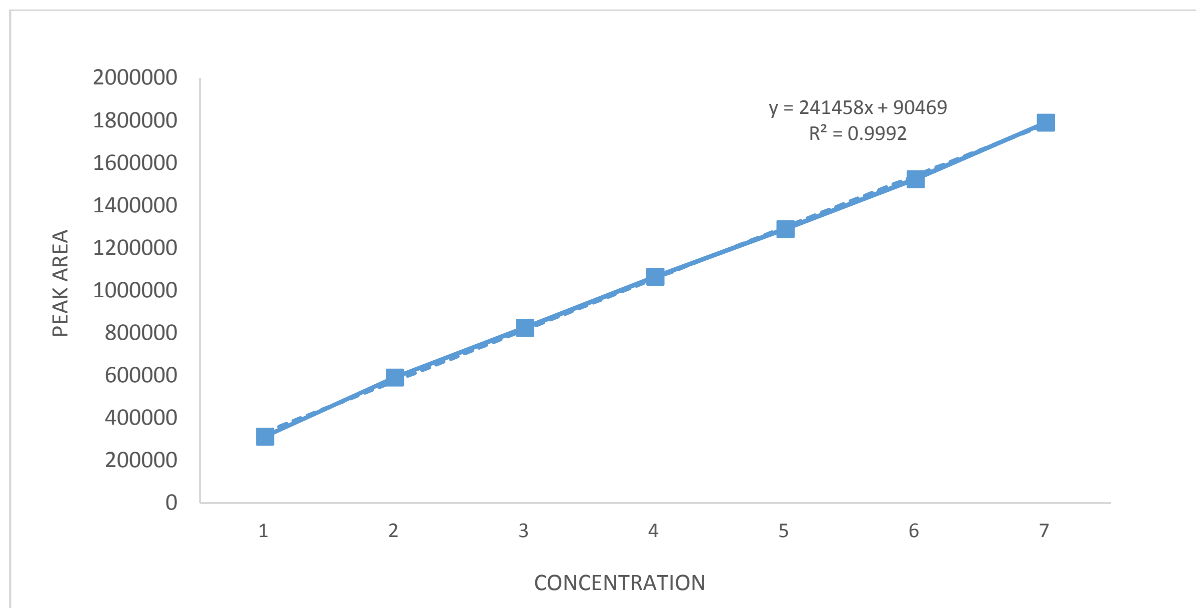


Figure : 11 Representative calibration curve plot for Niacin

TABLE : 3

Back calculated concentration for calibration curves of Niacin

Sample Level	Analyte Concentration (µg/ml)	Back Calculated Concentration (µg/ml)	Accuracy (%)
1	3.74	3.72	99.46
2	7.19	7.32	101.81
3	10.79	10.83	100.37
4	15.03	14.82	98.60
5	17.95	17.56	97.76
6	21.67	21.26	98.11
7	25.53	25.34	99.25

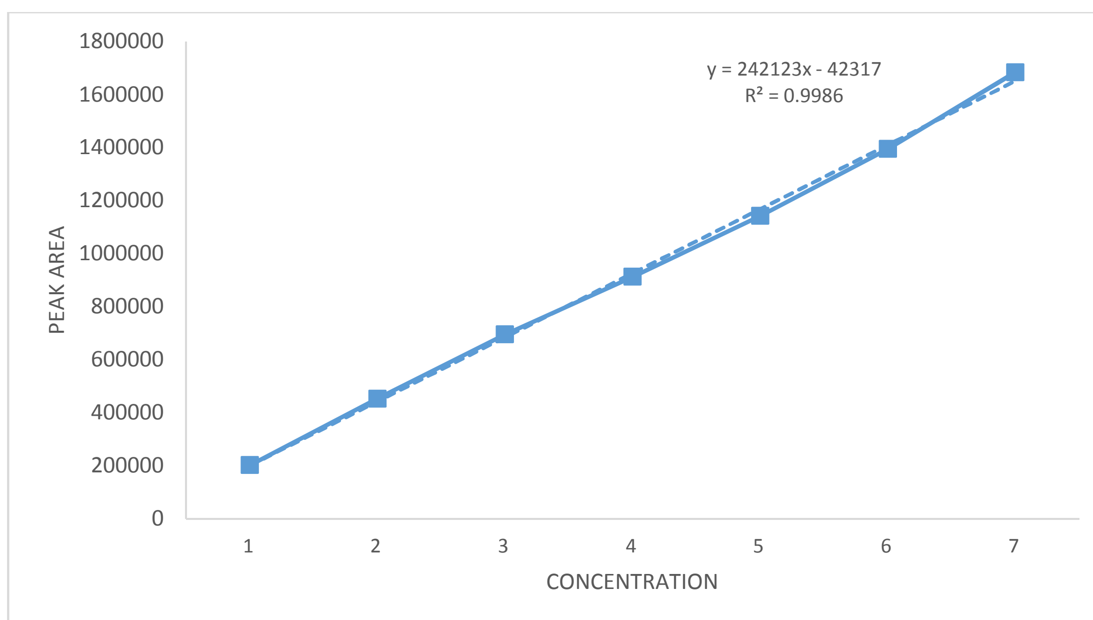


Figure : 12 Representative calibration curve plot for Resveratrol

TABLE : 4

Back calculated concentration for calibration curves of Resveratrol

Sample Level	Analyte Concentration (µg/ml)	Back Calculated Concentration (µg/ml)	Accuracy (%)
1	1.01	0.99	98.02
2	208	2.07	99.52
3	3.01	3.06	101.66
4	4.15	4.12	99.28
5	5.19	5.16	99.42
6	6.03	6.06	99.54
7	7.35	7.23	98.36

Sensitivity:

Sensitivity is measured using lower limit of quantification (LLOQ) is the lower concentration of the standard curve is measured with the acceptable accuracy and precision. LLOQ was established using five samples independent of standards and determining the coefficient of variation and confidence interval. Blank plasma and lowest level of calibration curve was processed in triplicate. The mean noise (H) for the blank plasma samples in terms of height is determined the mean signal (H) for the peaks of the samples for the lowest calibration level in terms of height was determined. Then the signal to noise for the peaks for the drugs was not more than the acceptance criteria.

Limit of Detection and Limit of Quantification :

The limit of detection (LOD) and limit of quantification (LOQ) of the developed method were established by injecting the lower concentration of standard solution using the developed RP-HPLC method. The limit of detection was found to be 0.05µg/ml and 0.03µg/ml and the limit of quantification was found to be 0.15µg/ml and 010µg/ml for Niacin and Resveratrol respectively.

Carry over:

Blank plasma screening during validation, blank plasma samples from 4 different lots were processed according to the extraction procedure and evaluate the interference at the retention times of analyte and internal standard. The 3 interference lots were selected. These do not have any carry over effect caused by injecting the highest concentration of the standard solution of the drug just before the plasma.

Recovery:

Transfer 50µl of the blank plasma, add 18µl and 5µl of drug mixture (NIA + RESV) from each level separately and vortex for 1 min, then add 20µl of the internal standard solution (caffeine) to a eppendorf vial and vortex for 1 min Then add 25µl as the extracting solvent and vortex for 3 mins. Kept at centrifuge at 4,000 rpm for 3 mins(at 40˚C). Separate the non -extracted samples were prepared and injected. This method was done by using liquid – liquid extraction technique which gave adequate recovery of the samples. The recovery was evaluated at three different levels of analyte such as low, medium and high quality control standards area were compared against the mean area of respective un-extracted quality standard area.

TABLE : 5

Observed recovery for Niacin

S.No	LQC			MQC			HQC		
	Area of Un-Extracted Standard	Area of Un-Extracted Standard	% Recovery	Area of Un-Extracted Standard	Area of Un-Extracted Standard	% Recovery	Area of Un-Extracted Standard	Area of Un-Extracted Standard	% Recovery
1	316729	243128	76.77	1245978	982496	78.85	1842359	1421378	77.14
2	325461	254682	78.25	1345573	1025649	76.22	1754892	1346920	76.75
3	304287	234692	77.13	1434673	1085436	75.65	1701258	1396348	76.20
4	294685	224591	76.21	1452246	1154237	79.48	1754693	1394583	79.47
5	314486	242187	77.01	1598425	1255469	78.54	1859637	1462109	78.62
6	294536	234082	79.47	1294678	1012458	78.20	1764238	1391325	78.86
Mean	304958.7		77.68	1424028		77.63	1790946		77.75
%CV	2.6		1.5	0.82		1.76	1.82		1.53

TABLE : 6

Observed recovery for Resveratrol

S.No	LQC			MQC			HQC		
	Area of Un-Extracted Standard	Area of Un-Extracted Standard	% Recov Ery	Area of Un-Extracted Standard	Area of Un-Extracted Standard	% Recovery	Area of Un-Extracted Standard	Area of Un-Extracted Standard	% Recovery
1	254697	201586	79.14	1189435	912543	76.72	2301894	1754201	76.20
2	282107	221058	78.35	1202548	929421	77.28	2296425	1820139	79.25
3	270245	215459	79.73	1184283	924589	78.07	2245812	1746245	77.75
4	259725	216365	77.95	1165421	894253	76.73	2145297	1684213	78.51
5	264581	204325	77.23	1146328	912743	79.61	2105483	1642126	77.80
6	201543	262468	76.78	1201546	942501	78.44	2315824	1825326	78.82
Mean	258816.3		78.19	1181594		77.80	2213458		78.05
%CV	1.86		1.43	1.52		0.85	0.92		1.37

Extraction efficiency from the human plasma was determined by comparing the responses obtained from extracted sample spiked with the known amount of Niacin and Resveratrol with the extracted responses. The quality control standards were compared against the mean area of respective un-extracted quality control standards. The mean recovery of Niacin and Resveratrol was found to be 77.68, 77.63, 77.50 and 78.19, 77.80, 78.05 repectively.

System suitability:

System suitability parameters were measured so as to verify the system performance. All important characteristics including the number of effective theoretical plates, resolution, asymmetry, retention time, detection limit and selectivity were measured and calculated by using SST solution injection in five replicates. It is the integral part of many analytical method.These test are carried out based on the concept that the equipment, analytical method and samples are integral part of system that can be evaluvated.System suitablity solution was prepared and injected six replicates in the system before each batch. Then coefficient of variation for peak area response and retention time for the peak of test substance determined (Piotr *et al.,* 2009).

The parameters used in system suitability test report are as follows;

- Number of theoretical plates and efficiency
- Capacity factor
- Seperation or relative retention
- Resolution
- Tailing factor
- Relative standard deviation

Stability :

To generate reproducible and reliable results, the samples, standards, reagents used for HPLC method must be stable for a reasonable time (eg one day, one week, one month, depending on need). Therefore a few hours of standard and sample solution stability can be required even short (10min) seperation. When more than one sample is analyzed (multiple lots of one sample or samples from different storage conditions from a single lot), automated, overnight runs often are performed for better lab efficiency. Such practices add requirements for greater solution stability. The acceptance criteria of validation for HPLC. Assessments of

analyte stability should be conducted in the matrices as that of the study samples. All stability determinations should use samples prepared from a freshly made stock solutions. Conditions used in these experiments should reflect situations likely to be encountered during actual sample handling and analysis such as

- Stock solution stability
- Short term stability
- post preparative stability
- Freeze-thaw stability

1. Stock solution stability:

The stability of stock solutions of drug and the internal standard should be evaluated at room temperature for at least 6 hours. If the stock solutions are refrigerated or frozen for the relevant period, the stability should be documented. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions.

2. Short term stability:

Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature from 4 to 24 hours (based on the expected duration that samples will be maintained at room temperature in the intended study) and analyzed.

TABLE : 7

Short Term Stability for Niacin

Nominal Concentration (µg/ml)	**LQC**		**HQC**	
	7.2		25.2	
S.No	Calculated Concentrations	Accuracy (%)	Calculated Concentrations	Accuracy (%)
1	7.18	99.73	25.28	100.32
2	7.25	100.69	24.23	96.15
3	7.12	98.89	25.16	99.84
4	7.06	98.05	24.58	97.53
5	7.01	97.36	24.86	98.65
6	6.98	96.94	25.04	99.36
Mean	7.10		24.85	
%CV	1.42		1.53	
%Nominal	98.61		98.64	

TABLE : 8

Short Term Stability for Resveratrol

Nominal Concentration (µg/ml)	**LQC**		**HQC**	
	2		7	
S.No	Calculated Concentrations	Accuracy (%)	Calculated Concentrations	Accuracy (%)
1	1.97	98.52	6.97	99.57
2	2.01	100.50	6.91	98.78
3	1.94	97.15	6.84	97.71
4	1.96	98.45	7.03	100.43
5	1.98	99.21	6.98	99.71
6	1.93	96.99	6.78	96.93
Mean	1.97		6.92	
%CV	1.47		0.09	
%Nominal	97.50		98.86	

3. Post Preparative Stability:

The stability of processed samples, including the resident time in the autosampler, should be determined. The stability of the drug and the internal standard should be assessed over the anticipated run time for the batch size in validation samples by determining concentrations on the basis of original calibration standards.

Long-term stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions as the study samples. The concentrations of all the stability samples should be compared to the mean of back-calculated values for the standards at the appropriate concentrations from the first day of long-term stability testing.

TABLE : 9

Post Preparative Stability for Niacin

Nominal Concentration (µg/ml)	**LQC**		**HQC**	
	7.2		25.2	
S.No	Calculated Concentrations	Accuracy (%)	Calculated Concentrations	Accuracy (%)
1	7.13	99.02	25.17	99.88
2	7.28	100.83	24.49	97.18
3	7.11	98.75	25.22	100.19
4	6.98	96.94	24.58	97.53
5	7.14	99.16	24.75	97.74
6	7.08	98.34	25.07	99.48
Mean	7.12		24.88	
%CV	1.36		1.26	
%Nominal	98.88		98.73	

TABLE : 10

Post Preparative Stability for Resveratrol

Nominal Concentration (µg/ml)	**LQC**		**HQC**	
	2		7	
S.No	Calculated Concentrations	Accuracy (%)	Calculated Concentrations	Accuracy (%)
1	1.97	98.52	6.97	99.57
2	2.01	100.50	6.91	98.78
3	1.94	97.15	6.84	97.71
4	1.96	98.45	7.03	100.43
5	1.98	99.21	6.98	99.71
6	1.93	96.99	6.78	96.93
Mean	1.96		6.95	
%CV	1.47		0.09	
%Nominal	98.02		99.28	

4. Freeze and thaw cycles:

Analyte stability should be determined after three freeze and thaw cycles. At least three aliquots at each of the low and high concentrations should be stored at the intended storage temperature for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples should be refrozen for 12 to 24 hours under the same conditions. The freeze–thaw cycle should be repeated two more times, then analyzed on the third cycle. If an analyte is unstable at the intended storage temperature, the stability sample should be frozen at -70ºC during the three freeze and thaw cycles.

TABLE : 11

Freeze and thaw cycles for Niacin

Nominal Concentration (µg/ml)	**LQC**		**HQC**	
	7.2		25.2	
S.No	Calculated Concentrations	Accuracy (%)	Calculated Concentrations	Accuracy (%)
1	7.22	100.27	25.05	99.40
2	7.13	99.02	24.58	97.53
3	7.15	99.31	25.12	99.68
4	6.98	96.94	24.84	98.57
5	7.33	101.80	24.39	96.78
6	7.08	98.34	25.28	100.31
Mean	7.23		24.96	
%CV	1.22		1.37	
%Nominal	100.41		99.04	

TABLE : 12

Freeze and thaw cycles for Resveratrol

Nominal Concentration (µg/ml)	**LQC**		**HQC**	
	2		7	
S.No	Calculated Concentrations	Accuracy (%)	Calculated Concentrations	Accuracy (%)
1	1.94	97.02	7.05	100.71
2	2.03	101.50	6.94	99.14
3	1.98	99.25	6.87	98.26
4	1.93	96.64	6.90	98.57
5	1.97	98.69	6.96	99.43
6	1.96	98.31	6.77	96.74
Mean	1.99		7.05	
%CV	1.14		0.95	
%Nominal	99.30		97.92	

The stability of the drug spiked human plasma samples at three levels were studied. The mean concentrations of the stability samples were compared to the theoretical concentrations. The responses found in short term, post preparative, freeze thaw stability and standard solution stability were evaluated.

DISCUSSION:

The developed method was found to be suitable for simultaneous estimation of Niacin and Resveratrol in human plasma using RP-HPLC with good peak shape, resolution and less tailing. The goodness of fit (correlation coefficient 'r^2') for both standard in mobile phase and plasma was greater than 0.99. This shows the better detector response in different concentration for both in aqueous as well as plasma. The proposed method was validation as per USFDA guidelines. LOD and LOQ indicates the sensitivity of the method. The coefficient variation was less than 5% which shows the method was precise. The extraction efficiency in plasma studies was found to be more than 76 – 78%.

Simple, sensitive, accurate and quick analytical method developed and validated as per FDA guidelines for the simultaneous estimation of Niacin and Resveratrol in human plasma by using RP-HPLC.

SUMMARY

In this project, the study includes the method development and validation of Niacin and Resveratrol in bio-analytical method using RP-HPLC.

The Niacin and Resveratrol were journalised individually. No combined method has been found so far. This method was very accurate, precise, robust and simple when compared to other methods.

The study has been summarised below:

The introduction explain about the Nutraceutical drugs developed in Bio-analytical method. As the Bio- analytical methods shows the Bioavailability of the drug present and reacted in our body. The method has been developed and optimized according to the ICH Q2 (R1) guidelines.

Literature survey has been reviewed with many techniques and they provided different results. Clinical study were also published and that to were helped in providing information. The drugs are developed combinedly for our optimised study.

The Aim and Plan of work defines about the project.

According to the drug profile, Proper selection of the method depends upon the nature of the sample, its molecular weight and solubility. The drugs selected in the present study are polar in nature and hence RP-HPLC method was used because of its simplicity and suitability.

Materials and Methods briefs out the preparation of standard and internal standard drugs, sample of dosage forms which are optimized. As the physical properties are same for Caffiene (IS) as that of the standard drugs. So, it was selected as the Internal standard.

The results (tables, graphs) were shown for the conclusion of our study which are developed and validated as per ICH guidelines:

UV spectrum was ranged between 200-400 nm. 214 and 306 nm showed maximum absorbance for Niacin and Resveratrol respectively. The 270 nm was found as the isobestic point for these two drugs.

With Phenomenex luna C_{18} column the drug was not eluted. No peaks were obtained. The column was changed to Sunfire C_{18}, initially methanol was used which gave high pressure which is beyond the limit.

The linearity was found to be 3.6-25.2µg/ml, 1-7µg/ml and correlation coefficient was found to be 0.999, 0.998 for Niacin and Resveratrol respectively.

The retention time was found to be 1.8, 2.6 and 5.1 mins for Niacin, Caffeine and Resveratrol respectively.

The accuracy and precision was found to be 98-99%. The recovery were found to be 76-78%.

The LOD and LOQ was found to be 0.05, 0.03 for Niacin and 0.15, 0.10 for Resveratrol.

The short term stability study was found to be 97.50-98.86%.

The post preparative stability study was found to be 98.02-99.28%

The freeze and thaw cycles was found to be 97.92-100.41%.

The result of dosage form analysis by developed method was compatible with the plasma. There was no interference of excipient for analysis of dosage forms.

The developed method can be applied for the studies of pharmacokinetics parameters of Anti- cholesterol drugs for Niacin and Resveratrol and its metabolites in human plasma. It can also be applied in therapeutic drug monitoring practices and clinical toxicological assays.

CONCLUSION

An isocratic novel method has been developed for simultaneous estimation of Niacin and Resveratrol in plasma by RP-HPLC coupled with PDA detector. The proposed method has the ability to separate the components within 10 minutes. This method is simple, rapid and time consuming when compared to other methods so it offers a significant advantage in short run time, economic, rapid extraction, easy sample preparation, good recovery and wide linearity range. All parameters such as precision, accuracy, linearity, robustness, LOD, LOQ and system suitability was validated as per ICH guidelines and the result shows that the method was reliable and acceptable. Therefore, we can conclude that the proposed chromatographic method can be used to analyze samples in routine assay of Niacin and Resveratrol in Bulk drugs and in Plasma.

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