

# [High performance liquid chromatography experiment](https://assignbuster.com/high-performance-liquid-chromatography-experiment/)

## INTRODUCTION

Pharmaceutical Analysis may be defined as the application of analytical procedures used to determine the purity, safety and quality of drugs and chemicals. The term “ Pharmaceutical analysis” is otherwise called quantitative pharmaceutical chemistry. Pharmaceutical analysis includes both qualitative and quantitative analysis of drugs and pharmaceutical substances starts from bulk drugs to the finished dosage forms. In the modern practice of medicine, the analytical methods are used in the analysis of chemical constituents found in human body whose altered concentrations during disease states serve as diagnostic aids and also used to analyze the medical agents and their metabolites found in biological system.

Qualitative inorganic analysis seeks to establish the presence of given element or inorganic compound in a sample.

Qualitative organic analysis seeks to establish the presence of a given functional group or organic compound in a sample.

## Quantitative

Quantitative analysis seeks to establish the amount of a given element or compound in a sample.

The term “ quality” as applied to a drug product has been defined as the sum of all factors, which contribute directly or indirectly to the safety, effectiveness and reliability of the product. These properties are built into drug products through research and during process by procedures collectively referred to as “ Quality control”. Quality control guarantees with in reasonable limits that a drug products

Is free of impurities.

Is physically and chemically stable

Contains the amount of active ingredients as stated on the label and

Provides optimal release of active ingredients when the product is administered.

Most modern analytical chemistry is categorized by two different approaches such as analytical targets or analytical methods.

## INTRODUCTION FOR CHROMATOGRAPHY:

High performance liquid chromatography is the process, which seperates mixture containing two or more components under high pressure. In this the stationary phase is packed in column one end of which is attached to a source of pressurized liquid mobile phase.

High performance liquid chromatography is the fasted growing analytical technique for the analysis of drug. Its simplicity, high specificity and wide range of sensitivity makes its ideal for the analysis of many drugs in both dosage form and biologic fluids.

HPLC is also known as High performance liquid chromatography. It is essential form column chromatography in which the stationary phase is consists of a small particles (3-5oµm) packing contained in a column with a small bore (2-5mm), one end of which is attached to source of pressurized liquid eluent(mobile phase).

Different Types of Principles: According to the phases involved, HPLC can be classified into several types, which are as follows:

* Normal Phase Chromatography (NPC)
* Reverse – Phase Chromatography (RPC)
* Liquid – Solid Chromatography or adsorption HPLC
* Liquid – Liquid Chromatography or Partition HPLC
* Ion exchange Chromatography or Ion exchange HPLC
* Size exclusion or gel permeation or steric exclusion HPLC

1. Normal Phase Chromatography (NPC): In normal phase chromatography, the stationary phase is more polar then the mobile phase, and the mobile phase is a mixture of organic solvents with out added water (e. g. isopropane with hexane) and the column packing is either an inorganic adsorbent (silica) are a polar bonded phase (cyanno, diol, amino) on a silica support. Sample retention in normal phase chromatography increases with the polarity of mobile phase decreases. They are eluted in the order of increasing polarities.

2. Reverse – Phase Chromatography (RPC): In reverse-phase chromatography, the stationary phase is less polar than the mobile phase and the mobile phase is a mixture of organic and aqueous phase. Reverse-phase chromatography is typically more convenient and rugged than the other forms of liquid chromatography and is more likely to result in a satisfactory final separation. High performance RPC columns are efficient, stable and reproducible. In this, the solutes are eluted in the order of their decreasing polarities. These are prepared by treating the surface silanol group of site with an organic chloro silane reagent.

## INSTRUMENTATION:

## RECORDER

SCHEMATIC DIAGRAM OF HPLC

a. Pumps: Pumps are required to deliver a constant flow of mobile phase at pressures ranging from 1 – 550 bar pumps capable of pressure up to 6000 psi provide a wide range of flow rates of mobile phase, typically from 0. 01-10ml min-1. Low flow rates (10-100ï­l min-1) are used with micro bore columns, intermediate flow rates (0. 5-2ml min-1) are used with conventional analytical HPLC columns, and fast flow rates are used for preparative or semi preparative columns and for slurry packing techniques.

Mechanical pumps of the reciprocating piston type view a pulsating supply of mobile phase. A damping device is there fore required to smooth out the pulses so that excessive noise at high levels of sensitivity or low pressure does not detract from detection of small quantities of sample. This type of pump is mostly used.

Dual – piston reciprocating pumps produce an almost pulse free flow because the two pistons are carefully faced so that as one is filling the other is pumping. These pumps are more expensive than single piston pumps but are of benefit when using a flow sensitive detector such as ultraviolet or refractive index detector.

b. Injection Systems:

Injection ports are of two basic types, (A) those in which the sample with injected directly into the column and (B) those in which the sample is deposited before the column inlet and then swept by a valving action into the column by the mobile phase.

c. Columns:

HPLC columns are made of high quality stainless steel, polish internally to a mirror finish. Standard analytical columns are 4-5 mm internal diameter and 10-30 cm in length. Shorted columns (3-6 cm) containing a smaller particles size packing material (3 or 5 ï­m) produce similar or better efficiencies, in terms of the number of theoretical plates (about 7000), that those of 20 cm columns containing 10 ï­m irregular particles and are used an short analysis time and highest throughput of samples are required. Micro bore columns of 1-2 mm internal diameter and 10-25 cm in length have certain advantages of lower detection limits and lower consumption of solvent, the latter being important if expensive HPLC – grade solvents are used. HPLC are also being carried out on the semi preparative scales by using columns of 7-10 mm or 20-40 mm internal diameter respectively.

d. Detectors:

The most widely used detectors for liquid chromatography are

## Detector

## Analytes

## Solvent Requirements

## Comments

UV-Visible

Any with chromophores

UV-grade non UV absorbing solvents

Has degree of selectivity and useful for many HPLC applications

Fluorescence

Fluorescent compounds

UV-grade non UV absorbing solvents

Highly selective and sensitive, often used to analyze derivitized compounds

Refractive index

Compounds with different RI than mobile phase

Cannot run mobile phase gradients

Limited sensitivity

Conductivity

Charged or polar compounds

Mobile phase must be conducting

Excellent for ion exchange compounds

Electrochemical

Readily oxidized or reduced compounds, specially biological samples

Mobile phase must be conducting

Very selective and sensitive

Mass-Spectrometer

Broad range compounds

Must use volatile solvents or volatile buffers

Highly sensitive. Many modes available. Needs trained person

## Theoretical principles of HPLC:

a. Retention time: The time is required between the injection point and the peak maximum is called the retention time. It is denoted as the Rt. It is mainly useful for the qualitative analysis for the identification of compound.

b. Capacity factor: It represents the molar ratio of the compound in the stationary phase and the mobile phase. It is independent of column length and mobile phase flow rate. It is denoted as the “ k”. It should be kept 1-10. If “ k” values are too low it is likely that the solutes may be adequately resolved and for high ‘ k’ values the analysis time is too long. It can be calculated by

tr – t0

k = —————-

t0

tr = Retention time, t0 = Dead time.

c. Tailing factor: Closer study of a chromatographic show that the Gaussian forms is usually not completely symmetrical. The graph spread out to a greater or lesser extent, forming a tail. It reduces the column plate number which intern influences the resolution. Tailing is mainly due to deteriorated column, overloading column, extra column-volumes, and incompatibility of sample with standard and/or mobile phase. Practically it can be calculated or determined at 10% of the total peak height. It must not be greater than 2. 0

d. Resolution: The degree of separation of one component from another is described by the resolution. It is generally denoted by ‘ Rs’. It is measured as the difference in retention time and the arithmetic mean of the two peak widths.

tr2 – tr1

Rs = ———————

0. 5(w1 + w2)

tr2 = Retention time of first peak w1 = width of first peak

tr1 = Retention time of second peak w2 = width of second peak

e. Theoretical plates: It is important property of the column. It reflects its quality of separation and its ability to produce sharp, narrow peak and achieving good resolution of peak. ‘ N’ denotes it.

3500 X L (cm)

Theoretical plates = ———————-

dp(µm)

L = length of the column – in cm, dp = diameter of the particle (µm)

It follows that if the exchange is fast and efficient, the theoretical plate will be small in size and there will be large number of plates in the column.

f. Height equivalent to theoretical plate (HETP): Number of plates directly proportional to the column length (L) and inversely proportional to the diameter of the particles (dp). The value of H is a criterion for the quality of a column. Lower the HETP, higher is the efficiency of the column.’ Its value depends upon particle size, flow rate, viscosity of mobile phase.

H = L/N

L = Length of column, N = No. of theoretical plate

## HPLC method development:

The wide variety of equipment, columns, eluent and operational parameters involved makes high performance liquid chromatography (HPLC) method development seem complex. The main objective of method development is to obtain a good separation with minimum time and effort. Based on the goal of separation, the method development is preceded. The steps involved are:

* Information on sample, define separation goals
* Need for special HPLC procedure, sample pretreatment, etc.
* Choose detector and detector settings
* Choose LC method, preliminary run;
* Estimate best separation conditions
* Optimize separation conditions
* Check for problems or requirement for special procedure
* Validation for release to routine laboratory

## The following must be considered when developing an HPLC method:

* Keep it simple
* Try the most common columns and stationary phases first
* Thoroughly investigate binary mobile phases before going on to ternary
* Think of the factors that are likely to be significant in achieving the desired resolution.

Mobile phase composition, for example, is the most powerful way of optimizing selectivity whereas temperature has a minor effect and would only achieve small selectivity changes. pH will only significantly affect the retention of weak acids and bases.

## VALIDATION OF ANALYTICAL METHOD IN PHARMACEUTICAL ANALYSIS:

Validation is documented evidence, which is completed to ensure that an analytical method is accurate, reproducible and robust over the specific range. The quality of the analytical data is a key factor in the success of a drug development program. The process of method development and validation has a direct impact on the quality of these data.

## Method validation:

Method validation is the process to confirm that analytical procedure employed for a specific test is suitable for its intended use. Method needs to be validated or revalidated

Before their introduction into routine use

Whenever the conditions changes for which the method has been validated , e. g., instrument with different characteristics

Whenever the method is changed, and the change is outside the original scope of the method.

Depending on the use of the assay, different parameters will have to be measured during the assay validation. ICH and several regulatory bodies and Pharmacopoeia have published information on the validation of analytical procedures

## METHOD VALIDATION PARAMETERS:

* SPECIFICITY.
* ACCURACY.
* PRECISION.
* LINEARITY.
* ROBUSTNESS.
* SOLUTION STABILITY.

The goal of the validation process is to challenge the method and determine the limit of allowed variability for the conditions needed to run the method. The following statistical parameters are to be determined to validate the developed method.

## Correlation coefficient(r):

When the changes in one variable are associated or followed by changes in the other, it is called correlation. The numerical measure of correlation is called the coefficient of correlation and is defined by the relation.

ï“ (x – x’) (y -y’)

r = —————————————————

âˆš ï“(x -x’) 2 ï“(y -y’

## Regression equation:

Regression equation= I + aC

Y2 – Y1

a = slope = —————

X2 – X1

I = Intercept = regression – a C

As a percentage of mean absorbance.

## 3. Standard Deviation:

S = âˆš ï“ (X- X!) 2/N – 1

Where, X = observed values

X! = Arithmetic mean = ï“ X/N

N = Number of deviations

For practical interpretation it is more convenient to express ‘ S’ in terms of percent of the approximate average of the range of analysis is used in the calculation of ‘ S’. This is called co-efficient of variation (C. V) or percent relative standard deviation (%RSD).

C. V OR %RSD = 100\* S/ X!

## Criteria for Validation of the Method

## CHARACTERISTICS

## ACCEPTABLE RANGE

Specificity

No Interference

Accuracy

Recovery (98-102%)

Precision

RSD < 2%

Linearity

Correlation Coefficient(r)> 0. 99

Range

80-120%

Stability

> 24h or > 12h

DRUG PROFILE

RIZATRIPTAN BENZOATE:

Structure:

Chemical name : N, N diethyl -5-(1H-1, 2, 4-triazol-1-1-ylmetyl)-1H

Indole-3 Ethanamine monobenzoate

Molecular Formula : C15H19N5. C6H5COOH

Molecular weight : 391. 47

Description: White crystalline powder

Melting point: 178-1800C

Solubility: Sparingly soluble in water and methanol

Storage: Air tight container protect from light.

Drug Category: Anti migraine drug

THERAPEUTIC RATIONAL

RIZATRIPTAN BENZOATE:

CLINICAL PHARMACOLOGY:

Mechanism of action:

Rizatriptan binds with high affinity to human 5-HTIB and 5-HTID receptors leading to cranial blood vessel constriction.

Pharmacokinetics:

Absorption:

Completely absorbed from GI tract, absolute bioavailability is 45% plasma peak concentration attained with in 1-1. 5 hours (conventional tablet )or 1. 6-2. 5 hours (orally disintegrating tablet)after oral administration.

Distribution:

Crosses placenta and is distributed in to milk in animal, no studies in pregnant or nursing women.

Metabolism:

Metabolized principally via oxidative deamination by Mao-A to an inactive indole acetic acid metabolite

Elimination:

Excreted principally in urine(14% of dose as unchanged drug and 51 % a indole acetic acid metabolite

Adverse effects:

* Dry mouth
* Dizziness
* Pain tightness/pressure in neck/throat/jaw.
* Nausea
* Chest pain
* Parasthesia
* Fatigue

Dosage and administration:

The dose range of Rizatriptan benzoate is 10-30mg orally once daily. Rizatriptan benzoate can be administer orally disintegrating tablet with out meals.

## LITERATURE REVIEW

Sasmitha Kumar et al: has been developed UV spectroscopic method for estimation of Rizatriptan benzoate. The drug shows maximum absorption at 277 nm and 281 nm and obeys beer-lamberts law in the concentration of 0. 5-20 µg/ml at 277 nm and 0. 5-80 µg/ml at 281 nm respectively. The percentage recovery was found to be 97-100%.

Madhukar et al; has been developed reverse phase high performance liquid chromatographic method for determination of Rizatriptan benzoate. The proposed method utilized column L1 inertsil ODS-3v, 250 nmx4. 6 mm having particle size, 5µm. The mobile phases were comprised of A, B of Acetonitrile and buffer pH 6. 5 at UV detection 225 nm. The method shows recovery 96. 64-97. 71

Sachin jagthap et al; has been developed stability indicating reversed phase high performance liquid chromatographic method for the determination of Rizatriptan benzoate in bulk powder and in pharmaceutical formulations. The method utilizes c18 column having dimension 250mmx4. 6 mm having particle size, 5. 0 µm using a mobile phase 0. 01M sodium dihydrogen phosphate buffer: Methanol , at a flow rate 1ml/min at ambient temperature and detected at 225 nm. and the method was validated according to ICH guidelines

Quizi zhang et al: has been developed, a high performance liquid chromatographic method for the determination of Rizatriptan benzoate in human plasma. using asingle step liqid liqid extraction with metyl tertiary butyl ether, the analytes separated usig amobile phase consisting of 0. 05%v/v triehylamine in water adjusting ph 2. 75 with 85% phosphoric acid and acetonitrile. fluroscence detection was performed at an excitation wavelength of 225 nm and an emission wavelength of 360 nm. The linearity for rizatriptan was within the concentration range of 0. 5-50ng/ml.

Rajendra Kumar et al: has been developed and validated stability a stability indicating high performance liquid chromatographic method for Rizatriptan benzoate. The force degradation studies were performed on bulk sample of Rizatriptan benzoate. The method utilizes a zorbax SB-CN column with dimension of 250 mmx4. 6 mm, 5um column. The mobile phase consists of a mixture of aqueous potassium dihydrogen ortho phosphate (ph3. 4), acetonitrile and methanol.

Rauza bagh et al: has been developed a spectroscopic method for analysis of Rizatriptan benzoate in bulk and tablet dosage form. The Rizatriptan benzoate shows maximum absorbance at 225 nm. Beer’s law was obeyed in the concentration range of 1-10µg/ml.

## AIM AND PLAN OF WORK

The present aim is to develop a new simple and rapid analytical method to estimate the Rizatriptan benzoate

The plan of the proposed work includes the following steps:

* To undertake solubility studies for analytical studies of Rosuvastatin calcium
* Develop initial chromatographic conditions.
* Setting up of initial chromatographic conditions for the assay of Rosuvastatin calcium Optimization of initial chromatographic conditions.
* Validation of the developed HPLC Analytical method according to ICH method validation parameters.

## EXPERIMENTAL

### NEW RP-HPLC METHOD FOR THE ESTIMATION OF RIZATRIPTAN BENZOATE IN TABLET DOSAGE FORM

A simple reverse phase HPLC methods was developed for the determination of Rizatriptan benzoate in tablet dosage form. Zorbax Eclipse XBD C18 (250 cm Ã- 4. 6 mm) column in isocratic mode with mobile phase Buffer ph 5. 0: Methanol (80: 20) was used and pH-3 adjusted with tri ethylamine. The flow rate was 1. 0 ml/min and UV detection at 225nm. The retention time 3. 0 min. The proposed method was also validated.

## EXPERIMENTAL

### 1. Instrumentation:

Shimadzu LC-10A HPLC

Vacuum pump – Gelmon science

Elico SL-164 double beam UV-Visible spectrophotometer

Ultra sonicator 3. 5L 100(pci)

### 2. Chemicals:

Water HPLC grade

Methanol HPLC grade (Merck)

Potassium dihydrogen orthophosphate(AR Grade)

Triethylamine (AR Grade)

## 5. 1 OPTIMIZATION:

### 1. Selection of wavelength:

After solubility study for the drug solvent was selected and appropriate concentration of Rizatriptan benzoate standards with solvent were prepared. The solution were then scanned by using doubl beam UV-Visible spectrophotometer the range between 200-400nm. The overlain spectra for the both drug were observed and maximum wavelength was finally selected.

### 2. Selection of mobile phase:

To develop a précised and robust HPLC method for determination of Rizatriptan benzoate , its standard solution were injected in the HPLC system. After literature survey and solubility data different composition of mobile phase of different flow rates were employed in order to determine the best condition for effective separation of drugs.

### 3. Selection of column:

Initially different C8 and C18 columns were tried for selected composition of mobile phase and quality of peaks were observed for the drugs. Finally the column was fixed upon the satisfactory results of various system suitability parameters such as column efficiency, retention time, tailing factor / peak asymmetry of the peaks.

Other parameters such as flow rate, column temperature etc. were selected by varying its value up to certain levels and results were observed. The value at satisfactory results were obtained has been selected for the method. The final selection of chromatographic conditions as follows

## Optimized chromatographic conditions

### Preparation of Buffer ph 5. 0:

Dissove 2. 76 gm of potassium dihydrogen orthophosphate in 1000ml of HPLC water plus 5. 0 mlof Triethylamine. Mix and adjust PH 5. 0 with orthophosporic acid. Filter with 0. 45u nylon filter.

### Preparation of mobile phase:

The mobile phase was prepared by mixing Buffer: Methanol (80: 20). the solution was then filtered through 0. 45Î¼m membrane filter and sonicated.

### Preparation of standard stock solution:

Standard solution of the pure drug was prepared by dissolving 73. 0 mg of Rizatriptan benzoate in 100ml volumetric flask. The drugs were dissolved by using mobile phase as a diluent. Add about 50ml of diluent and sonicate to dissolve. Make up the volume with diluent. Mix well. Further dilute 5. 0ml of the above solution to 250ml with diluent, mix well.

### Preparation of sample solution:

Weight and transfer 10 intact tablet in into a100ml volumetric flask. Add about 50ml of diluent and sonicate for 15 min and make up the volume with diluent. Mix well, filter through 25 mm 0. 45 u nylon , discard 4ml filtrate. Further dilute 5ml of the solution to 250 ml with diluent and mix well.

## CONCLUSION

The evaluation of obtained values suggests that the proposed HPLC methods provide simple, precise, rapid and robust quantitative analytical method for determination of Rizatriptan benzoate in tablet dosage form. The mobile phase is simple to prepare and economical. After validating proposed method as per ICH guidelines and correlating obtained values with the standard values, satisfactory results were obtained.

Hence, the method can be easily and conveniently adopted for routine estimation of Rizatriptan benzoate in tablet dosage form.