

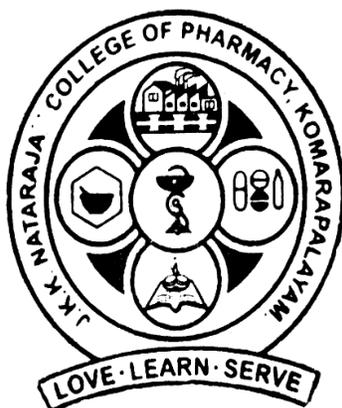
**QUANTITATIVE ANALYSIS OF GALLIC ACID AND ELLAGIC  
ACID IN TRIPHALA CHURNAM TABLET  
FORMULATION BY RP-HPLC**

Dissertation submitted to *The Tamil Nadu Dr. M. G.R. Medical University*  
Chennai-32

In partial fulfillment for the award of the degree of

**MASTER OF PHARMACY  
in  
PHARMACEUTICAL ANALYSIS**

Under the guidance of  
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**CERTIFICATE**

This is to certify that the work embodied in the dissertation “QUANTITATIVE ANALYSIS OF GALLIC ACID AND ELLAGIC ACID IN TRIPHALA CHURNAM TABLET FORMULATION BY RP-HPLC” submitted to *The TamilNadu Dr. M. G. R. Medical University*, Chennai, was carried out by V. V. Suri Babu Mummidi [Registration No. 26074959], for the partial fulfillment of degree of MASTER OF PHARMACY in the branch of Analysis under direct supervision of Mr. G. Babu. M. Pharm., Asst. Professor, Department of Analysis, J. K. K. Nataraja College of Pharmacy, Komarapalayam, during the academic year 2008-2009.

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This dissertation is now ready for examination.

[G. BABU]

**Place:**

**Date:**

## **DECLARATION**

The work presented in this dissertation entitled “QUANTITATIVE ANALYSIS OF GALLIC ACID AND ELLAGIC ACID IN TRIPHALA CHURNAM TABLET FORMULATION BY RP-HPLC”, was carried out by me, under the direct supervision of Mr. G. Babu. M. Pharm, Asst. Professor, Department of Analysis, J. K .K. Nataraja College of Pharmacy, Komarapalayam.

I further declare that, this work is original and has not been submitted in part or full for the award of any other degree or diploma in any other university.

**V. V. SURIBABU.MUMMIDI**

Place:

Date:

## ACKNOWLEDGEMENT

My dissertation entitled 'Quantitative Analysis of Gallic Acid and Ellagic Acid in Triphala Churnam Tablet Formulation by RP-HPLC' would not have been a feasible one without the grace of God Almighty who gave me morale till the completion of the work.

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**V. V. SURIBABU MUMMIDI**

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## INTRODUCTION

Pharmaceutical industry in India is one of the fastest growing in the world. Exporting a significant percentage of generic drugs makes the pharmaceutical industry a leading player in the world market along with its application in the Research and Development (R&D). Research and Development in the pharma industry is multifaceted and draws upon the expertise of chemists, chemical engineers, pharmacologists and medical practitioners. The different activities of R&D include drug development (synthesis and manufacture), formulation, clinical trials, evaluations and finally launching. Closely associated with these are regulatory and quality assurance functions. The above functions require different manpower as synthetic chemists, analytical and quality control chemists, process development and manufacturing personnel (chemists and chemical engineers), pharmacologists for evaluation of drug delivery systems and finally doctors for clinical trials<sup>4</sup>.

### **New drug discovery**

New drugs have been discovered from two major sources

- Synthetic chemicals
- Natural products including plants, animals and microbes

The drug discovery process is responsible not only for the introduction of many new drugs to the market but also for the improvements in the therapy of many diseases. The drug development starts from receptor mapping, target identification and optimization, pharmacodynamic screening, pharmacokinetic optimization and ends in pre-clinical assessment to clinical assessment of the drug.<sup>1</sup>

### **Techniques used**

Mass spectroscopy was used for the accurate determination of molecular mass in structural determination of chemical compounds. Non-validated screening methods are used to monitor the synthesis of active ingredients or to confirm their identity during discovery and preclinical research. Analytical methods are progressively optimized and a preliminary validation package is furnished as a part of the Investigational New Drug (IND) application before phase I safety trials are initiated.

Recently the protein analysis was carried out by two-dimensional gel electrophoresis. The viable alternative approach is chromatographic separation of

peptides with MS detection. Nanotechnology can provide substantial benefits to the drug discovery process by improving the understanding of materials/chemicals at the cellular/molecular level, enhanced detection, increased throughput, shorter time for the discovery of new drugs and precise use (nano litre scale) of precious reagents required to carry out screening of potential drugs, etc.

Nano particles of Quantum Dots (QDs), Gold colloids and nano shells are used in detection of different biological components like proteins, strands of DNA with specific colours, immunoglobulin in whole blood, that possess the targets in drug discoveries.<sup>2-5</sup>

In new drug discovery process the analytical R&D and analytical chemist play a key role. In R&D the chemists are involved in a wide range of activities like,

- Characterization, optimization, selection of new drug candidates in the interface between drug discovery and the early phase of drug development.
- Development and analysis of new formulations and drug delivery systems.
- New analytical method development for the Active Pharmaceutical Ingredient (API)
- Predicting shelf life of products
- Development and optimization of new formulas and technical processes for the pharmaceutical formulations required for clinical trials and the market.<sup>6</sup>

### **The method development and validation process for pharmaceutical dosage form**

Analytical methods development and validation play important roles in the new drug discovery, development and manufacture of pharmaceuticals.

The steps of method development and method validation depend upon the type of method being developed. However, the following steps are common to most types of analytical methods.

- Method development plan definition
- Background information gathering (nature of the sample, size of the sample, proportion of the constituent to be determined)
- Laboratory method development
- Generation of test procedure
- Methods validation protocol definition
- Laboratory methods validation

- Validated test method generation
- Validation report

A well-developed method should be easy to validate. A method should be developed with the goal to rapidly test preclinical samples, formulation prototypes and commercial samples. Ideally, the validation protocol should be written only following a thorough understanding of the method's capabilities and intended use. The validation protocol will list the acceptance criteria that the method can meet. Any failure to meet the criteria will require that a formal investigation be conducted. The required validation parameters, also termed analytical performance characteristics, depend upon the type of analytical method.

Pharmaceutical analytical methods are categorized into five general types:

- Identification tests
- Potency assays
- Impurity tests: quantitative
- Impurity tests: limit
- Specific tests

The first four tests are universal tests, but the specific tests such as particle-size analysis and x-ray diffraction are used to control specific properties of the API or the drug product.<sup>4</sup>

Recent progress in method development has been largely based on the result of improvements in analytical instrumentation. This is especially true for chromatographs and detectors. Isocratic and gradient reverse-phase HPLC have evolved as the primary techniques for the analysis of non-volatile APIs. The HPLC detector of choice for many types of methods development is the photo-diode array (PDA) detector because it can be used for both quantitative and qualitative analysis. The use of a PDA detector to determine peak purity of the active ingredient in stressed samples greatly facilitates the development of stability-indicating assays.

Gas chromatography remains the method of choice for the analysis of volatile compounds. Gas chromatography with mass spectroscopy detection is increased being used to identify impurities and to determine active ingredient peak purity in stressed samples. Advances in the use of non-destructive infrared (IR) and near infrared (near IR) spectroscopy and NMR techniques are particularly for method development scientists.

The emphasis on the identification of trace impurities and degradants has led to the increased use of hyphenated techniques such as liquid chromatography – mass spectroscopy (LC-MS) and liquid chromatography – nuclear magnetic resonance spectroscopy (LC-NMR).<sup>9</sup>

According to ICH guidelines the parameters including *specificity*, *accuracy*, *linearity*, *precision*, *range*, *detection limit*, *quantification limit*, and *robustness* are required to validate a newly developed method.

Only specificity is needed for an identification test. However, the full range of specificity, accuracy, linearity, and range, limit of detection (LOD), limit of quantification (LOQ), precision and robustness testing is needed for more complex methods such as quantitative impurity methods.

### **Challenges in method development**

Common challenges that are encountered during method development and validation are

- Lacking in robustness and the loss of efficiency during routine QC testing.
- No sound knowledge in design and execution of forced - degradation stability studies.
- Lack in up-gradation of current regulatory standards
- Insufficient monitoring and training programs.<sup>4,7</sup>

### **Validation**

The word “Validation” means “Assessment” of validity or action of proving effectiveness.<sup>13</sup>

### **Definitions**

FDA defines validation as “establish the documented evidence which provides a high degree of assurance that a specific process will consistently produce a product of predetermined specifications and quantity attributes”.

EUGMP define validation as “action of proving in accordance with the principle of Good Manufacturing Practice (GMP), that any material, activity or system actually lead to expected result”.

AUSTRALIAN GMP defines validation as “the action of proving that any material, process, activity, procedure, system, equipment or mechanism used in

manufacture or control can and will be reliable and achieve the desired and intended result”.

### **Analytical Method Validation**

Method validation is the documented successful evaluation of an analytical method that provides a high level of assurance that such method will consistently yield results that are accurate within previously established specifications.

### **Types of Validation**

The following are frequently required to be validated on a pharmaceutical process; Equipment, Environment, Materials, Methods, Controls, Process, Personnel's, Facilities and Operating procedure. Based on these, the validation program comprises,

- Equipment validation
- Facility validation
- Process validation
- Cleaning validation
- Analytical method validation

### **Analytical Method Validation**

Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Analytical testing of a pharmaceutical product is necessary to ensure its purity, stability, safety and efficacy. Analytical method validation is an integral part of the quality control system.

Although a thorough validation cannot rule out all potential problems, the process of method development and validation should address the most common ones.

### **Parameters used for assay validation**

The validation of the assay procedure was carried using the following parameters;

#### **1. Specificity**

##### **Definition**

Specificity is the ability to assess unequivocally the analyte in the presence of impurities, degradants, matrix etc (components) which may be expected to be present.

Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedures.

### **Determination**

The demonstration of specificity requires that the procedure is unaffected by the presence of impurities or excipients. In practice this can be done by spiking the drug substance or product with appropriate levels of impurities or excipients and demonstrating that the assay result is unaffected by the presence of these extraneous materials.

### **ICH Requirement**

The ICH documents state that when chromatographic procedures are used, representative chromatograms should be presented to demonstrate the degree of selectivity, and peaks should be appropriately labeled. Peak purity tests (e.g., using diode array or mass spectrometry) may be useful to show that the analyte chromatographic peak is not attributable to more than one component.

## **2. Accuracy**

### **Definition**

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the value found.

### **Determination**

In case of assay of a drug in a formulated product, accuracy may be determined by application of the analytical method to synthetic mixtures of the drug product components to which the known amount of analyte have been added within the range of the method. If it is not possible to obtain all drug product components, it may be acceptable either to add known quantities of the analyte to the drug product or to compare results with those of a second, well characterized method, the accuracy of which has been stated or defined.

### **ICH Requirements**

The ICH documents recommended that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels,

covering the specified range (i.e., three concentrations and three replicates of each concentration).

### **3. Precision**

#### **Definition**

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

#### **Determination**

The precision of an analytical method is determined by assaying a sufficient number of aliquots of a homogeneous sample to be able to calculate statistically valid estimates of standard deviation or relative standard deviation.

#### **ICH Requirements**

The ICH documents recommended that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure (i.e., three concentrations and three replicates of each concentration or using a minimum of six determinations at 100 % of the test concentration).

### **4. Linearity**

#### **Definition**

The linearity of an analytical procedure is its ability (within a given range) to obtain the test results which are directly proportional to the concentration (amount) of analyte in the sample.

#### **Determination**

Linearity of an analytical procedure is established, using a minimum of five concentrations. It is established initially by visual examination of a plot of signals as a function of analyte concentration or content. If there appears to be a linear relationship, test results are established by appropriate statistical methods. (i.e., by calculation of a regression line by the method of least squares).

## **ICH Requirements**

ICH recommends that, for the establishment of linearity, a minimum of five concentrations normally be used. It is also recommended that the following minimum specified ranges should be considered.

Assay of drug substance (or a finished product): from 80% to 120% of the test concentration.

## **5. Limit of Detection (LOD)**

### **Definition**

LOD is the lowest concentration of the substance that the method can detect but not necessarily quantify. LOD simply indicates that the sample is below or above a certain level.

### **Determination**

For non-instrumental methods, the detection limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

### **ICH Requirement**

The ICH describes a common approach, which is to compare measured signal from samples with known low concentrations of analyte with those of blank samples. The minimum concentration at which the analyte can reliably be detected is established.

Typically acceptable signal-to-noise ratios are 2:1 or 3:1.

## **6. Limit of Quantification (LOQ)**

### **Definition**

LOQ is the lowest concentration of the substance that can be estimated quantitatively with acceptable precision, accuracy and reliability by the proposed method. LOQ is determined by analysis of samples containing decreasing known quantity of the substance and determining the lowest level at which acceptable level of accuracy and precision is attained.

### **ICH Requirement**

The ICH describes a common approach, which is to compare measured signal from samples with known low concentrations of analyte with those of blank samples.

The minimum concentration at which the analyte can reliably be quantified is established. Typical acceptable signal-to-noise ratios are 10:1.

## **7. Range**

### **Definition**

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

### **Determination**

The range of the method is validated by verifying that the analytical method provides acceptable precision, accuracy and linearity when applied to samples containing analyte at the extremes of the range as well as within the range.

## **8. Robustness**

### **Definition**

The robustness of an analytical procedure is a measure of its capacity to remain unchanged by small but deliberate variations in methods parameters and provides an indication of its reliability during normal usage.

### **Determination**

The robustness of method is determined by performing the assay by deliberately altering parameters (-change in flow rate-  $\pm 10\%$ , change in pH of mobile phase-  $\pm 0.2\%$ , change in wave length detection-  $\pm 5\text{nm}$ ) that the results are not influenced by the changes in the above parameters.

## **9. Ruggedness**

### **Definition**

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

### **Determination**

The ruggedness of an analytical method is determined by the analysis of aliquots from homogeneous lots in different laboratories, by different analysts, using

operational and environmental condition that may differ but are still within the specified parameters of the assay. The degree of reproducibility of the result is then determined as a function of assay variables. This reproducibility may be compared to the precision of assay under normal condition to obtain a measure of the ruggedness of the analytical method.

### **High performance liquid chromatography (HPLC)**

High performance liquid chromatography (HPLC) developed during 1960s as a direct offshoot of classical column liquid chromatography through improvements in the technology of columns and instrumental components (pumps, injection valves and detectors). Advantages of HPLC over traditional low-pressure column liquid chromatography include,

1. Speed and improved resolution
2. Greater sensitivity
3. Re-usable columns
4. Ideal for ionic species and large molecules (substances of low volatility)
5. Easy sample recovery<sup>2,9,10</sup>

HPLC has many modes (adsorption, partition, ion-exchange, ion-pair, size-exclusion, chiral, affinity) of separation which are selected based on component nature and type of analysis. Though many modes are available in HPLC, the RP-HPLC is widely used in pharma industry as most of API is polar in nature.

### **Method development in RP-HPLC**

For reverse phase chromatography (RPC), the stationary phase is less polar than the mobile phase. The mobile phase is a mixture of organic and aqueous phase. In RPC, the solutes are eluted in order of their decreasing polarities. These are prepared by treating the surface of silanol group with an organo-chloro silane reagent. It includes,

Retention in RP-HPLC

Selectivity in RP-HPLC

The RPC retention of a compound is determined by its polarity, experimental conditions, mobile phase, column, temperature<sup>3,4</sup>

Three main variables can be used in RPC to change selectivity ( $\alpha$ ) for neutral samples, i.e.

Mobile phase composition

Solvent strength selectivity

Solvent type selectivity

Column type

Temperature

The four primary techniques for quantification are,

- i. Normalized peak area method
- ii. External standard method
- iii. Internal standard method
- iv. Method of standard addition
- v. Calibration by standards<sup>3,4</sup>

### **Normalized peak area method**

The area percent of any individual peak is referred to the normalized peak area. This technique is widely used to estimate the relative amounts of small impurities or degradation compounds in a purified material and in this method; the response factor for each component is identical.

### **External standard method**

This method includes injection of both standard solution and unknown sample. The concentration of unknown is determined graphically from a calibration plot or numerically using response factors.

A response factor ( $R_f$ ) (sometimes called a sensitivity factor) can be determined for each standard as follows.

$$R_f = \frac{\text{Standard area (peak height)}}{\text{Standard concentration}}$$

The external standard approach is preferred for most samples in HPLC that do not require extensive sample preparation. For good quantitation using external standards, the Chromatographic conditions must remain constant during the separation of all standards and samples. External standards are often used to ensure that the total Chromatographic system (equipment, column conditions) is performing properly and can provide reliable results.

### **Internal standard method**

The internal standard is a different compound from the analyte but one that is well resolved in separation. The internal standard should be chosen to mimic the behaviour of the sample compound. One of the main reasons for using internal standard is for samples requiring significant pre-treatment or preparation.

### **Method of standard addition**

The method of standard addition can be used to provide a calibration plot for quantitative analysis. It is most often used in trace analysis. An important aspect of the method of standard addition is that the response prior to spiking additional analytes should be high enough to provide reasonable S/N ratio (>10), otherwise the result will have poor precision.<sup>5,8</sup>

### **Calibration by standards**

Calibration curves for each component are prepared from pure standards, using identical injection volumes of operating conditions for standards and samples. The concentration of solute is read from its curve if the curve is linear.

$$X = K \times \text{Peak Area}$$

Where, X = Concentration

K = Proportionality constant

In this evaluation method, only the area of the peaks of interest is measured. Relative response factors must be considered when converting areas to volume and when the response of a given detector differs for each molecular type of compounds.

#### **LITERATURE REVIEW**

1. Jadhav PD, Laddha KS, Estimation of gallic acid and ellagic acid from Terminalia chebula Retz, Indian Drugs, 41 (4), April 2004, 200-206<sup>26</sup>.

They described a RP-HPLC method to estimate the gallic acid and ellagic acid in Terminalia chebula, fruit extract, by using acetonitrile: water (0.1% O-phosphoric acid) in the ratio of (2: 8) v/v. the peak of gallic acid and ellagic acid were eluted at 2.325 and 5.75 min respectively.

2. Gulcan O, Nilgun GB, A direct RP-HPLC determination of phenolic compounds in Turkish Red Wines, Akdeniz Universitesi Ziraat Fakultesi Dergisi, 19 (2), 2006, 229-234.

They described a RP-HPLC method to determine phenolic compounds including gallic acid using acetic-water: methanol (2: 98) v/v. The peak of gallic acid was eluted at 6.17 minute<sup>14</sup>.

3. Carlin AW, Soong YY, Philip JB, Method development for the optimization of solid-phase extraction of mango seed kernel extracts.

They described a method for analyzing gallic acid and ellagic acid using HPLC. 90 % v/v methanol acidified with 2% v/v acetic acid was used as mobile phase. A flow rate of 1 ml/min was used<sup>15</sup>.

4. Liudas I, Valdas J, Jolita R, Audronis L, Algirdas B, Evaluation of phenolic acids and polypropanoids in the crude drugs, *Medicina*, (Kaunas), 44(1), 2008, 48-55.

They described a method for determining gallic acid using HPLC. In this method, methanol and 0.5% v/v acetic acid in water was used as mobile phase. Gallic acid was eluted at 3.9 min<sup>16</sup>.

5. Jaganathan NS, Kannan K, HPTLC method for estimation of ellagic acid and gallic acid in Triphalachurnam formulation, *Research J. of Phytochemistry*, 2 (1), 2008, 1-9.

They described a HPTLC method using toluene: ethyl acetate: formic acid: methanol (3: 3: 0.8: 0.2 % v/v). The detection was done at 280 nm. The R<sub>f</sub> values were 0.47 and 0.56 for ellagic acid and gallic acid respectively.<sup>17</sup>

6. Jiri D, et al., Rapid detection of gallic acid esters using centrifugal chromatography, *J. of Chrom*, 11 (1963), 419-420.

They suggested a centrifugal chromatographic method for the determination of gallic acid and other ethyl gallates using many mobile phase combinations.<sup>18</sup>

7. Ying JW, Lian WQ, Ping L, Hou WL, Ling Y, Liang H, Sheng, Improved quality control method for Fufang Danshen preparation through simultaneous determination of phenolic acids, saponins and diterpenoid quinines by HPLC coupled with diode array and evaporative light scattering detectors, *Journal of Pharmaceutical and Biomedical Analysis*, 45 (5) 2007, 775-784.

In this they developed an improved quality control method for those herb medicines. A simultaneous separation and quantification of the 12 components was performed on a C-18 column, in which the mobile phase consisted of (A) 0.1% aqueous formic acid and (B) acetonitrile using a gradient elution and the wavelength at 281nm and ELSD temperature at 131 °C, at flow rate 3.1L/min, and the gain =4<sup>19</sup>.

8. Qu JX, Wenquan W, Yanqing Z, Yin B, Yang, Simultaneous analysis of glycyrrhizin, paeoniflorin, quercetin, ferulic acid, liquiritin, formononetin, benzoic acid and isoliquiritigenin in the Chinese proprietary medicine by HPLC, *Journal of Pharmaceutical and biomedical analysis*, 45 (3), 2007, 450-455.

In this analysis they performed by reverse phase gradient elution, used an aqueous mobile phase (contain 0.1% phosphoric acid) modified by acetonitrile and detection made simultaneously at four wavelengths<sup>20</sup>.

9. Susanne K, Ulrich HE, Determination of flavonols, theogallin gallic acid and caffeine in tea using HPLC, *Zeitschrift fur lebensmitteluntersuchung und-forschung A*, 192, 6, 1991, 526-529.

In this method HPLC separation was performed on a hypersil- ODS column using a gradient method with 2 % acetic acid (aq) and acetonitrile as solvents<sup>21</sup>.

10. Yean YS, Philip BJ, Quantification of gallic acid and ellagic acid from longan (*Dimocarpus Longan* Lour.) seed and mango (*mangifera indica* L.) kernel and their effects on anti-oxidant activity, *Food Chemistry*, 97(3), 2006, 524-530<sup>23</sup>.

11. Huaifu W, Keith H, Xiaoqing Y, Isocratic elution system for the determination of catechins, caffeine and gallic acid in green tea using HPLC, *Food Chemistry*, 68, (1), 2000, 115-121<sup>22</sup>.

## **AIM AND PLAN OF WORK**

### **Aim**

Ayurveda, the ancient system of herbal medicine is growing popular worldwide, but a number of factors such as standardization and stability studies are still at an infant stage for an ayurvedic product. The drug analysis plays an important role in the development of drugs, their manufacture and therapeutic use. These drugs may be in single component or multi-component dosage forms. The multi-component dosage forms prove to be effective due to the combined mode of action on the body. The complexity of dosage forms including multiple drug entities possesses considerable

challenge to the analytical chemist during the development of assay procedures for ayurvedic formulations.

For the estimation of drugs present in multi-component ayurvedic formulations, chromatographic methods are considered to be more suitable than spectrophotometric methods due to its very high sensitivity.

The present study deals with the development of new RP-HPLC method for the estimation of gallic acid and ellagic acid in the triphala churnam tablet formulation and then validation of the method as per ICH guidelines (International Conference on Harmonization of Technical Requirement for Registration of Pharmaceuticals for Human use).

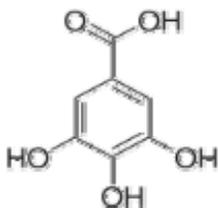
### **Plan**

1. Study the physicochemical properties of drug (pH, solubility and molecular weight).
2. Selection of chromatographic conditions (mobile phase, column, flow rate, etc.)
3. Optimization of the method.
4. Study of system suitability parameters.
5. Validation of proposed method.
6. Applying developed method to marketed formulation.

## **DRUG PROFILE**

### **GALLIC ACID<sup>26-28</sup>**

#### **Chemical structure**



**Molecular formula:** C<sub>7</sub>H<sub>6</sub>O<sub>5</sub>.

**IUPAC name:** 3, 4, 5-Trihydroxybenzoic acid.

**Molecular weight:** 170.12  
**Density:** 1.7g/cm<sup>3</sup>(anhydrous)  
**Melting point:** 250 °C, 523 K, 482 °F.  
**Stability:** Stable under ordinary conditions of use and storage.

**Description:**

Pure gallic acid is colorless crystalline organic powder. Gallic acid occurs as a free molecule or as part of a tannin molecule.

**Distribution:**

Gallic acid is found in almost all plants. Plants known for their high gallic acid content include gallnuts, grapes, tea, hops and oak bark.

**Protein binding:**

Gallic acid does not combine with protein and has therefore no astringent taste.

**Solubility in water:**

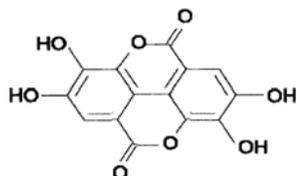
It is freely soluble in water at 20 °C and soluble in methanol (when gallic acid treated with methanol it undergoes methanolysis and forms methylgallates).

**Properties:**

Gallic acid seems to have anti-fungal and anti-viral properties. Gallic acid acts as an antioxidant and helps to protect our cells against oxidative damage. Gallic acid was found to show cytotoxicity against cancer cells, without harming healthy cells. Gallic acid is used as a remote astringent in case of internal haemorrhage. Gallic acid is also used to treat albuminuria and diabetes. Some ointment to treat psoriasis and external haemorrhoids contains gallic acid.

**ELLAGIC ACID**<sup>29-33</sup>

**Chemical structure**



**Molecular formula:** C<sub>14</sub>H<sub>6</sub>O<sub>8</sub>

**IUPAC name:** 4, 4', 5, 5', 6, 6'-hexahydrodiphenic acid 2, 6, 2', 6'-di-lactone

**Molecular weight:** 302.197

<b>Description:</b>	Gray to slightly beige crystalline powder
<b>Density:</b>	1.67 g/cm <sup>3</sup>
<b>Melting point:</b>	> 350 °C
<b>Solubility:</b>	Soluble in methanol, and slightly soluble in water.
<b>Stability:</b>	Stable under normal conditions.
<b>Purity (HPLC):</b>	95.0% min

**Properties:**

Ellagic acid has antioxidant, anti-mutagen and anti-cancer properties. Studies have shown the anti-cancer activity on cancer cells of the breast, oesophagus, skin, colon, prostate and pancreas. More specifically, ellagic acid prevents the destruction of P53 gene by cancer cells. Ellagic acid can bind with cancer causing molecules, thereby making them inactive. In their study The effects of dietary ellagic acid on rat hepatic and esophageal mucosal cytochromes P450 and phase II enzymes. Ahn D et al showed that ellagic acid causes a decrease in total hepatic mucosal cytochromes and an increase in some hepatic phase II enzyme activities, thereby enhancing the ability of the target tissues to detoxify the reactive intermediates. Ellagic acid showed also a chemoprotective effect against various chemically induced cancers. A study by Thresiamma KC and Kuttan R.Indian (Indian Journal Physiology and Pharmacology, 1996 October) indicate that oral administration of ellagic acid by rats can circumvent the carbon tetrachloride toxicity and subsequent fibrosis of the liver.

**Mode of action:**

Ellagic acid is a scavenger of cancer-causing chemicals, making them inactive, inhibiting the ability of other chemicals to cause mutations in bacteria. Additionally, Ellagic Acid prevents binding of carcinogens to DNA, and reduces the incidence of cancer in cultured human cells exposed to carcinogens.

## **MATERIALS AND INSTRUMENTS**

### **Instruments**

UV-Visible spectrometer:	Elico-sl 164
HPLC:	Schimadzu, prominence
Sonicator:	Bandelin
Rotatory shaker:	Remi instrument ltd
Centrifuge apparatus:	Remi instrument
Analytical balance:	Metler
P <sup>H</sup> meter:	Elico P <sup>H</sup> meter Li 127

### **HPLC Instrument description:**

Reverse phase HPLC was performed on isocratic HPLC pump.

### **Materials**

Gallic acid marker compound: Indofine, Inc, USA.

Ellagic acid marker compound: Indofine, Inc, USA.

Triphala churnam uncoated tablets 500 mg: Procured from local market

### **Chemicals**

Methanol HPLC grade

Ortho phosphoric acid AR grade

Water Milli-Q-water

### **Method development for assay:**

The plan of work was designed into following parts.

### **HPLC Method Development:**

#### **Material and method employed**

The method development and optimization of chromatographic condition were

Sample type

Solubility

Selection of wavelength

Selection of initial condition for separation

Optimization of chromatographic condition

Effect of ratio of mobile phase and its strength

Effect of column change

## **METHOD DEVELOPMENT**

### **Method development and optimization of chromatographic conditions for Gallic acid:**

#### **Sample type**

Gallic acid is a natural product having a molecular weight of 170.12 and contain COOH group in structure.

#### **Solubility**

Gallic acid is freely soluble in water and soluble in methanol.

When gallic acid is treated with methanol it converts to methylgallate. This phenomenon is suitable for only less amounts of gallic acid. But this is inconvenient for screening large numbers of samples and cannot be employed in labs due to high pressure reaction condition.<sup>26</sup>

Then different dilutions were checked for solubility of gallic acid in water. Finally water was selected to make dilution for assay method in the present work.

### **Selection of wavelength**

The sensitivity of HPLC depends upon proper selection of wave length of detection. An ideal wavelength is one that gives good response for the drug to be detected. In order to determine the proper wave length of gallic acid in diluents, spectra were scanned on UV-Visible spectrometer in the range of 200 nm- 400 nm against diluents as blank. The  $\lambda_{\max}$  was found to be 270 nm.

### **Selection of initial chromatographic conditions**

Appropriate selection of chromatographic method depends upon the characteristic nature of the sample (ionic or ionisable or neutral), its molecular weight and solubility. The nature of gallic acid is organic acid. Hence reverse phase chromatography is used. The reverse phase HPLC was selected for initial chromatographic condition because of its simplicity and suitability.

### **Initial chromatographic conditions**

Columns: 4.6 x 250 mm column containing packing of octadecylsilane chemically bounded to porous silica or ceramic micro particles, of 5  $\mu\text{m}$ .

Temperature: Room temperature

Flow rate: 1 ml/min

Injection volume: 20  $\mu\text{l}$

Wave length: 270 nm

In RP-HPLC pH of the mobile phase ranges in between 2 to 8, in this case pH of gallic acid was 4.5 and according to Handerson Hasselbalch equation pH of the mobile phase should be adjusted above 2 of the pKa value of the drug substance. Here pKa of the gallic acid 4.5 was hence buffer pH was adjusted to 5.1 as 97.5% of gallic acid was ionisable under RP condition and eluted was carried out by using phosphate buffer.

### Mobile phase selection

The main requirement of mobile phase is that it has to dissolve the analytes upto the concentration suitable for detection. Acetonitrile having a UV cut off < 200 nm, refractive index – 1.3441, viscosity- 0.38, boiling point- 81.6 °C, polarity value- 5.8 and solvent miscibility number of 11 as it should not be greater than 17.

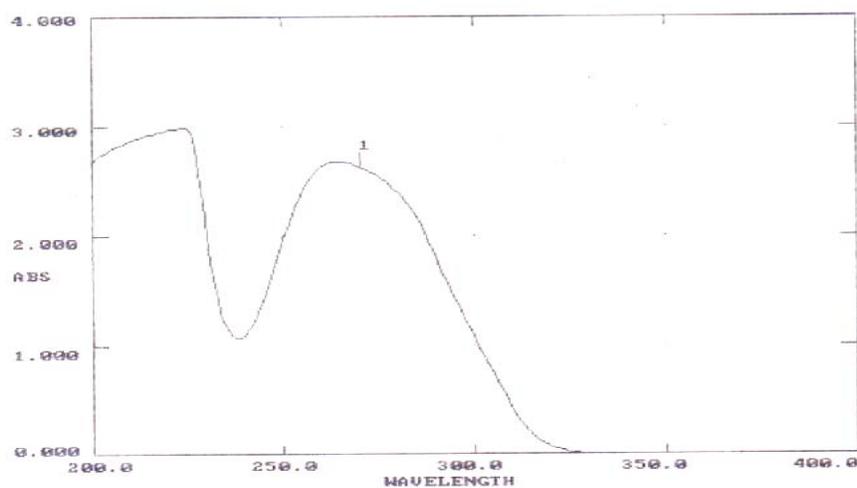
### Selection of buffer

In reverse phase chromatography mobile phase pH values are usually between 2 and 7.2. as gallic acid was ionizable under reverse phase condition having –COOH as a functional group with pKa between 4.5 to 10.

### Selection of column

In this method we were used Symmetry Shield™ RP 18, C 18 4.6 X 250 mm, 5 µm column.

### UV- Spectra



### Trial-I

**Objective:** To develop a method for the assay of gallic acid by HPLC.

**Chromatographic conditions:**

Column: 4.6 X 250 mm column containing packing of octadecyl silane chemically bounded to porous silica or ceramic micro particles, 5 µm.

Column temperature: Room temperature.

Flow rate: 1.0 ml/min.

Injection volume: 20 µl.

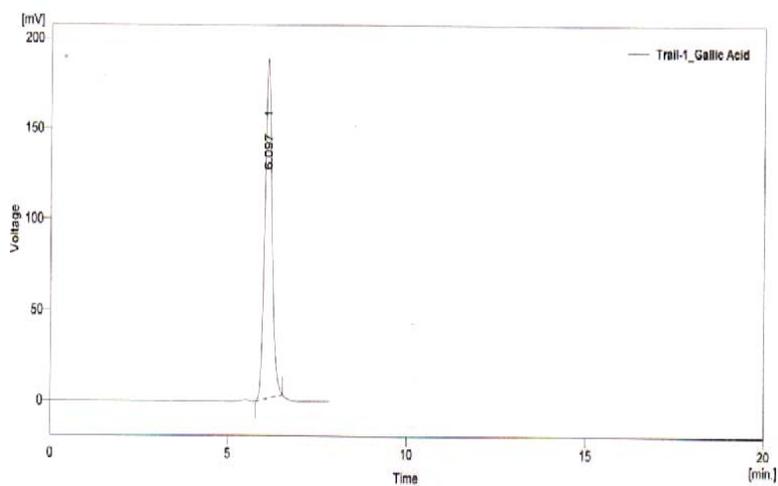
Wavelength: 270 nm.

Mobile phase: Acetonitrile: buffer (0.01 %v/v O-phosphoric acid in water)  
(20: 80).

### Observation

In this trial symmetry shield was taken, gallic acid peak eluted at 6.097<sup>th</sup> minute. Tailing was observed with the analyte peak.

### Chromatogram



### Trial-II

**Objective:** to develop a method for the assay of gallic acid by HPLC.

#### Chromatographic conditions

Column: 4.6 X 250 mm column containing packing of octadecyl silane chemically bounded to porous silica or ceramic micro particles, 5  $\mu$ m.

Column temperature: Room temperature.

Flow rate: 1.0 ml/min.

Injection volume: 20  $\mu$ l.

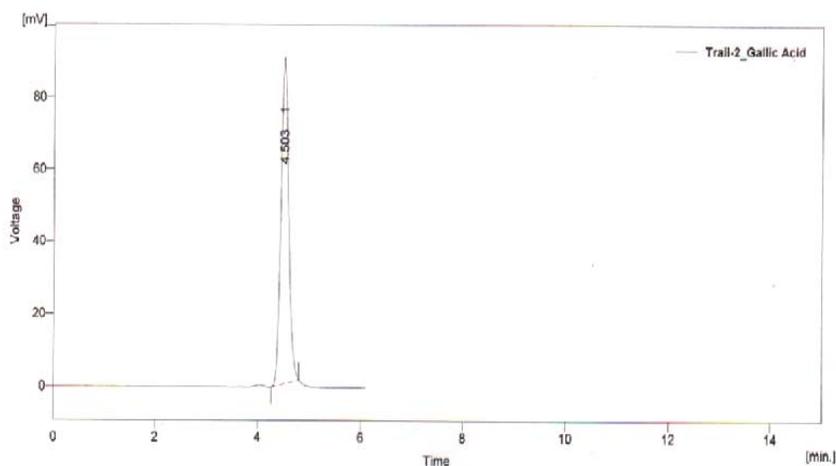
Wavelength: 270 nm.

Mobile phase: Acetonitrile: buffer (0.01 %v/v O-phosphoric acid in water)  
(40: 60).

### Observation

In this trial symmetry shield column C 18 was taken, gallic acid eluted at 4.503<sup>th</sup> minute but tailing also increased to 1.9 and hence gone for further trial as it was not satisfactory.

### Chromatogram



### Trial-III

**Objective:** To develop a method for the assay of gallic acid by HPLC.

#### Chromatographic conditions:

Column: 4.6 X 250 mm column containing packing of octadecyl silane chemically bounded to porous silica or ceramic micro particles, 5 µm.

Column temperature: room temperature.

Flow rate: 1.0 ml/min.

Injection volume: 20 µl.

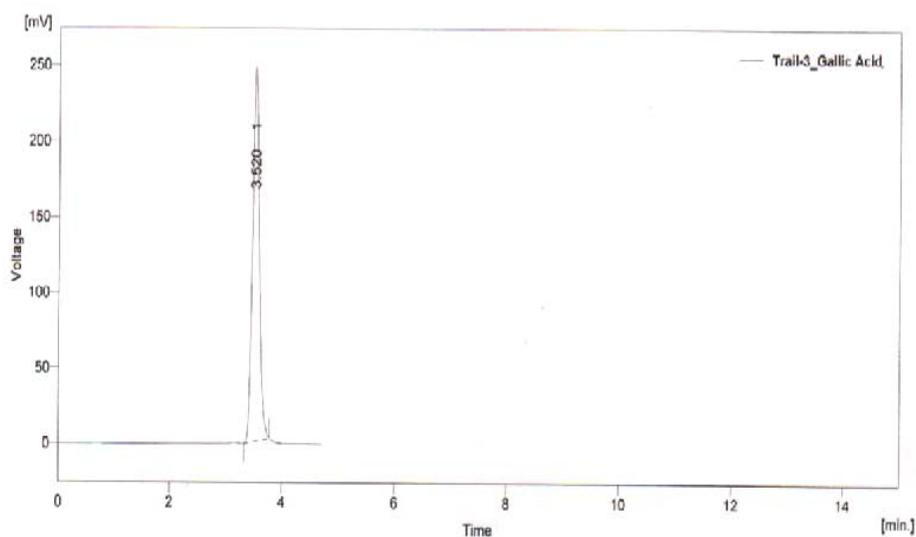
Wavelength: 270 nm.

Mobile phase: Acetonitrile: buffer (0.01 %v/v O-phosphoric acid in water)  
(60: 40).

### Observation:

In this trail we had taken symmetry shield column C18, gallic acid peak was eluted at 3.52<sup>th</sup> minute but elution of solute was not satisfactory hence underwent for further trail.

Chromatogram:



### Trial-IV

**Objective:** to develop a method for the assay of gallic acid by HPLC.

#### Chromatographic conditions:

Column: 4.6 X 250 mm column containing packing of octadecyl silane chemically bounded to porous silica or ceramic micro particles, 5  $\mu$ m.

Column temperature: Room temperature.

Flow rate: 1.0 ml/min.

Injection volume: 20  $\mu$ l.

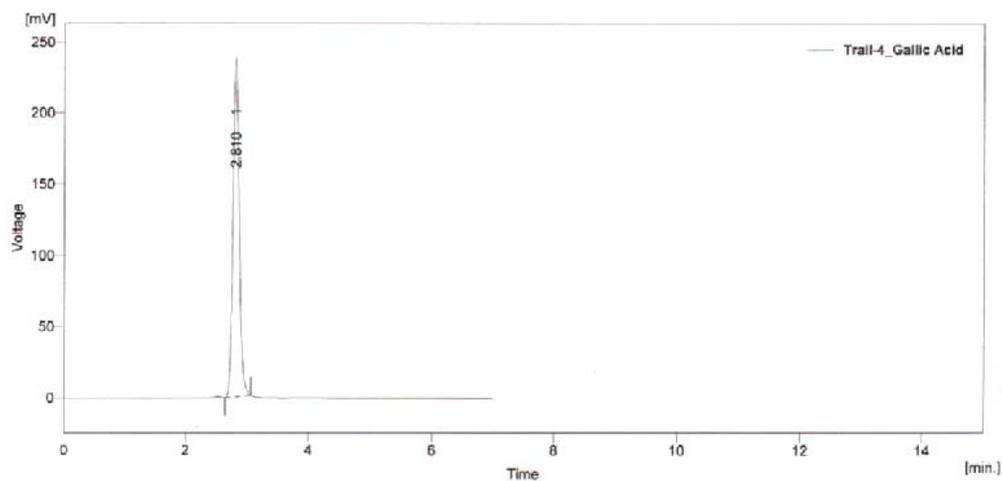
Wavelength: 270 nm.

Mobile phase: Acetonitrile: buffer (0.01 %v/v O-phosphoric acid in water)  
(80: 20).

### Observation:

In this trail we had taken symmetry shield column C-18, the peak was eluted at 2.81<sup>th</sup> minute which showed very sharp peak and hence we proceed further with this method.

### Chromatogram



### Method development and optimization of chromatographic conditions for Ellagic acid

#### Sample type

Ellagic acid is a natural product having a molecular weight 302.197 and contain C=O group in structure.

#### Solubility

According to literature survey, ellagic acid is freely soluble in methanol and slightly soluble in water. Then different dilution of was check for solubility of ellagic

acid in methanol. Finally methanol was selected to make dilution for assay method in the present work.

### **Selection of wavelength**

The sensitivity of HPLC depends upon proper selection of wave length of detection. An ideal wavelength is one that gives good response for the drug to be detected. In order to determine the proper wave length of ellagic acid in diluents, spectra were scanned on UV-Visible spectrometer in the range of 200 nm- 400 nm against diluents as blank. The  $\lambda_{\max}$  was found to be 254 nm.

### **Selection of initial chromatographic conditions**

Appropriate selection of chromatographic method depends upon the characteristic nature of the sample (ionic or ionisable or neutral), its molecular weight and solubility. The nature of ellagic acid is organic acid. Hence reverse phase chromatography is used. The reverse phase HPLC was selected for initial chromatographic condition because of its simplicity and suitability.

### **Initial chromatographic conditions:**

Columns: 4.6 X 250 mm column containing packing of octadecyl silane chemically bounded to porous silica or ceramic micro particles, 5  $\mu\text{m}$ .

Temperature: Room temperature

Flow rate: 1 ml/min

Injection volume: 20  $\mu\text{l}$

Wave length: 254 nm

In RP-HPLC pH of the mobile phase ranges in between 2 to 8, in this case pH of ellagic acid was 5 - 6 and according to Handerson Hasselbalch equation pH of the mobile phase should be adjusted above 2 of the pKa value of the drug substance. Here pKa of the ellagic acid 5-10 was hence buffer pH was adjusted to 5.1 as 95 % of ellagic acid was ionisable under RP condition and eluted was carried out by using phosphate buffer.

### **Mobile phase selection**

The main requirement of mobile phase is that it has to dissolve the analytes upto the concentration suitable for detection. Acetonitrile having a UV cut off < 200 nm,

refractive index – 1.3441, viscosity- 0.38, boiling point- 81.6 °C, polarity value- 5.8 and solvent miscibility number of 11 as it should not be greater than 17.

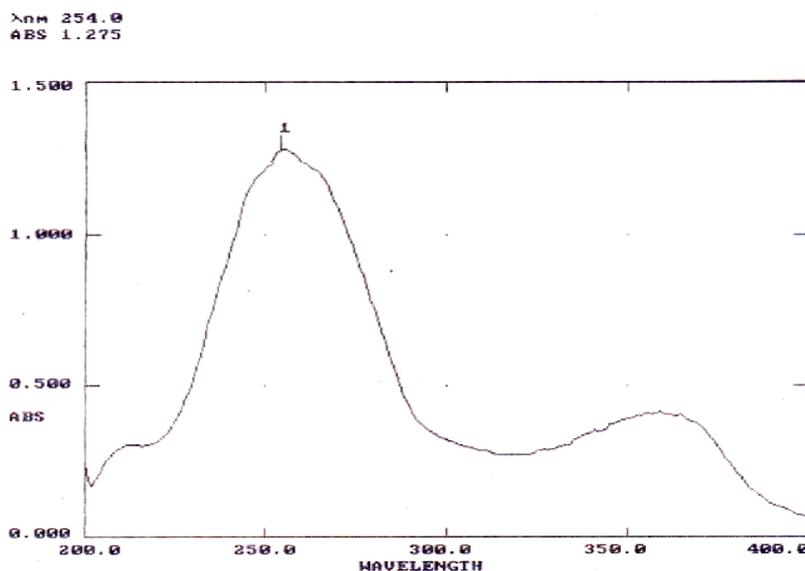
### **Selection of buffer**

In reverse phase chromatography mobile phase pH values are usually between 2 and 7.2. As ellagic acid was ionizable under reverse phase condition having –COOH as a functional group with pKa between 4.5 to 10

### **Selection of column**

In this method we were used Symmetry Shield™ RP 18, C 18 4.6 X 250 mm, 5 µm column.

### **UV- Spectra**



### **Trial-I**

**Objective:** To develop a method for the assay of ellagic acid by HPLC.

#### **Chromatographic conditions**

Column: 4.6 X 250 mm column containing packing of octadecyl silane chemically bounded to porous silica or ceramic micro particles, 5 µm.

Column temperature: Room temperature.

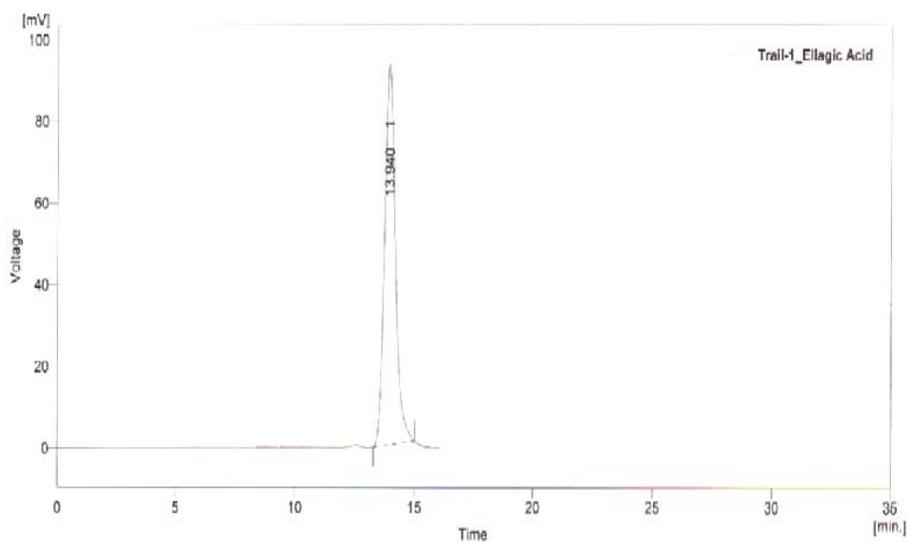
Flow rate: 1.0 ml/min.

Injection volume: 20  $\mu$ l.  
Wavelength: 254 nm.  
Mobile phase: Acetonitrile: buffer (0.01 %v/v O-phosphoric acid in water)  
(20: 80).

### Observation

In this we trail symmetry shield was taken, ellagic acid peak eluted at 13.94<sup>th</sup> minute. Tailing was observed with the analyte peak.

### Chromatogram



### Trial-II

**Objective:** to develop a method for the assay of ellagic acid by HPLC.

**Chromatographic conditions:**

Column: 4.6 X 250 mm column containing packing of octadecyl silane chemically bounded to porous silica or ceramic micro particles, 5  $\mu$ m.

Column temperature: Room temperature.

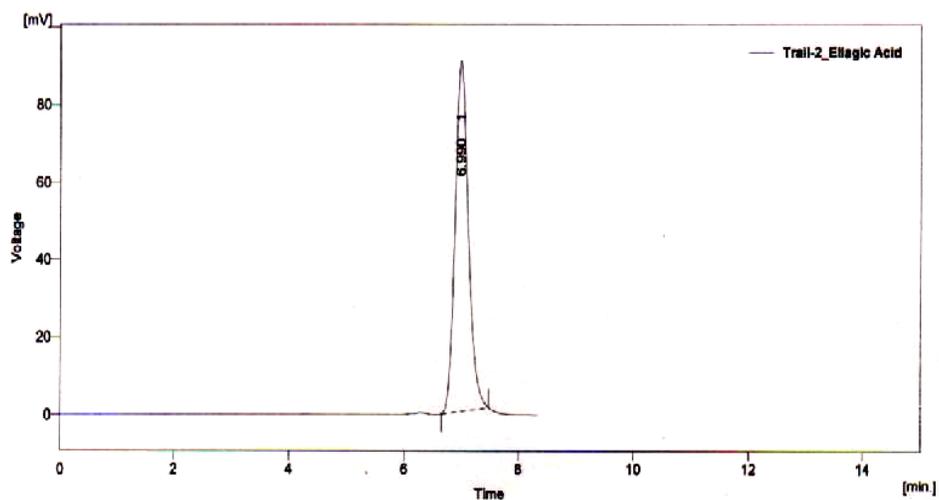
Flow rate: 1.0 ml/min.

Injection volume: 20  $\mu$ l.  
Wavelength: 254 nm.  
Mobile phase: Acetonitrile: buffer (0.01 %v/v O-phosphoric acid in water)  
(40: 60).

### Observation

In this trail symmetry shield column C 18 was taken, ellagic acid eluted at 6.99<sup>th</sup> minute but tailing also increased to 1.5 and hence gone for further trail as it was not satisfactory.

### Chromatogram



### Trial-III

**Objective:** to develop a method for the assay of ellagic acid by HPLC.

**Chromatographic conditions:**

Column: 4.6 X 250 mm column containing packing of octadecyl silane chemically bounded to porous silica or ceramic micro particles, 5  $\mu$ m.

Column temperature: Room temperature.

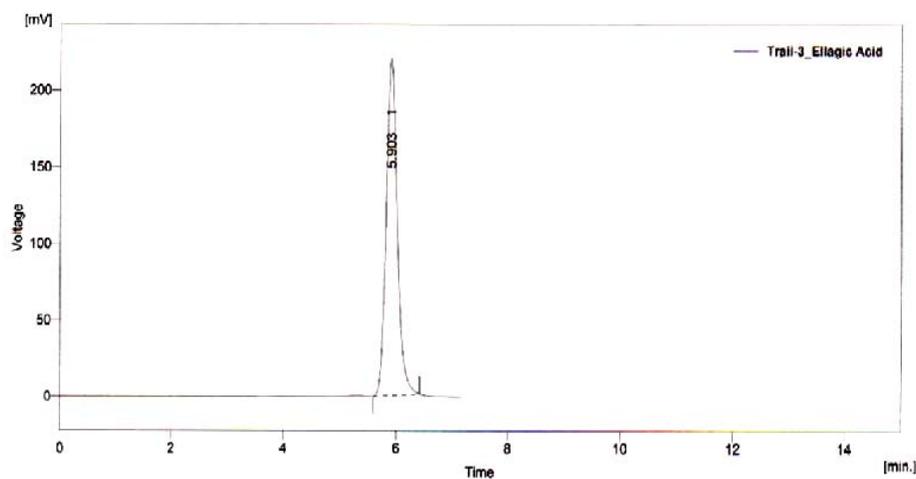
Flow rate: 1.0 ml/min.

Injection volume: 20  $\mu$ l.  
Wavelength: 254 nm.  
Mobile phase: Acetonitrile: buffer (0.01 %v/v O-phosphoric acid in water)  
(60: 40).

### Observation

In this trail we had taken symmetry shield column C18, ellagic acid peak was eluted at 5.903<sup>th</sup> minute but elution of solute was not satisfactory hence underwent for further trail.

### Chromatogram



### Trial-IV

**Objective:** to develop a method for the assay of ellagic acid by HPLC.

#### Chromatographic conditions

Column: 4.6 X 250 mm column containing packing of octadecyl silane chemically bounded to porous silica or ceramic micro particles, 5  $\mu$ m.

Column temperature: Room temperature.

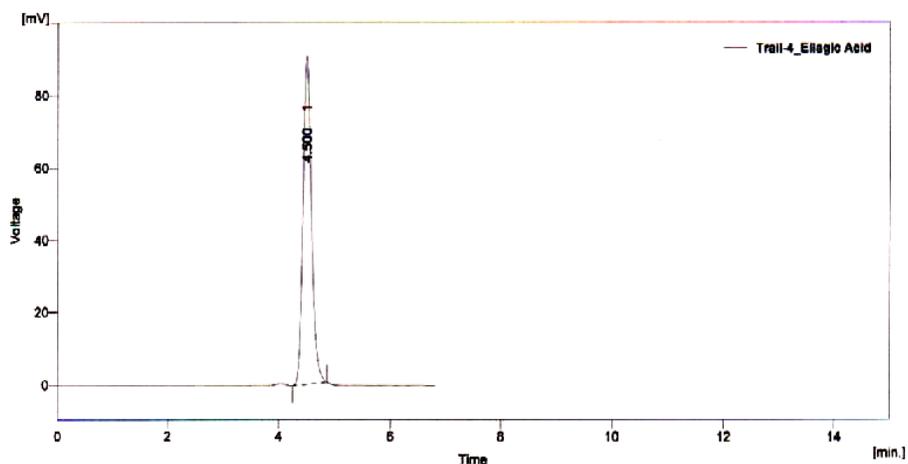
Flow rate: 1.0 ml/min.

Injection volume: 20  $\mu$ l.  
Wavelength: 254 nm.  
Mobile phase: Acetonitrile: buffer (0.01 %v/v O-phosphoric acid in water)  
(80: 20).

### Observation

In this trail we had taken symmetry shield column C-18, the peak was eluted at 4.50<sup>th</sup> minute which showed very sharp peak and hence we proceed further with this method.

### Chromatogram



## QUANTITATION

### For Gallic acid

The fixed chromatographic conditions were applied for the estimation of gallic acid in formulation by RP-HPLC method.

### Preparation of mobile phase:

1 ml of ortho-phosphoric acid was dissolved in 1000 ml of milli-Q-water. Filter and degassed by sonication and mixed the Acetonitrile and buffer in the ratio of 80:20 v/v.

#### **Preparation of standard solution**

10 mg of gallic acid were taken in a 10 ml standard flask and dissolved in 5mL of milli-Q-water. The volume was then made up to 10 ml with milli-Q-water to get a concentration of 1mg/ml. Further dilutions were made by taking 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml, 1 ml and 1.2 ml of working standard solution of gallic acid and then making up the volume to 10 ml with mobile phase. This resulted in concentrations of 20µg/ml, 40µg/ml, 60µg/ml, 80µg/ml, 100µg/ml and 120µg/ml of gallic acid respectively.

#### **Preparation of formulation solution**

20 tablets were weighed and powdered. From that 1.6403 gm of powder was weighed into a 10 ml standard flask and extracted with 10 ml of milli-Q-water by sonication for 20 minutes. From that 0.8 ml was diluted to 10 ml with mobile phase.

#### **Recording of chromatograms**

The standard solutions were injected after a steady baseline was obtained. The standard chromatograms were recorded and the peak areas of standard chromatograms were noted. A calibration graph was plotted using peak area Vs concentration.

Then the formulation solution was injected and amount of gallic acid present in the formulation was calculated from the calibration curve. The amount of gallic acid present in each tablet formulation was found to be 3.06 mg.

#### **For Ellagic acid**

The fixed chromatographic conditions were applied for the estimation of ellagic acid in formulation by RP-HPLC method.

#### **Preparation of mobile phase:**

1 ml of ortho-phosphoric acid was dissolved in 1000 ml of milli-Q-water. Filter and degassed by sonication and mixed the Acetonitrile and buffer in the ratio of 80:

20 v/v.

### **Preparation of standard solution**

10 mg of ellagic acid were taken in a 10 ml standard flask and dissolved in 5mL of methanol. The volume was then made up to 10 ml with methanol to get a concentration of 1mg/ml. Further dilutions were made by taking 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml, 1 ml and 1.2 ml of working standard solution of ellagic acid and then making up the volume to 10 ml with mobile phase. This resulted in concentrations of 20µg/ml, 40µg/ml, 60µg/ml, 80µg/ml, 100µg/ml and 120µg/ml of ellagic acid respectively.

### **Preparation of formulation solution**

20 tablets were weighed and powdered. From that 5.191 gm of powder was weighed into a 10 ml standard flask and extracted with 10 ml of methanol by sonication for 20 minutes. From that 0.8 ml was diluted to 10 ml with mobile phase.

### **Recording of chromatograms**

The standard solutions were injected after a steady baseline was obtained. The standard chromatograms were recorded and the peak areas of standard chromatograms were noted. A calibration graph was plotted using peak area Vs concentration.

Then the formulation solution was injected and amount of ellagic acid present in the formulation was calculated from the calibration curve. The amount of ellagic acid present in each tablet formulation was found to be 0.955 mg.

## **VALIDATION**

### **Validation of the developed method**

#### **For Gallic acid**

The RP-HPLC method was validated in terms of parameters like accuracy, linearity, precision, range, detection limit, quantification limit, robustness, ruggedness, system suitability, etc. For all the parameters percentage relative standard deviation values were calculated.

### System suitability

The system suitability studies were carried out. The parameters include asymmetry factor, column efficiency, number of theoretical plates, etc. These parameters are shown in table.

S. NO:	RT(min)	Peak area	Asymmetry
1	2.733	2570175	1.636
2	2.737	2571245	1.636
3	2.727	2590146	1.636
4	2.723	2580176	1.591
5	2.710	2591215	1.636
AVG	2.726	2580591	1.627
SD	0.016263	10002.25	0.020125
% RSD	0.596605	0.387595	1.236

### Specificity

A study of establish the interferences effecting the elution of peak of gallic acid was conducted. By injecting blank, standard solution and sample solutions to HPLC, the resultant chromatograms were not showing any interference to elution of gallic acid peak at 2.81 min. this indicates that the other ingredients present in the formulation do not interfere in estimation of gallic acid by this method.

### Accuracy

The accuracy of the method was checked by intercepting calibration curve (which is plotted between the area under curve on y- axis and concentration of standard solutions on x-axis) with the sample area under curve which is obtained when injecting the 80 µg/ml, 100 µg/ml and 120 µg/ml standard solutions to HPLC. The % recovery for gallic acid was found to be 98.5%, 99.5% and 99.15% respectively.

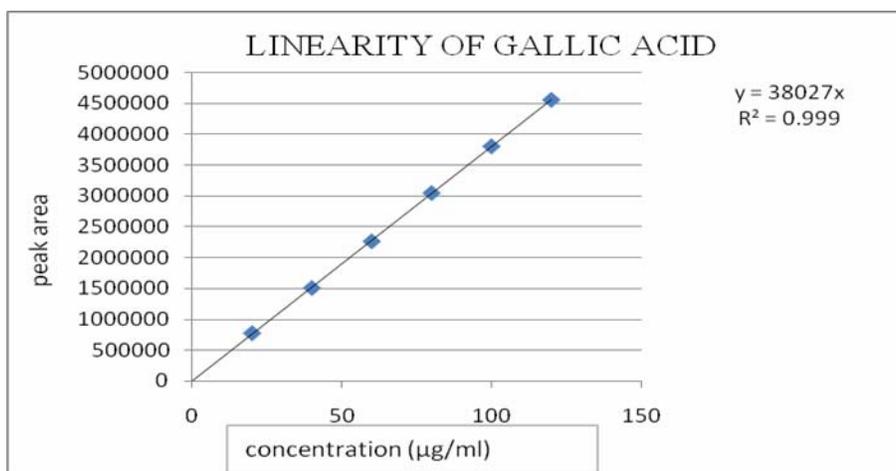
Conc (µg)	Peak area	Average	Recovery (µg)	% Recovery
80	2994344	2995397	78.8	98.5
	2995545			
	2996303			
100	3782275	3782854	99.5	99.5
	3781005			
	3785282			

120	4522803	4523479	118.98	99.15
	4524971			
	4522664			

### Linearity

The standard stock solution was diluted further to get a concentration in the range of 20µg/ml to 120µg/ml of gallic acid. Each concentration was injected in triplicate and the average area was calculated. A calibration curve was plotted using peak area Vs concentration. The calibration plot is shown in figure-1. The correlation coefficient was found to be 0.999.

Conc (µg/ml)	Peak area
20	777838
40	1510959
60	2266923
80	2990856
100	3807734
120	4561012
AVG	2662554
SD	1421352



Correlation coefficient ( $R^2$ ) = 0.999  
Slope = 38027  
Straight line equation (y) = 38027x

### **Precision**

Precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly.

### **Repeatability of injections**

Repeatability studies were done by consequently injecting the standard drug solution of gallic acid. % RSD of peak areas was calculated and shown in the table.

<b>S.No</b>	<b>RT(min)</b>	<b>Peak area</b>
1	2.81	3025854
2	2.81	3024905
3	2.81	3025478
4	2.81	3023431
5	2.813	3025171
AVR	2.8106	3024968
SD	0.001342	0.929064
% RSD	0.047735	0.030713

### **Limit of detection**

The lowest detection limit was calculated from the slope and was found to be 6.13 $\mu$ g/ml.

### **Limit of quantification**

The lowest limit of quantification was calculated from the slope and was found to be 18.57 $\mu$ g/ml.

### **Robustness**

For demonstrating the robustness of the method, the following optimized

conditions were slightly changed. By changing  $\pm 3$  units in temperature (27 °C).

Temp (°C)	RT	Peak area
24	3.117	4364371
27	2.813	3931075
30	2.567	3597353

### **Ruggedness**

For demonstrating ruggedness of the method, the standard drug solution was injected in triplicate by two different analysts.

Analyst	Peak area	Avarage	% RSD
1	3948644	3950977	0.059
2	3953310		

### **For ellagic acid**

The RP-HPLC method was validated in terms of parameters like accuracy, linearity, precision, range, detection limit, quantification limit, robustness, ruggedness, system suitability, etc. For all the parameters percentage relative standard deviation values were calculated.

### **System suitability**

The system suitability studies were carried out. The parameters include asymmetry factor, column efficiency, number of theoretical plates, etc. These parameters are shown in table.

S. No	RT (min)	Peak area	Asymmetry
1	4.533	3247279	1.813
2	4.54	3241193	1.727
3	4.54	3203093	1.727
4	4.55	3182422	1.727
5	4.553	3193179	1.697
AVG	4.5432	3213433	1.7382
SD	0.014142	38254.48	0.043786
% RSD	0.311281	1.190	2.519

### Specificity

A study of establish the interferences effecting the elution of peak of gallic acid was conducted. By injecting blank, standard solution and sample solutions to HPLC, the resultant chromatograms were not showing any interference to elution of gallic acid peak at 2.81 min. this indicates that the other ingredients present in the formulation do not interfere in estimation of gallic acid by this method.

### Accuracy

The accuracy of the method was checked by interception of calibration curve (which is plotted between the area under curve on y- axis and concentration of standard solutions on x-axis) with the sample area under curve (which is obtained when injecting the sample solution to HPLC). The % recoveries for ellagic acid were 98.95%, 100.2%, 100.15%.

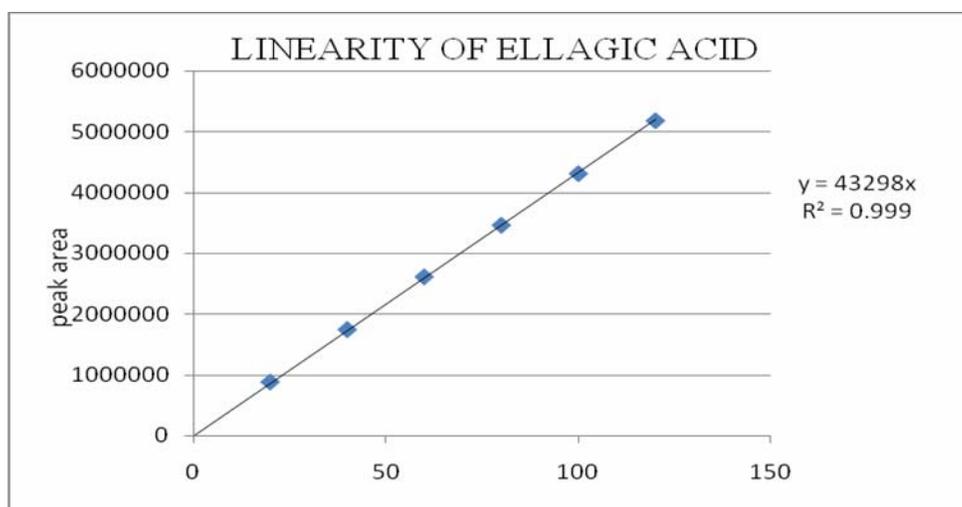
Conc. (µg/ml)	Peak area	Average	Recovery (µg)	% Recovery
80	3426566	3427519	79.16	98.95
	3428197			
	3427794			
100	4335685	4336858	100.2	100.2
	4343386			
	4331503			
120	5204539	5203447	120.18	100.15

	5204397			
	5201406			

### Linearity

The standard stock solution was diluted further to get a concentration in the range of 20µg/ml to 120µg/ml of ellagic acid. Each concentration was injected in triplicate and the average area was calculated. A calibration curve was plotted using peak area Vs concentration. The calibration plot is shown in figure. The correlation coefficient was found to be 0.999.

Conc (µg/ml)	Peak area
20	882749
40	1745738
60	2616383
80	3466229
100	4316739
120	5188323
AVG	3036027
SD	1608430



Correlation coefficient ( $R^2$ ) = 0.999  
Slope = 43298  
Straight line equation  $y = 43298x$

### **Precision**

Precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly.

### **Repeatability of injections**

Repeatability studies were done by consequently injecting the standard drug solution of ellagic acid. % RSD of peak areas was calculated and shown in the table.

<b>S. No</b>	<b>RT (min)</b>	<b>Peak area</b>
1	2.81	3025854
2	2.81	3024905
3	2.81	3025478
4	2.81	3023431
5	2.813	3025171
AVG	2.8106	3024968
SD	0.001342	929.0644
% RSD	0.047735	0.0307

### **Limit of detection**

The lowest detection limit was calculated from the slope and was found to be 3.744 $\mu$ g/mL.

### **Limit of quantification**

The lowest limit of quantification was calculated from the slope and was found to be 16.84 $\mu$ g/mL.

### **Robustness**

For demonstrating the robustness of the method, the following optimized conditions were slightly changed. By changing the temperature (27<sup>0</sup> c)  $\pm$ 3 units.

Temp (°C)	RT (min)	Peak area
24	5.417	5538435
27	4.547	4728177
30	3.917	4103664

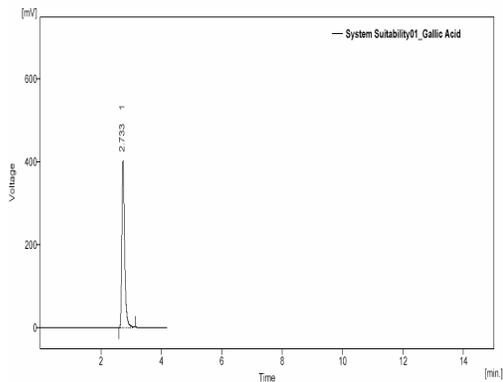
### Ruggedness

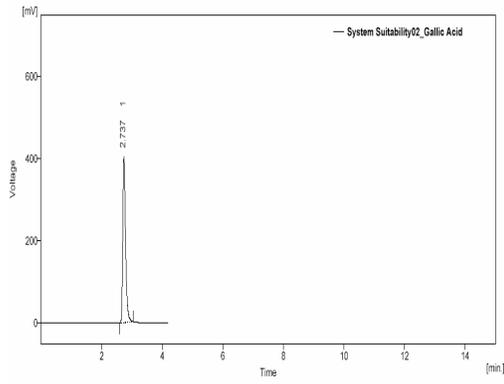
For demonstrating ruggedness of the method, the standard drug solution was injected in triplicate by two different analysts.

Analyst	Peak area	Avarage	% RSD
1	8809092	8807978	0.01788
2	8806864		

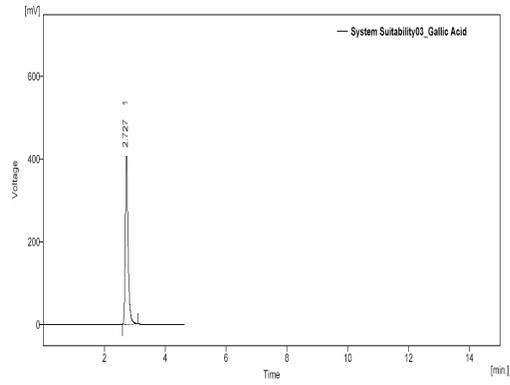
## CHROMATOGRAMS

### For Gallic acid System suitability

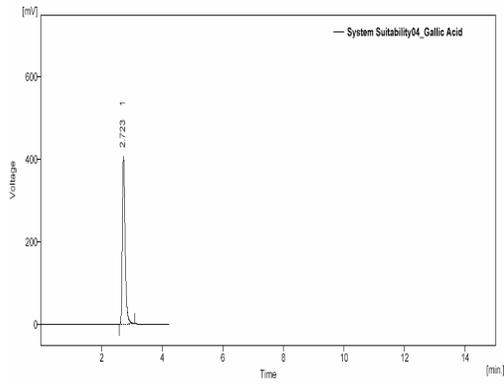




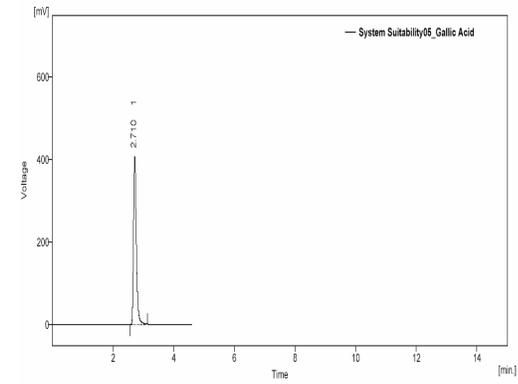
**inj-01\_System suitability**



**inj-02\_System suitability**

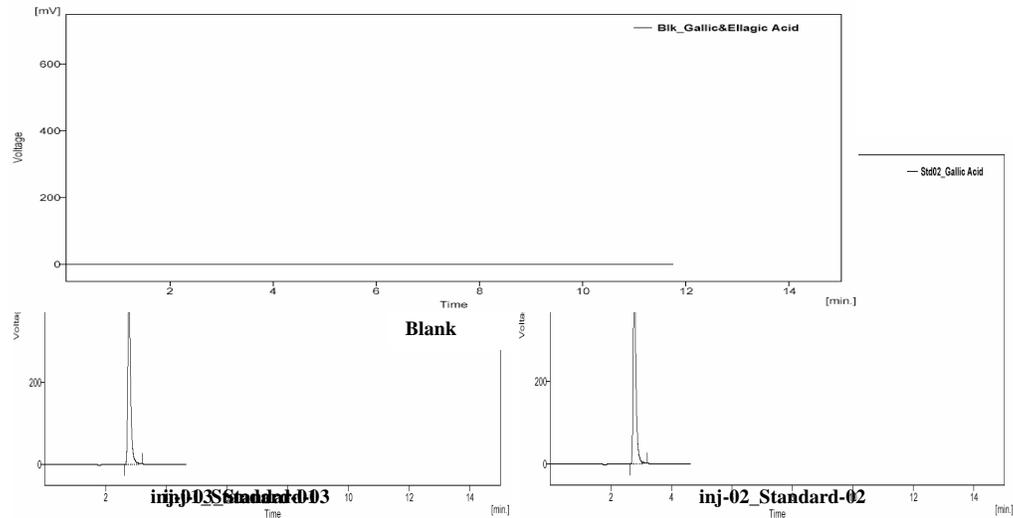


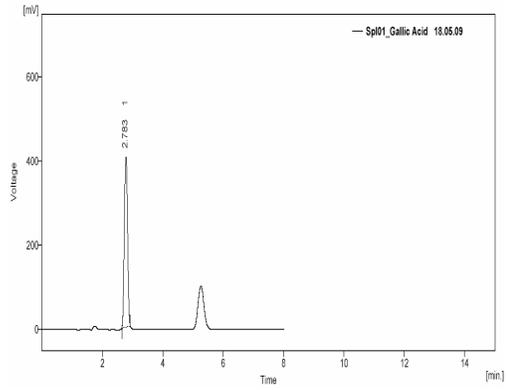
**inj-03\_System suitability**



**inj-05\_System suitability**

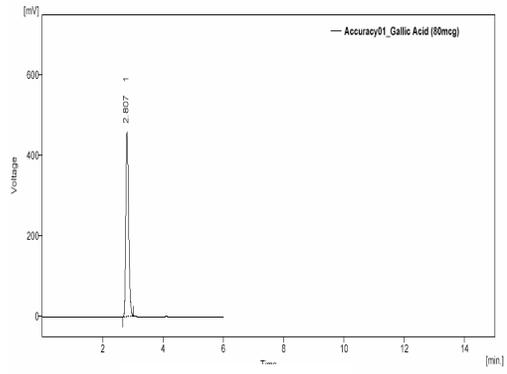
**Specificity:**



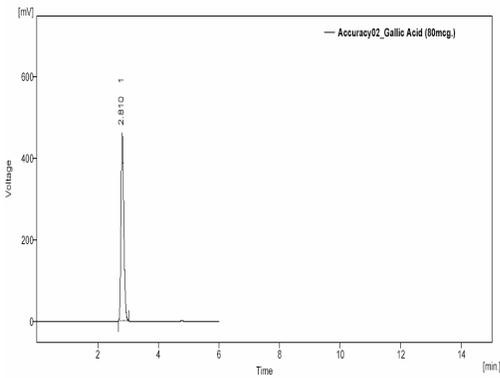


**inj-01\_Sample**

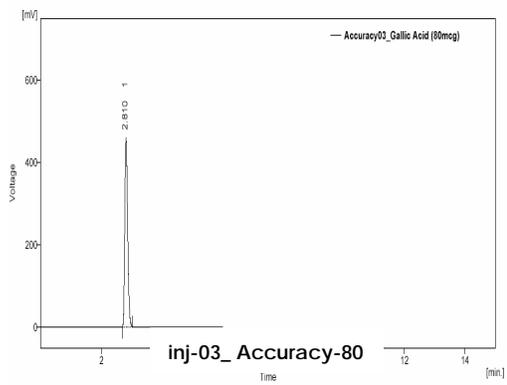
**Accuracy:**



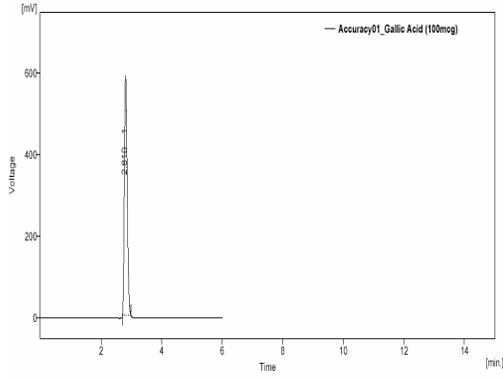
**inj-01\_Accuracy-80**



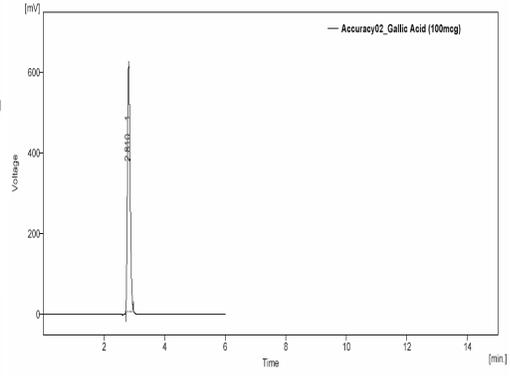
**inj-02\_Accuracy-**



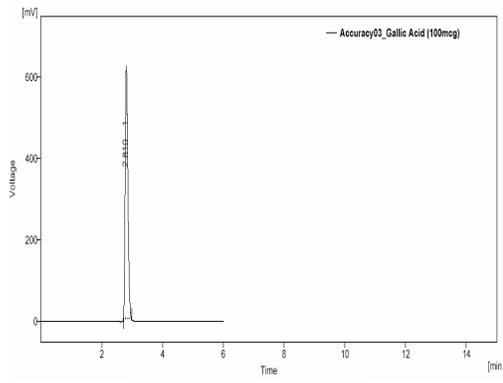
**inj-03\_Accuracy-80**



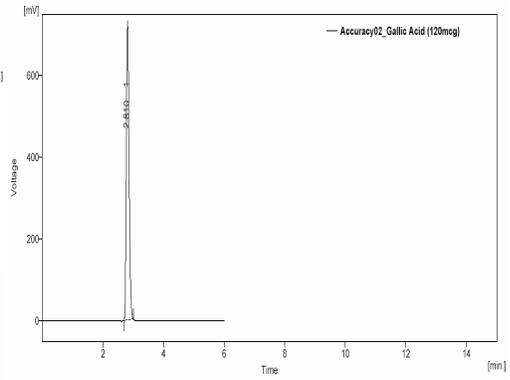
inj-01\_Accuracy-100



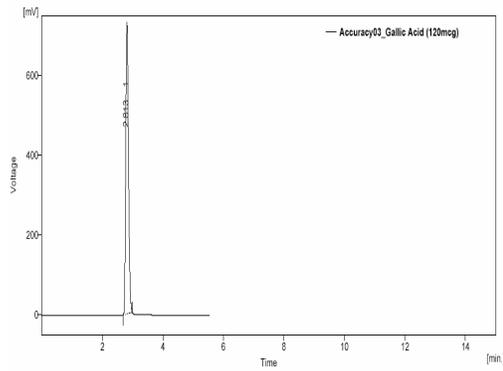
inj-02\_Accuracy-100



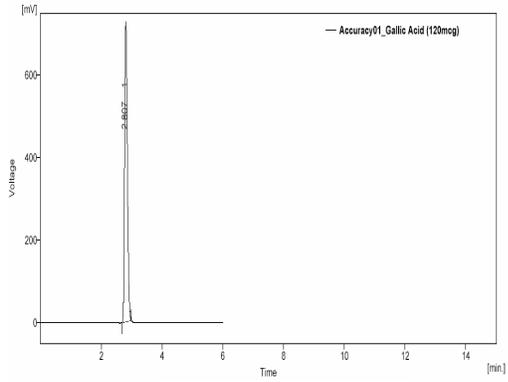
inj-03\_Accuracy-100



inj-01\_Accuracy-

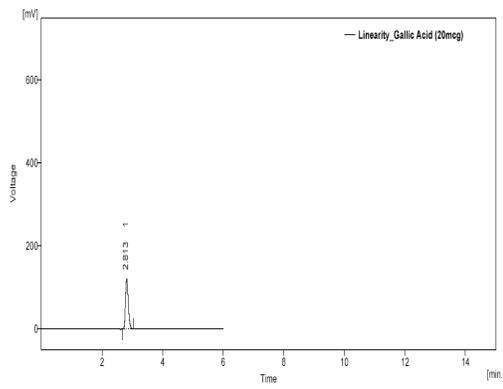


inj-02\_Accuracy-120

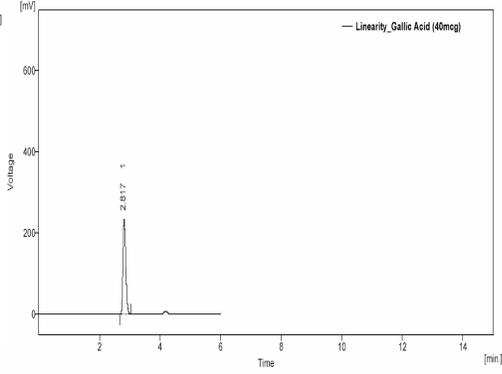


inj-03\_Accuracy-120

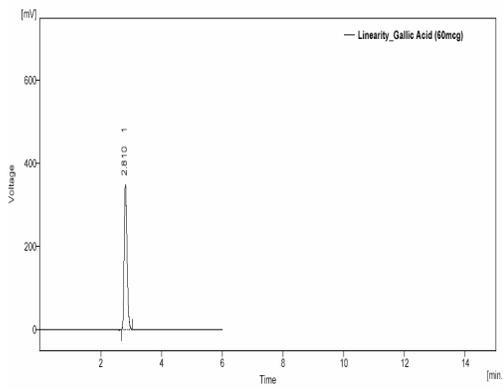
## Linearity



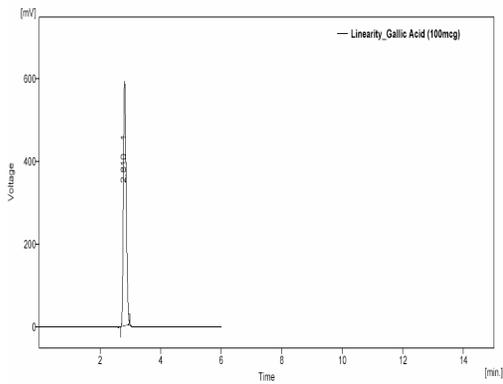
inj-01\_Linearity-20



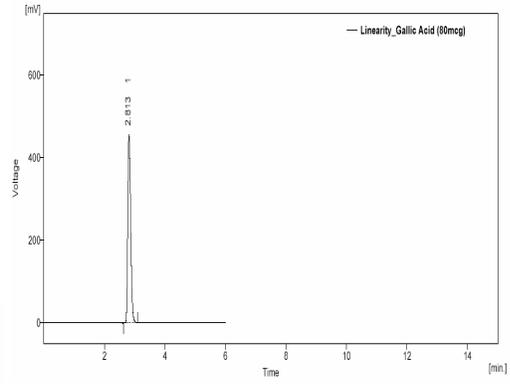
inj-02\_Linearity-40  $\mu\text{g}$



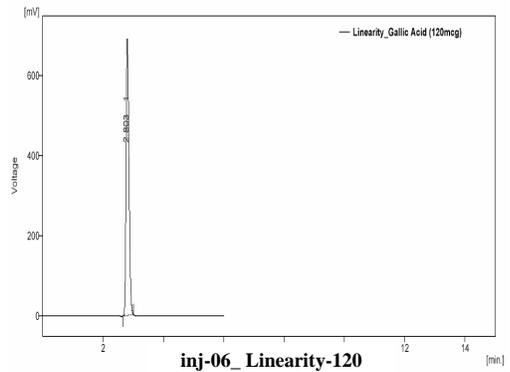
inj-03\_Linearity-60  $\mu\text{g}$



**inj-05\_Linearity-100**

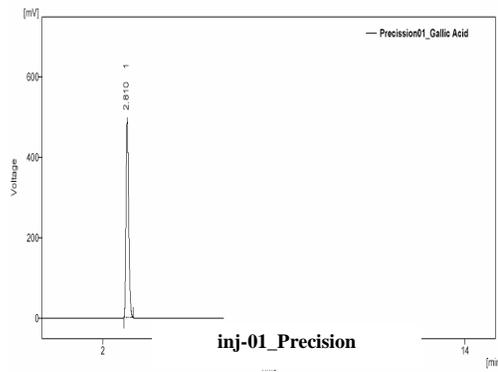


**inj-04\_Linearity-80  $\mu$ g**

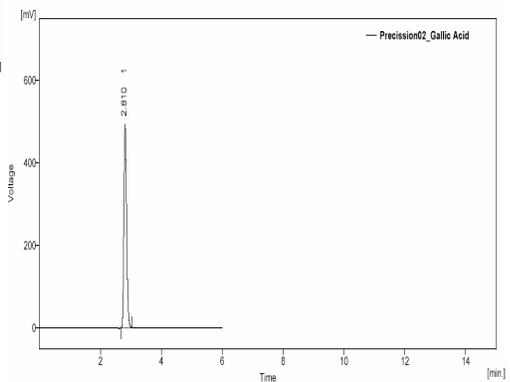


**inj-06\_Linearity-120**

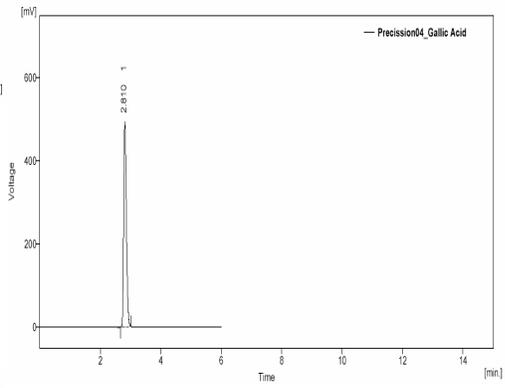
**Precision**



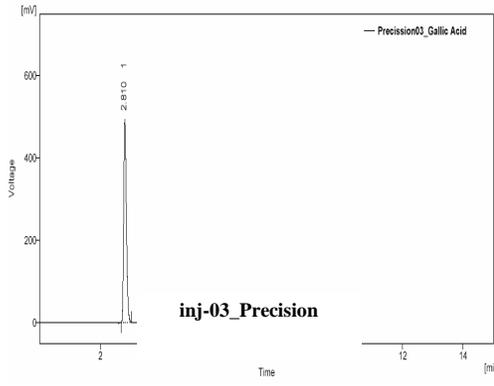
**inj-01\_Precision**



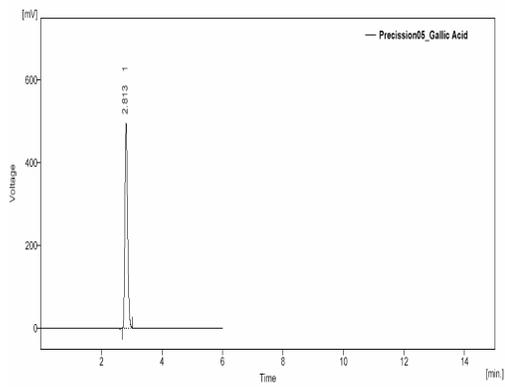
**inj-02\_Precision**



**inj-04\_Precision**

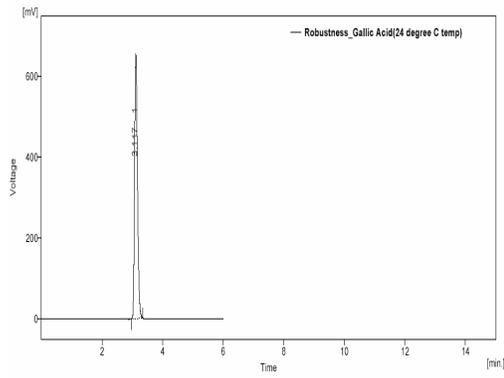


**inj-03\_Precision**

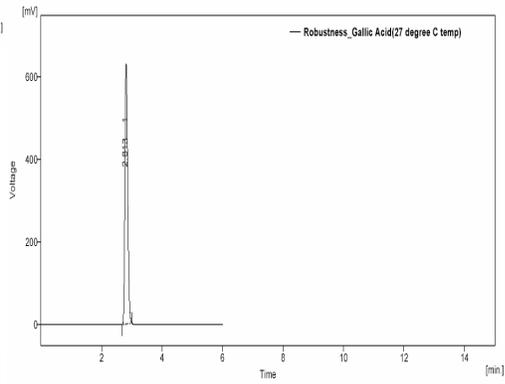


**inj-05\_Precision**

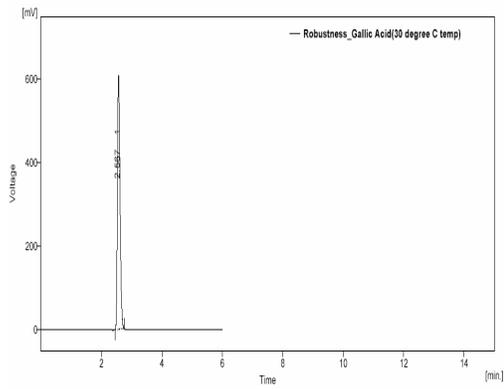
## Robustness:



inj-01\_24deg C

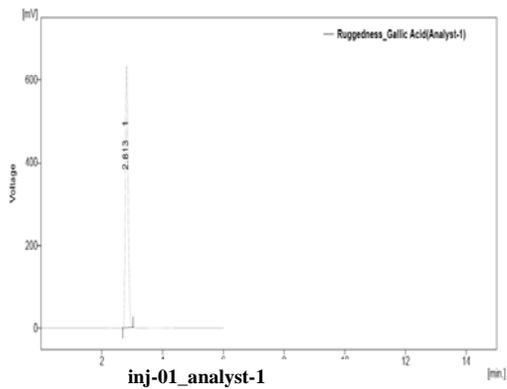


inj-02\_27deg C

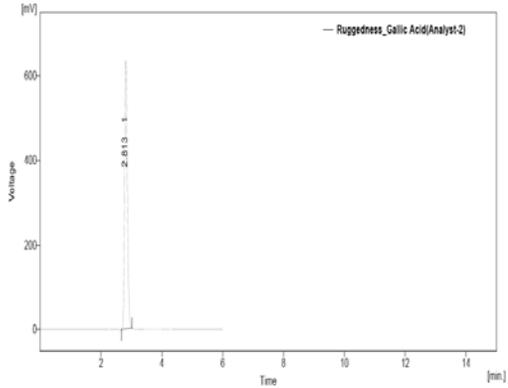


inj-03\_30deg C

## Ruggedness

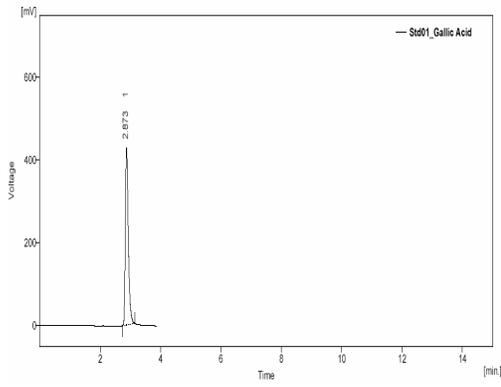


inj-01\_analyst-1

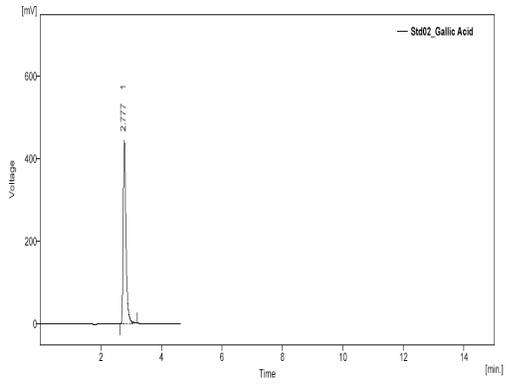


**inj-02\_analyst-2**

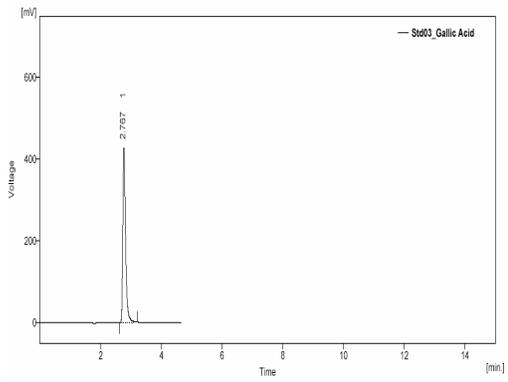
## Standards



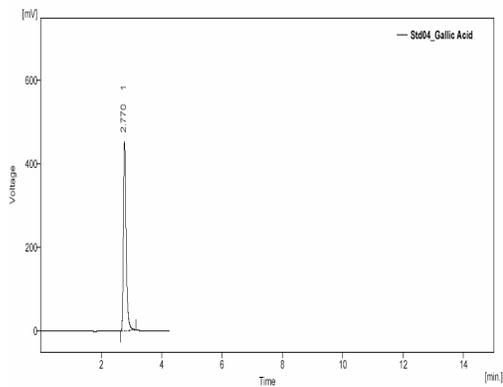
**inj-01\_Standard-01**



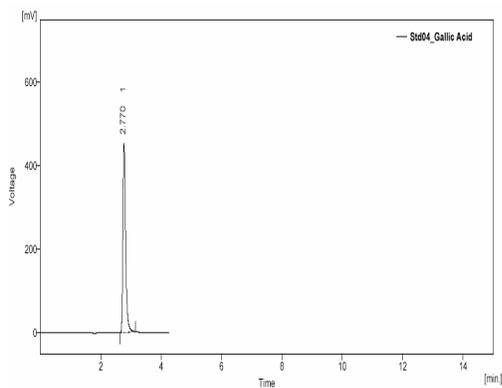
**inj-02\_Standard-02**



**inj-03\_Standard-03**

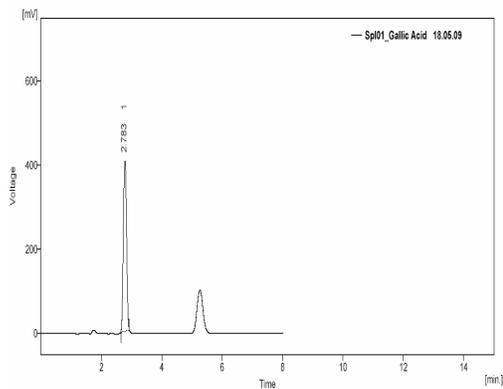


**inj-04\_Standard-04**

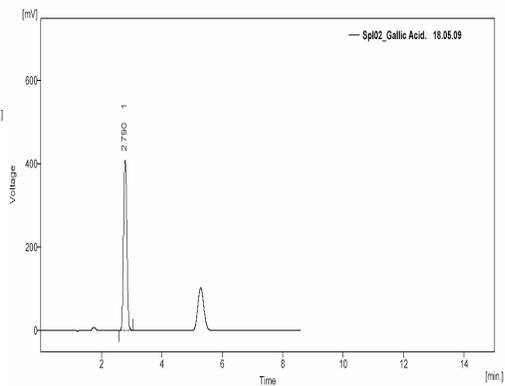


**inj-05\_Standard-05**

### Assay of samples



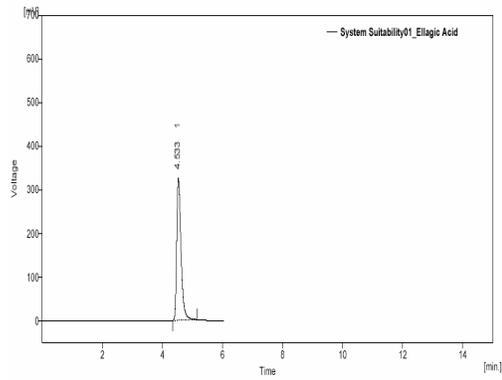
**inj-01\_Sample-01**



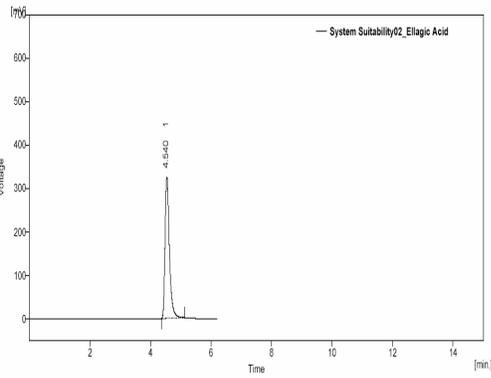
**inj-01\_sample-02**

# For Ellagic acid

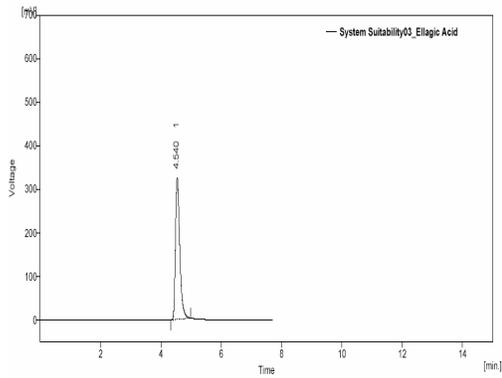
## System suitability:



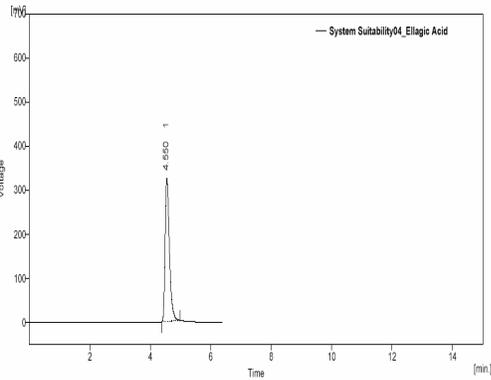
inj-01\_System suitability-01



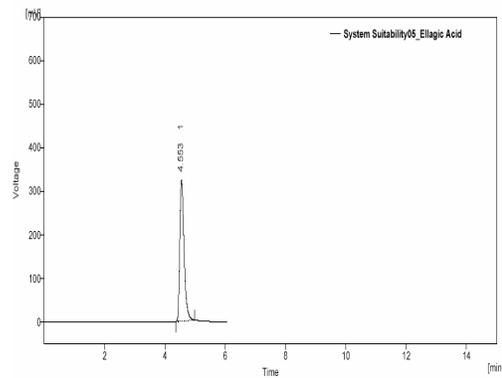
inj-02\_System suitability--01



inj-03\_System suitability--03

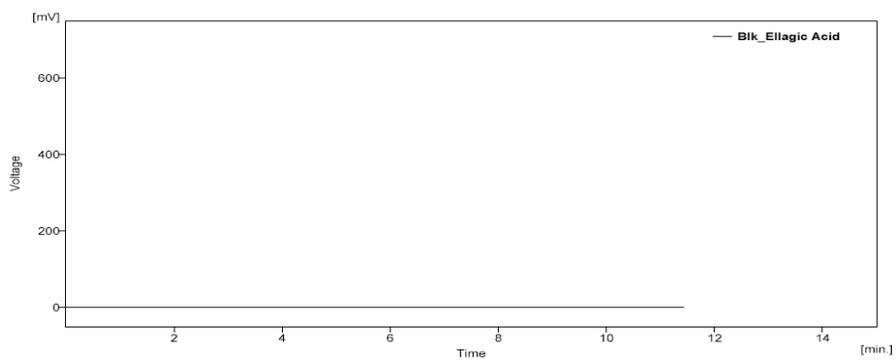


inj-04\_System suitability--04

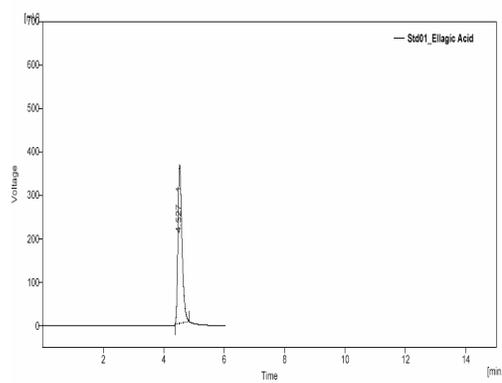


inj-05\_System suitability--05

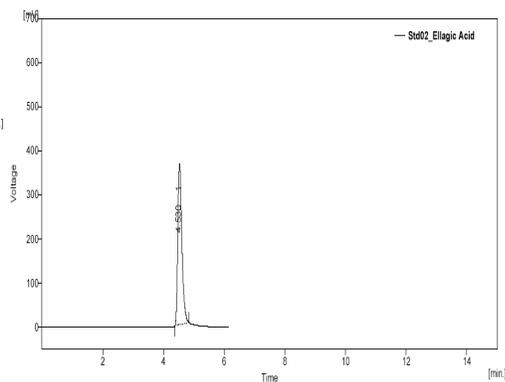
## Specificity:



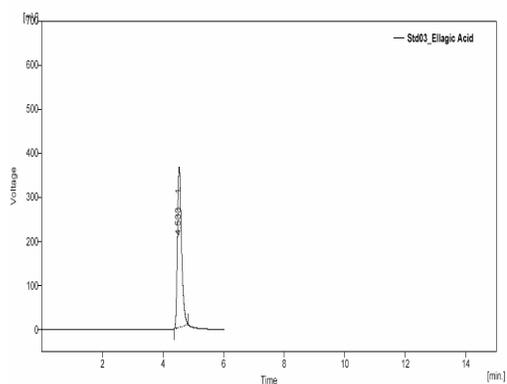
**inj-01\_Blank**



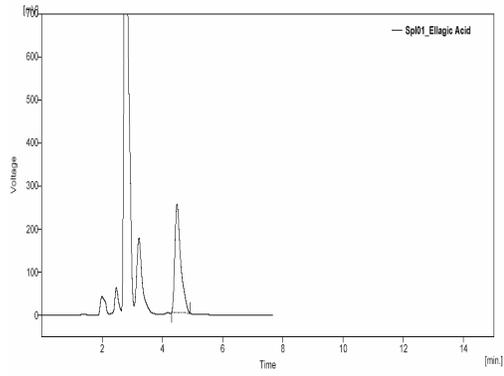
**inj-01\_Standard-01**



**inj-02\_Standard-02**

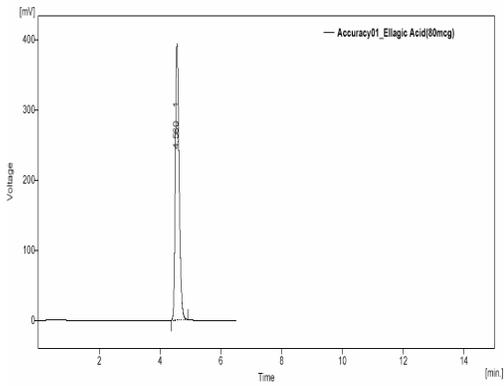


**inj-03\_Standard-03**

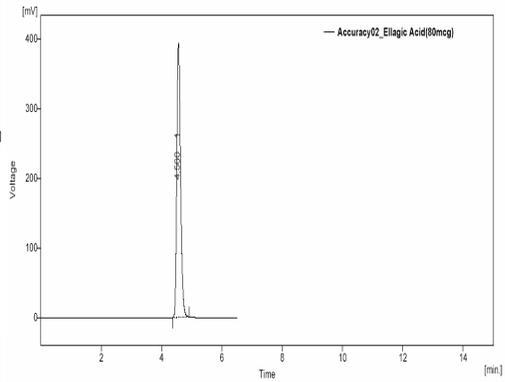


**inj-01\_spl-01**

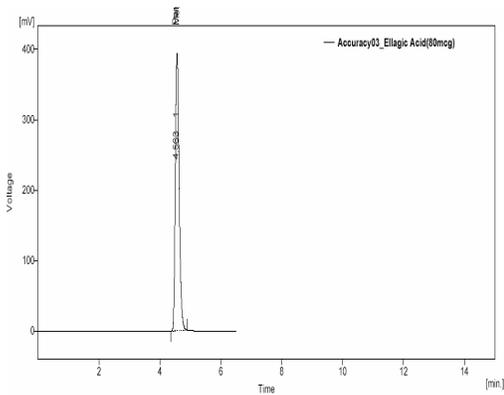
**Accuracy:**



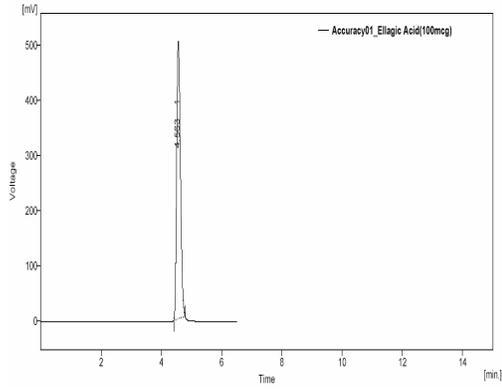
**inj-01\_Accuracy-80mcg**



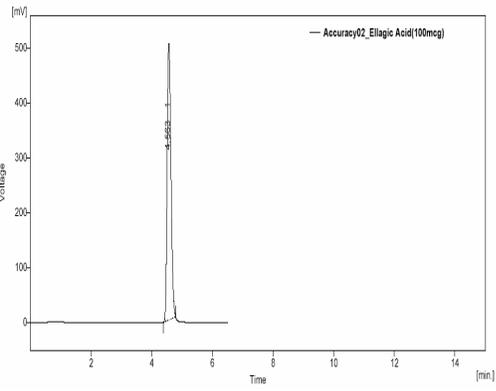
**inj-02\_Accuracy-80mcg**



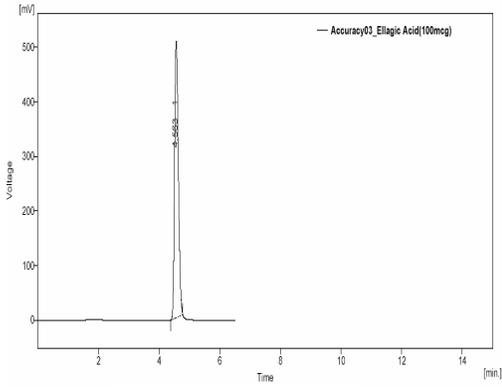
**inj-03\_Accuracy-80mcg**



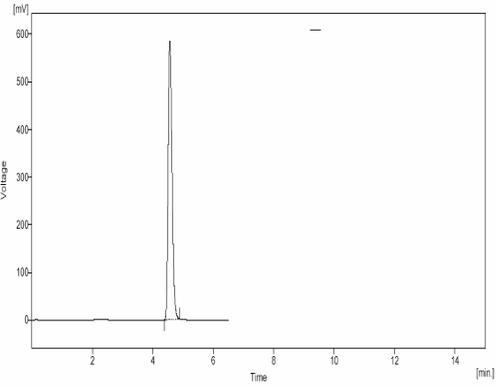
**inj-01\_Accuracy-100mcg**



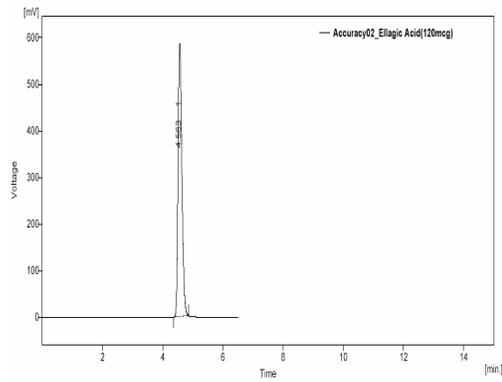
**inj-02\_Accuracy-100mcg**



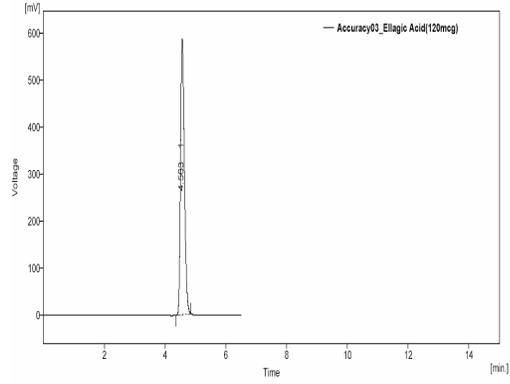
**inj-03\_Accuracy-100mcg**



**inj-01\_Accuracy-120mcg**

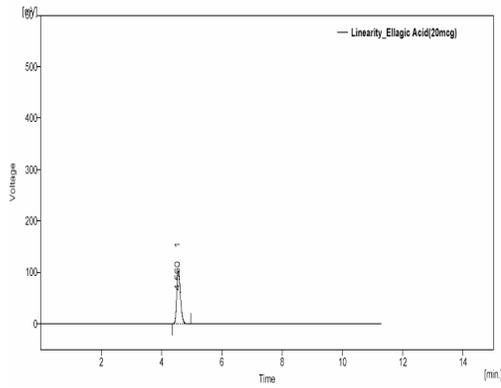


**inj-02\_Accuracy-120**

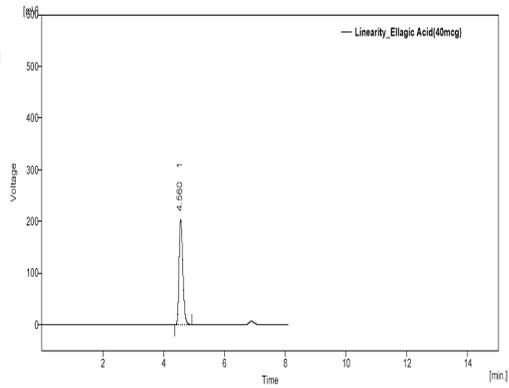


**inj-03\_Accuracy-120mcg**

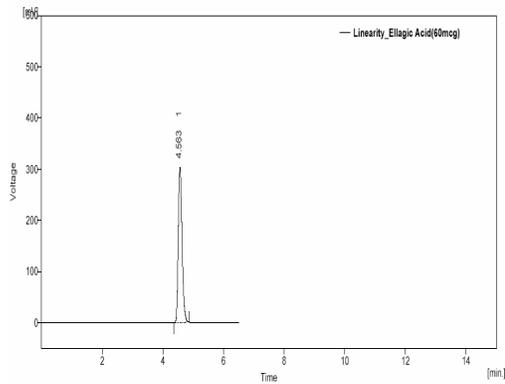
**Linearity:**



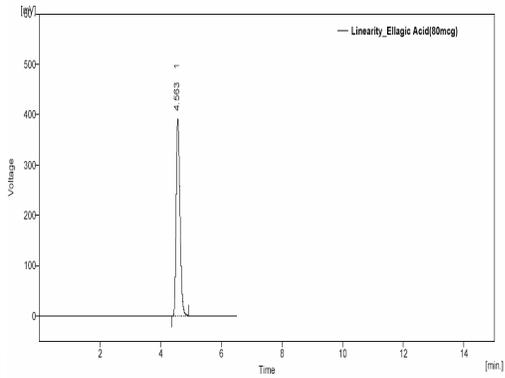
**inj-01\_Linearity-20mcg**



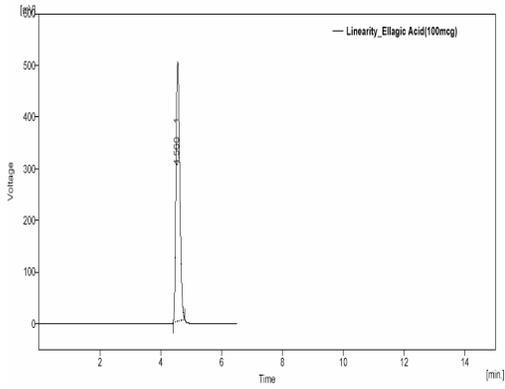
**inj-02\_Linearity-40mcg**



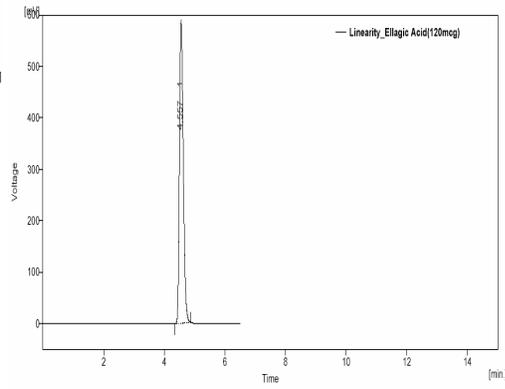
**inj-03\_Linearity-60mcg**



**inj-04\_Linearity-80mcg**

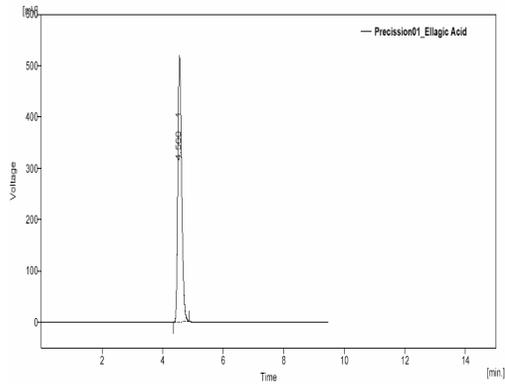


**inj-05\_Linearity-100mcg**

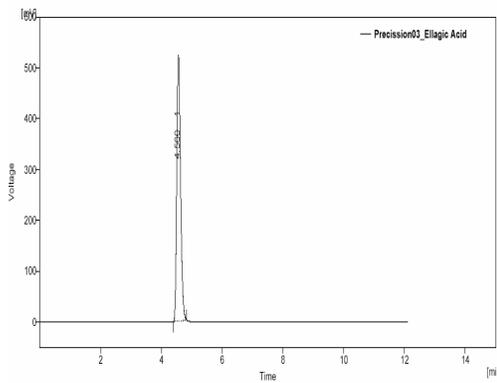


**inj-06\_Linearity-120mcg**

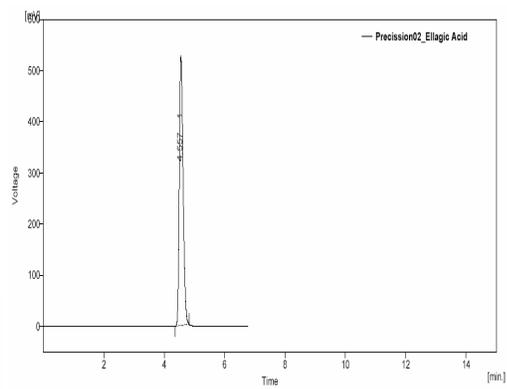
**Precision:**



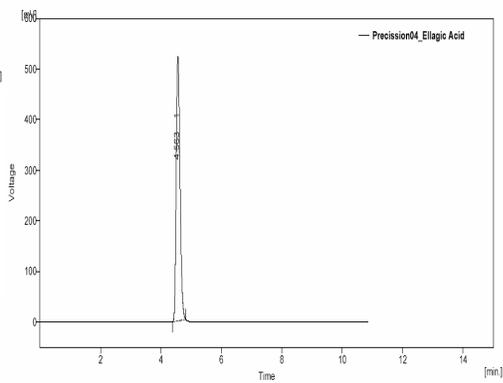
**inj-01\_Precision**



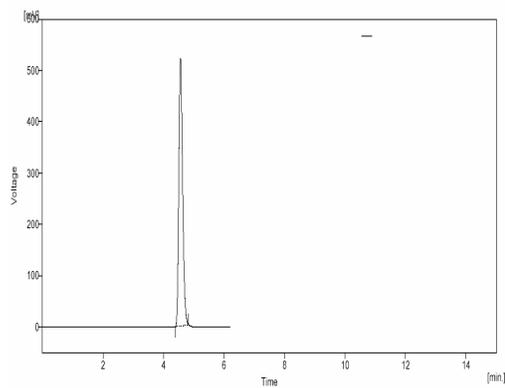
**inj-03\_Precision**



**inj-02\_Precision**

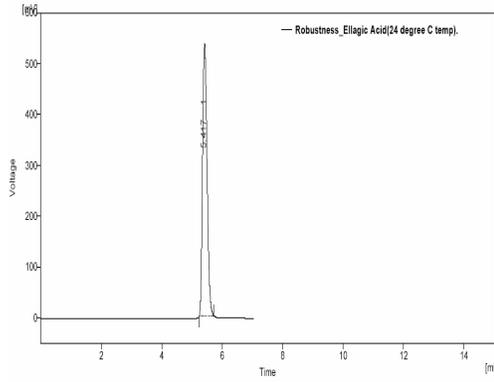


**inj-04\_Precision**

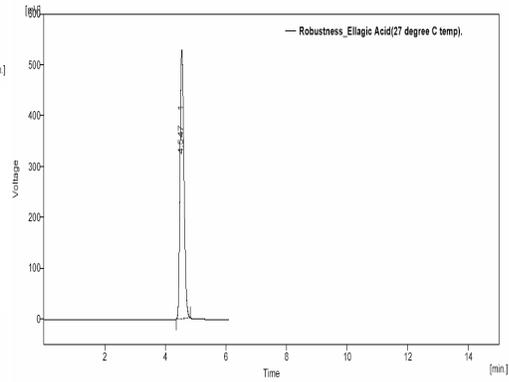


**inj-05\_Precision**

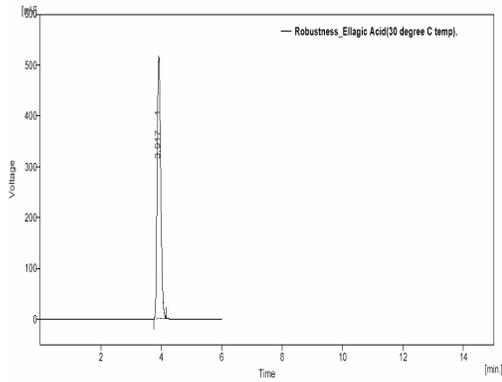
## Robustness:



inj-01\_24deg C

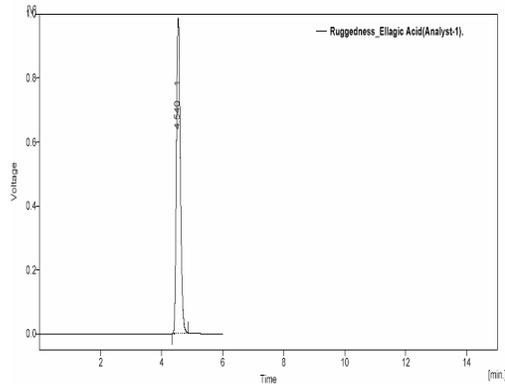


inj-01\_27deg C

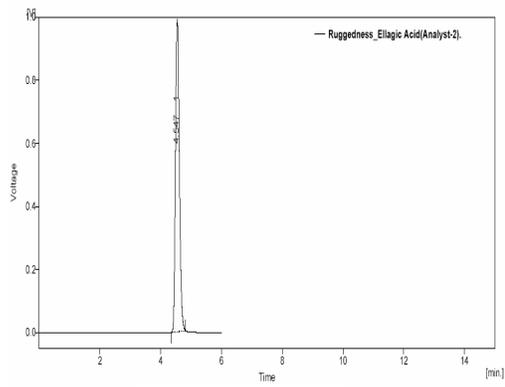


inj-01\_30deg C

## Ruggedness:

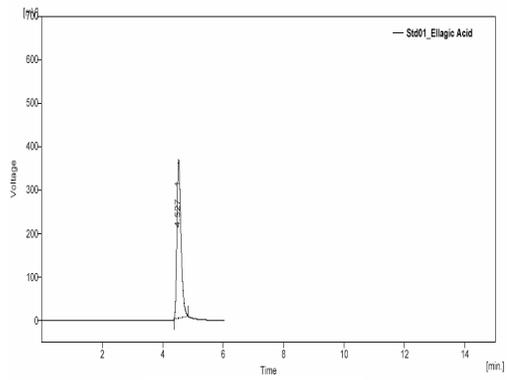


inj-01\_analyst-01

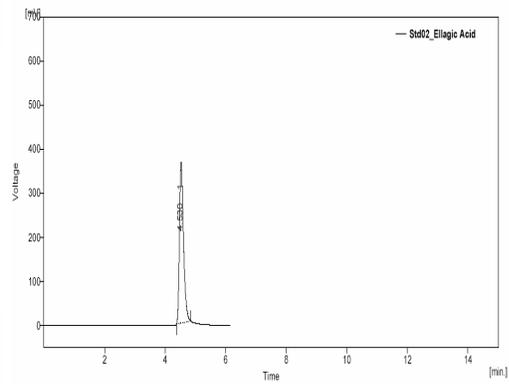


**inj-02\_analyst-02**

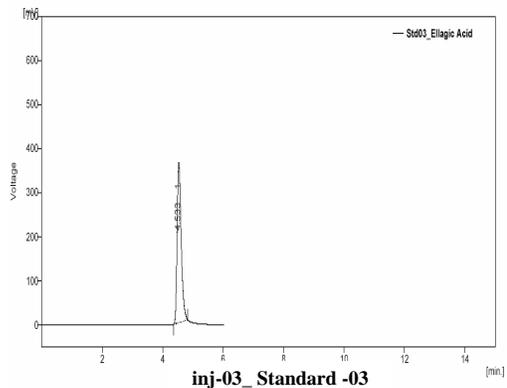
**Standard:**



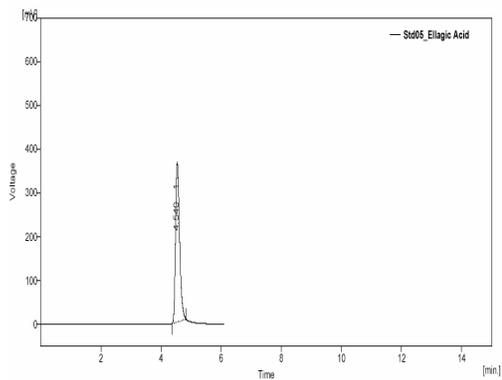
**inj-01\_Standard-01**



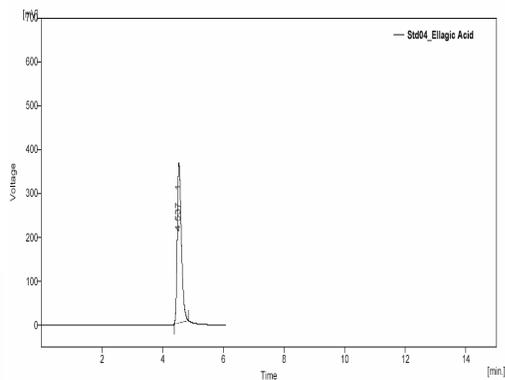
**inj-02\_Standard -02**



**inj-03\_Standard -03**

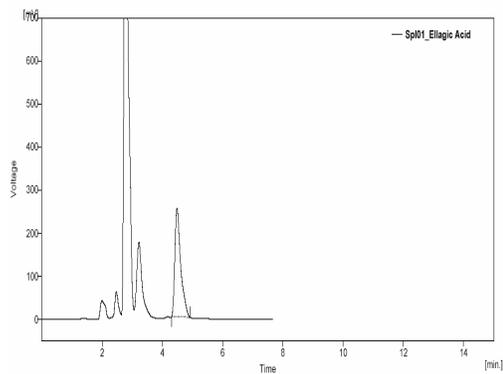


**inj-05\_Standard -05**

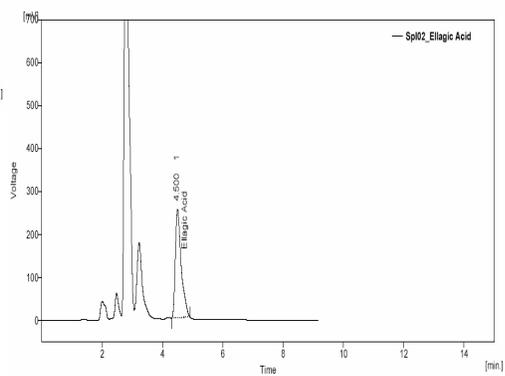


**inj-04\_Standard -04**

**Assay of samples:**



**inj-01\_Sample-01**



**inj-01\_Sample-02**

## RESULTS AND DISCUSSION

### For gallic acid

Of the various proportions of acetonitrile and buffer (0.1% orthophosphoric acid in milli-Q-water) tried, that containing 80: 20 v/v of acetonitrile and buffer was found to be most suitable one. In this system, gallic acid was eluted ( $R_t = 2.81$  min) in the presence of other compounds in the sample extract. The absorbance maximum ( $\lambda_{max}$ ) for gallic acid was 270 nm.

### Validation

The HPLC method was validated in terms of precision, repeatability and accuracy. The method is specific as it is well-resolved gallic acid with a retention time ( $R_t$ ) value of 2.81 min in the presence of other components in the sample of triphala churnam tablet formulation.

The relationship between the concentration of standard solution and the peak response was linear with in the concentration range of 20 $\mu$ g/ml to 120 $\mu$ g/ml with a correlation coefficient of 0.999.

$$y = 38027x$$

The precision was studied by repeated injections of the same concentration five times (% CV = 0.0307).

Accuracy of the developed method was determined at three levels (80%, 100% and 120%). The % recoveries were found to be 98.5%, 99.5% and 99.15%. The limit of detection for gallic acid was calculated from the standard deviation of the linear curve and the slope. The value was 6.13 $\mu$ g/ml.

The limit of quantification for gallic acid was calculated from the standard deviation of the linear curve and the slope. The value was 18.57 $\mu$ g/ml.

There is allowable variation in temperature which indicates that method is robust enough.

The low RSD value (0.32) for percent assay of test preparation revealed that the proposed method is rugged. The chromatogram of sample showed a single peak at the retention time of gallic acid indicating that there is no interference of the changing the persons for injecting the sample to the instrument.

The amount of gallic acid present in the formulation was found to be 3.06 mg.

### **For ellagic acid**

Of the various proportions of acetonitrile and buffer (0.1% orthophosphoric acid in milli-Q-water) tried, that containing 80: 20 v/v of acetonitrile and buffer was found to be most suitable one. In this system, ellagic acid was eluted ( $R_t = 4.5$  min) in the presence of other compounds in the sample extract. The absorbance maximum ( $\lambda_{max}$ ) for ellagic acid was 254 nm.

### **Validation**

The HPLC method was validated in terms of precision, repeatability and accuracy. The method is specific as it is well-resolved ellagic acid with a retention time ( $R_t$ ) value of 4.5 min in the presence of other components in the sample of triphala churnam tablet formulation.

The relationship between the concentration of standard solution and the peak response was linear with in the concentration range of 20  $\mu$ g/ml to 120  $\mu$ g/ml with a correlation coefficient of 0.999.

$$y = 43298x$$

The precision was studied by repeated injections of the same concentration 5 times (% CV = 0.0571).

Accuracy of the developed method was determined at three levels (80%, 100% and 120%). The recoveries were found to be 98.95%, 100.2% and 100.15%.

The limit of detection for the ellagic acid was calculated from the standard deviation of the linear curve and slope. That was found to be 3.744  $\mu$ g/mL.

The limit of quantification for the ellagic acid was calculated from the standard deviation of the linear curve and slope. That was found to be 16.84  $\mu$ g/ml.

There is allowable variation in temperature, which indicates that method is robust enough.

The low RSD value (0.0179) for percent assay of test preparation revealed that the proposed method is rugged. The chromatogram of sample showed a single peak at the retention time of ellagic acid indicating that there is no interference of the changing the persons for injecting the sample to the instrument.

The amount of ellagic acid present in the formulation was found to be 0.955 mg.

**Method validation parameters for the estimation of gallic acid and ellagic acid by the HPLC method**

<b>S. No</b>	<b>Validation Parameters</b>	<b>Gallic acid</b>	<b>Ellagic acid</b>
1.	System suitability (asymmetry % CV)	1.236	2.52
2.	Specificity	Specific	Specific
3.	Linearity (correlation coefficient R <sup>2</sup> )	0.999	0.999
4.	Precision (% CV) (n = 5)	0.0307	0.0571
5.	Limit Of Detection (LOD)	6.13 µg	3.744 µg
6.	Limit Of Quantitation (LOQ)	18.57 µg	16.84 µg
7.	Robustness (variation in temperature)	Robust	Robust
8.	Ruggedness (% CV)	0.32	0.0179

## SUMMARY AND CONCLUSION

### **For gallic acid**

A simple reverse phase high performance liquid chromatographic method employing symmetry shield C-18 column has been developed for analysis of gallic acid. Good separation was achieved by employing an isocratic system using acetonitrile and 0.01 % phosphate buffer. The detection was carried out at 270 nm. The method was validated for linearity, accuracy, precision, robustness and ruggedness. The elution was carried out with a mixture of acetonitrile and phosphate buffer pH adjusted to 4.5 and the ratio 80: 20 at a constant flow rate 1 ml/min. the analytical wavelength was 270 nm.

The developed HPLC method is simple, accurate and reproducible the following advantages. It uses simple symmetry shield C-18 column under less consumption of the mobile phase consist of acetonitrile and phosphate buffer (pH 4.5) and also less run time. So this method can be used as a routine analytical tool for the analysis of gallic acid in herbal formulations. In the present study the gallic acid content of 3.06 mg per tablet was observed in triphala churnam tablet formulation.

### **For ellagic acid**

A simple reverse phase high performance liquid chromatographic method employing symmetry shield C-18 column has been developed for analysis of ellagic acid. Good separation was achieved by employing an isocratic system using acetonitrile and 0.01 % phosphate buffer. The detection was carried out at 254 nm. The method was validated for linearity, accuracy, precision, robustness and ruggedness. The elution was carried out with a mixture of acetonitrile and phosphate buffer pH adjusted to 4.5 and the ratio 80: 20 at a constant flow rate 1 ml/min. the analytical wavelength was 254 nm.

The developed HPLC method is simple, accurate and reproducible the following advantages. It uses simple symmetry shield C-18 column under the less consumption of mobile phase consist of acetonitrile and phosphate buffer (pH 4.5) and also less run time. So this method can be used as a routine analytical tool for the analysis of ellagic acid in herbal formulations. In the present study the ellagic acid content of 0.955 mg per tablet was observed in triphala churnam tablet formulation.

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