

HPLC Method Development and Validation: A Review

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Abstract: Analytical methods development and validation play important roles in new discovery, development, manufacture of pharmaceutical drugs and various other studies related to humans and animals. Chromatography is the backbone of separation science and is being used in all research laboratories and pharmaceutical industries universally. High-performance liquid chromatography (HPLC) is dominant separation technique where analytes are separated by passage through a column packed with micro meter-sized particles. Now a day reversed-phase chromatography is the most commonly used separation technique in HPLC. This review covers the principle, types, instrumentation, validation, application of HPLC. Validation of HPLC method gives information about various stages and parameters like accuracy, precision, linearity, Limit of detection, Limit of quantification, specificity and robustness. Validation should be done as per regulatory guidelines such as ICH guidelines.

Keywords: Analytical, Chromatography HPLC, Method, Validation

I. INTRODUCTION

Analytical chemistry is a branch of chemistry concerned with the identification of components in substances, samples, and mixtures on a qualitative and quantitative level. The two types of analysis are qualitative and quantitative. Qualitative analysis is used to identify the components or analytes of a mixture or sample. Quantitative analysis determines the quantity of components or analytes in a mixture or sample^[1]. Analytical technique development and validation are crucial in the discovery, development, and production of pharmaceuticals. Every year, a considerable number of new medications enter the market, necessitating the development of innovative drug analysis processes. After development, validation of the novel analytical method is required^[2]. Analytical processes are designed and verified for active pharmaceutical ingredients (API), excipients, drug products, degradation products and related substances, residual solvents, and other chemicals. As a result, it has become a necessary component of regulatory requirements. Analytical methodologies lead to the establishment of official test methods. These methods are used in quality control laboratories to ensure the identity, purity, safety, efficacy, and performance of drug goods. During validation, an analytical method's accuracy, precision, linearity, Limit of Detection, Limit of Quantification, specificity, range, and robustness are all assessed. Validation should be done in compliance with regulatory norms like the ICH guidelines^[3].

II. CHROMATOGRAPHY^[4]

A method of separating and identifying the components of a complex mixture by differential movement through a two-phase system, in which the movement is effected by a flow of a liquid or a gas (mobile phase) which percolates through an adsorbent (stationary phase) or a second liquid phase.

2.1 Types of Chromatography

- Paper chromatography
- Column chromatography
- Thin layer chromatography
- Gas chromatography
- Ion exchange chromatography
- Two-dimensional chromatography

- High performance (pressure) liquid chromatography
- High pressure thin layer chromatography

2.2 HPLC

HPLC (high-performance liquid chromatography) or HPLC (high-pressure liquid chromatography) is an acronym for high-performance liquid chromatography or high-pressure liquid chromatography. Any sample that can be dissolved in liquid can be separated, identified, and quantified using HPLC [5]. Adsorption is the most important principle in liquid chromatography. It's a type of chromatography in which the mobile phase is liquid. The sample is presented as a liquid solution. The sample is injected into a porous material column (stationary phase) and a liquid phase column (mobile phase). High pressure generated by a pump moves the sample through the column with the mobile phase. The affinity of sample components for the stationary phase determines how far they go. The component that is more attracted to the stationary phase travels more slowly. The component that has a lower attraction for the stationary phase travels more quickly. The elements are isolated from one another [6]. N-hexane, methylene chloride, chloroform, methyl-t-butyl ether, Tetrahydrofuran (THF), Isopropanol (IPA), Acetonitrile (MeCN or CAN), Methanol (MeOH), and water are the most frequent solvents used in HPLC [7]. Efficiency (number of theoretical plates), retention factor, selectivity, resolution, and pressure are all important chromatographic parameters [8]. Chemical separation, purification, and identification are all applications of HPLC. Pharmaceuticals, environmental applications, forensics, clinical, food, and flavor are some of the other applications of HPLC [9].

2.3 Instrumentation of HPLC

Components of the HPLC system:

1. Solvent reservoir, mixing system and degassing system
2. High pressure pump
3. Sample injector
4. Column
5. Detector
6. Data Recording System

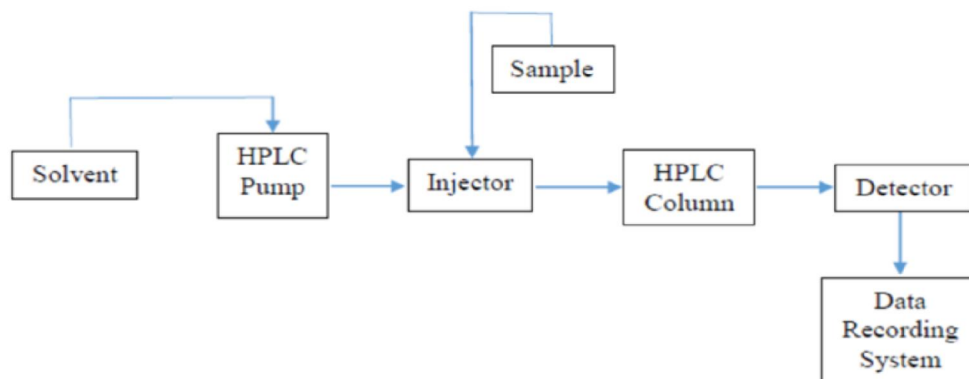


Figure 1: Instrumentation of HPLC

2.4 Solvent Reservoir, Mixing System, and Degassing System

The solvent is stored in the solvent reservoir (mobile phase). These are containers made of glass or stainless steel that does not discolour. Glass bottles are the most prevalent type of solvent reservoir [10]. Aside from delivering mobile phase, the pump must also combine solvents with extreme accuracy and precision. Low pressure mixing and high pressure mixing are the two types of mixing units [11]. The degassing mechanism eliminates air bubbles that have become trapped in the solution. Degassing is accomplished using ultrasonication and filtration techniques [10].

A. High-Pressure Pump

A pump's job is to push a liquid to flow at a certain rate. Millilitres per minute (ml/min) is the unit of measurement for flow rate. 1-2 ml/min is a typical flow rate. The pressure range of the pump is 6000-9000 psi (400-600 bar) ^[5]. Constant pressure pumps, syringe type pumps, and reciprocating piston pumps are all commonly used pump types ^[12].

B. Sample Injector

The sample injector introduces the liquid sample into the mobile phase. Between the pump and the column is a sample valve ^[10]. An injector (auto sampler) can inject the sample into the continuously flowing mobile phase stream, which transports it to the HPLC column. 5-20 microliters (l) is a typical sample volume ^[5]. Manual injectors and automatic injectors are the two types of injectors ^[12].

C. Column

A column is a location where actual component separation takes place. Stainless steel is used to construct the column. It has a length of 5-25 cm and a diameter of 2-4.6 cm on the inside.^[5]

D. Detector

The detector can identify and convert the various components that elute from the column into an electrical signal ^[5]. Specific detectors and bulk property detectors are the two types of detectors employed. UV-VIS detectors, photo diode array detectors, fluorescence detectors, and mass spectrometric detectors are examples of specific detectors. Refractive index detectors, electrochemical detectors, and light scattering detectors are examples of bulk property detectors ^[12].

E. Data Recording System^[9]

The output is recorded as a succession of peaks, with the area beneath each peak automatically calculated by the computer connected to the display.

2.3 HPLC Types ^[13, 14]

The phase system employed in the process determines the type of HPLC. The following HPLC types are commonly used in analysis:

A. Normal phase chromatography (NP-HPLC)

This approach separates analytes based on polarity and is also known as normal phase HPLC (NP-HPLC). A polar stationary phase and a non-polar mobile phase are used in NP-HPLC. The polar analyte interacts with the polar stationary phase and is held by it. As the polarity of the analyte rises, so does the adsorption strength, and the interaction between the polar analyte and the polar stationary phase lengthens the elution time.

B. Reversed Phase Chromatography

Reversed phase chromatography (RP-HPLC or RPC) uses an aqueous, moderately polar mobile phase and a non-polar stationary phase. The theory of RPC is based on hydrophobic interactions, which are caused by repulsive forces between a polar eluent, a comparatively non-polar analyte, and a non-polar stationary phase. The contact surface area around the non-polar section of the analyte molecule after association with the ligand in the aqueous eluent is proportional to the analyte's binding to the stationary phase.

C. Size Exclusion Chromatography

Size exclusion chromatography (SEC), commonly known as gel permeation chromatography or gel filtration chromatography, is a technique for separating particles based on their size. It can also be used to figure out the quaternary and tertiary structures of proteins and amino acids. This method is commonly used to determine the molecular weight of polysaccharides.

D. Ion Exchange Chromatography

The attraction between solute ions and charged sites bound to the stationary phase is the basis for retention in ion-exchange chromatography. Ions with the same charge are not allowed to mix. Purification of water, ligand-exchange chromatography, protein ion-exchange chromatography, high-pH anion-exchange chromatography of carbohydrates and oligosaccharides, and other applications use this type of chromatography.

E. Bio-affinity Chromatography

Separation based on reversible interactions between proteins and ligands. Ligands are covalently bonded to solid support on a bio-affinity matrix, which helps to keep proteins that interact with the column-bound ligands in place. Bioaffinity chromatography can result in very high purity in a single stage due to the specificity of the contact (10 - 1000-fold). There are two methods for eluting proteins coupled to a bioaffinity column:

1. **Biospecific elution:** elution buffer contains free ligand that competes with column bound ligand.
2. **Aspecific elution:** a change in pH, salt, or other factors that impair the interaction between the protein and the column-bound substrate.

III. STEPS IN METHOD DEVELOPMENT ^[15]

1. Physicochemical Properties of drug molecule
2. Selection of Chromatographic conditions
3. Developing the approach of analysis
4. Preparation of sample
5. Method Optimization
6. Method validation

3.1 Physico-Chemical Properties of Drug Molecule

A. Chemical Properties

The known and anticipated product's chemical structure Chemical structures are useful starting points for method development since they provide a scientific approach. Compare and contrast impurities, raw materials, by-products, intermediate, and degradation products with final products. The best input for selecting the common diluent for all compounds is to perform a solubility study at various pH values for all targeted molecules. The polarity of molecules must be considered while choosing a polar or non-polar HPLC column^[15]

B. pH and pKa Value of Compound:

Based on pH and pKa values, the compound's nature and polarity are assumed. When the pH equals pKa, the chemical is 50% ionised. Almost all pH-related changes take place within 1.5 units of pKa values. The molecule is either ionised or non-ionized outside of the range, and its retention does not alter substantially with pH ^[16].

3.2 Selection of Chromatographic Conditions

A set of initial conditions (column, mobile phase, and detectors) is chosen during technique development. In the majority of cases, reversed phase separations on a C18 column with UV detection are used. At this stage, you should decide whether to design an isocratic or a gradient technique.

A. Selection of Column

A column is the most important component of an HPLC system. During technique development, changing a column will have the biggest impact on analyte resolution. A properly chosen column can enable accurate and reliable chromatographic separation and analysis. A polar eluent and nonpolar (hydrophobic) stationary phase are used in reversed phase separation. The hydrophobic layer (or phase) is adhered to a robust framework that can withstand the high pressures seen in HPLC. Until recently, silica-based stationary phases were used in around 80% of all HPLC procedures ^[17].

- For initial rapid method development with usual sample types, C18 and C8 bonded phases are the best.

- Shortening method development time by selecting the most sample-appropriate bonded phase and using special, targeted bonded phases, such as SB-Aq for polar, difficult-to-retain chemicals.
- To shorten method development time, rapid resolution columns are required.
- Rapid resolution columns allow for high throughput rapid analysis by reducing both gradient and isocratic analysis time ^[18].

B. Chromatographic Conditions

The next step in the technique development process is to choose appropriate chromatographic conditions. This includes things like temperature, mobile phase composition, pH, and flow rate, among other things. At this stage, you should decide whether to design an isocratic or a gradient technique. In the majority of situations, reversed-phase separations on a C18 column with UV detection are used ^[19].

C. Optimization of Mobile Phase

The mobile phase pH might be one of the most critical variables in the control of retention in a reversed-phase HPLC (RP-HPLC) separation when samples contain ionizable chemicals. pH, on the other hand, can cause a slew of issues if it isn't correctly managed. Most mobile phases require pH regulation because most substances evaluated by RP-HPLC have one or more acidic or basic functional groups. As a result, buffers are commonly used [20]. Water and buffer are weak solvents in RP chromatography, while CAN, methanol, and THF are strong solvents. At high flow rates, methanol's high viscosity may limit the usage of smaller particle size or longer columns. Acetonitrile is a somewhat expensive substance. UV absorbance at low wavelengths; high viscosity ^[21].

D. Column Temperature

Temperature is a challenge to employ in HPLC technique development since it can have unforeseen impacts on selectivity. The usage of higher temperatures will accomplish the following: Reduce the viscosity and back-pressure of the mobile phase. This may enable you to use higher flow rates or longer columns with smaller particle sizes. Eliminate elution time. Improve the method's repeatability (as opposed to operating at room temperature). However, it is impossible to say whether using higher temperatures will aid or impede a particular separation. Improvements in one area of the chromatogram are almost invariably accompanied by losses in another part of the same chromatogram for complex separations ^[22].

E. Selection of Wavelength and Detector

To pick the UV detector nm for all mentioned compounds, they must have UV/Visible and FT-IR spectrums. The key source for understanding the activity of functional groups is FTIR spectral data ^[25].

3.3 Sample Preparation

In a perfect world, the sample would disintegrate in the first mobile phase. If stability or solubility issues prevent this, formic acid, acetic acid, or salt might be added to the sample to promote solubility. As long as the volume of the sample added is modest in comparison to the column volume, these additions have no effect on separation. When high sample volumes are used, the only effect is that an extra peak or two may elute in the void volume following sample injection. Sample preparation is an important aspect of HPLC analysis because it ensures a consistent and homogeneous solution that can be injected onto the column. The goal of sample preparation is to provide a sample aliquot that is reasonably free of interferences, won't harm the column, and is compatible with the desired HPLC procedure, meaning that the sample solvent will dissolve in the mobile phase without impacting sample retention or resolution. Sample preparation begins with the collection of the sample and continues with the injection of the sample onto the HPLC column. All of these procedures are necessary for sample preparation and have a significant impact on the method's accuracy, precision, and ease of use ^[23, 24].

3.4 Method Optimization

After obtaining adequate separations, the experimental settings should be tweaked to obtain desired separations and sensitivity. Planned/systemic inspection of parameters such as pH (if ionic), mobile phase components and ratios, gradient, flow rate, temperature, sample quantities, injection volume, and diluents solvent type will be used to establish stability

indicating assay experimental circumstances. The compositions of the mobile and stationary phases must be considered. Mobile phase parameter optimization is always prioritised over stationary phase parameter optimization since it is more easier and more convenient. Only the parameters that are likely to have a substantial effect on selectivity in the optimization must be studied to reduce the number of trial chromatograms involved [25].

3.5 Method Validation

Analytical method validation is defined as "documented evidence that a certain process will consistently generate a product that meets its set criteria and quality features."

A. Accuracy

Trueness is a term used to describe it. Accuracy is a measure of how near the measured and real values are. When compared to the reference standard, The analyte was spiked into a blank matrix, then the analyte was recovered using the standard addition method. For drug substances and products, the reported accuracy limitations are 98.0 – 102.0 % and 97.0 – 103.0 %, respectively. A range of 50 to 150 % average recovery may be tolerated for impurity determination [26].

B. Precision

The closeness of agreement between a set of measurements obtained from multiple sampling of the same homogeneous sample under the stipulated requirements of repeatability, intermediate precision, and reproducibility is expressed as the precision of an analytical technique [27,29]. RSD should be 2 % to assure technique precision for key analytes. RSD of 5-10% is usually acceptable for low-level contaminants.

- **Repeatability:** The term "repeatability" refers to how well a method works in one lab and on one instrument on a particular day. Calculating the 'Repeatability limit' is useful. Preparing a minimum of 6 determinations at 100% of the test concentration is required.
- **Reproducibility:** In both qualitative and quantitative aspects, reproducibility relates to how well a procedure operates from lab to lab, day to day, analyst to analyst, and instrument to instrument. Calculating the reproducibility limit, 'R', is useful.
- **Intermediate precision:** Intermediate precision refers to how well a method works qualitatively and quantitatively inside a single lab, but now also from instrument to instrument and day to day, as measured by the % RSD of assays.

B. Linearity

The linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of analyte in the sample. Test results should be evaluated by appropriate statistical methods. For example, by calculation of a regression line by the method of least squares. [28]

Table 1: Linearity ranges and acceptance criteria for various pharmaceutical methods

Level Acceptance criteria Assay (5 levels, 50-150% of label claim)	Correlation coefficient, $R^2 = 0.999$
Dissolution (5-8 levels, 10-150% of label claim)	% y intercept NMT 2.0%, $R^2 = 0.99$
Related substances (5 levels, LOQ to acceptance)	% y intercept NMT 5.0%, $R^2 = 0.99$

C. Detection Limit: [29, 30]

The lowest amount of analyte in a sample that can be detected but not necessarily quantified as an exact number is the detection limit of an individual analytical method.

The formula for calculating LOD is,

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

Where, δ = standard deviation of intercepts of calibration curves.

S = the slope of linearity plot.

Visually, the detection limit can be identified. ratio of signal to noise the response's standard deviation and the slope.

D. Quantitation Limit: ^[30]

The lowest amount of analyte in a sample that can be quantitatively measured with sufficient precision and accuracy is the quantitation limit of a particular analytical process. The quantitation limit is a parameter of quantitative tests for low amounts of chemicals in sample matrices, and it is used to determine contaminants and degradation products in particular. It can be determined by following formula,

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

Where, δ = standard deviation of response.

S = Mean of slopes of the calibration curves.

E. Specificity

The capacity to measure the intended analyte in the presence of components that are expected to be present is referred to as specificity. Impurities, degradants, matrix, and other materials are common. In every scenario, the peak purity value must be greater than 0.999 (for Agilent systems) or the purity angle must be smaller than the threshold (for Waters systems)^[31].

F. Range

The range of an analytical technique is the distance between the sample's upper and lower concentrations of analyte for which the analytical procedure has been shown to have a satisfactory level of precision, accuracy, and linearity. ^[31]

G. Robustness

The robustness of an analytical procedure is a measure of its ability to remain unaffected by modest but deliberate changes in method parameters, and it indicates its reliability in routine use. Flow rate, column temperature, sample temperature, pH, and mobile phase composition are some of the variable process parameters in HPLC. ^[32, 33]

H. System suitability

Liquid chromatographic procedures include system appropriateness tests as a standard procedure. They are used to ensure that the chromatographic system's detection sensitivity, resolution, and reproducibility are sufficient for the analysis. The tests are founded on the idea that the equipment, electronics, analytical activities, and samples to be studied are all part of a larger system that may be evaluated. The adequacy of the used approach was determined by measuring factors such as peak resolution, number of theoretical plates, peak tailing, and capacity. ^[33]

Table 2: System suitability parameters and its limits

Parameter	Limits
Resolution (Rs)	>2.0
Repeatability (RSD)	<1.0 For Five Replicates
Plate Count (N)	>2000
Tailing Factor(TF)	2.0
Separation Factor(SF)	>1.0

Parameters used in the system suitability tests:

- Number of theoretical plates or Efficiency (N)
- Capacity factor (K)
- Separation or Relative retention (α)
- Resolution (Rs)
- Tailing factor (T)
- Relative Standard Deviation (RSD)

I. Force Degradation

Force degradation of the drug material can aid in identifying the most likely degradation products, which can then aid in determining the molecule's degradation pathways and inherent stability. The parts of force degradation are as follow:

- Acidic hydrolysis
- Alkaline hydrolysis
- Hydrolytic
- Oxidative degradation
- Solid state stability
- Thermal degradation
- Photolytic degradation

IV. ADVANTAGES OF HPLC

- It is exceptionally fast and efficient, as well as accurate and repeatable.
- The same column can be used for repetitive, repeatable analysis.
- It allows for a great degree of selectivity in some analyses.
- It provides a better level of resolution.
- It is largely automated, thus basic HPLC runs can be conducted with minimal training.
- It is versatile and exceedingly precise when it comes to detecting and quantifying chemical components.

REFERENCES

- [1]. Sharma S, Goyal S, Chauhan. A review on analytical method development and validation. International Journal of Applied pharmaceuticals Nov 2018;10(6):8
- [2]. Rina R, Baile M, Jain A. A Review: Analytical method development and validation. Sys Rev Pharm 2021; 12(8): 450-454
- [3]. Ravisankar P, Gowthami S, Devlala G . A review on analytical method development. IJR PB 2014 ;2(3)1183
- [4]. Dr.Ravisankar S., Text book of Pharmaceutical analysis, Rx Publications, 3rd Edition, 2.2-2.5, 13.1, 17.14-17.18 & 18.2-18.6.
- [5]. Chawla G, Chaudhary KK. A review of HPLC technique covering its pharmaceutical, environmental, forensic, clinical and other applications. Int J Pharm Chem Anal. 2019; 6(2): 27-39.
- [6]. Yadav V, Bharkatiya M. A review on HPLC method development and validation. Res J Life Sci. 2017; 2(6): 166-178.
- [7]. Mc Polin . An introduction to HPLC for pharmaceutical analysis. Lulu. 2009.
- [8]. Ravisankar P, Gowthami S, Rao GD. A review on analytical method development. Indian J Res Pharm Biotech. 2014; 2(3): 1183.
- [9]. Malviya R, Bansal V, Pal OP, Sharma PK. High performance liquid chromatography: a short review. J Glob Pharma Technol 2010; 2(5): 22-26.
- [10]. Jena AK. HPLC: highly accessible instrument in pharmaceutical industry for effective method development. Pharm Anal Acta 2011; 3.
- [11]. Agilent Technologies. The LC Handbook Guide to LC Columns and Method Development. Agilent Technologies. 2016; 16.
- [12]. Hamilton RJ, Sewell PA. Introduction to high performance liquid chromatography. Springer. 1982; 1-12.
- [13]. Abidi,S.L. High-performance liquid chromatography of phosphatidic acids and related polar lipids. J. Chromatogr. 1991; 587: 193-203.
- [14]. Hearn M.T.W. Ion-pair chromatography on normal and reversed-phase systems. Adv. Chromatogr. 1980; 18: 59–100.
- [15]. Phani.R.S.Ch, K.R.S. Prasad and Useni Reddy Mallu; Scientific approach for RP-HPLC method development: complete review; International Journal of Science Innovations and Discoveries; 2012, 2 (6): 218-228.
- [16]. ZirChrom®-EZ & ZirChrom®-MS; Method Development Guide; Column Use Tip;

- [17]. www.zirchrom.com. 1-28.
- [18]. M.W. Dong, Modern HPLC for practicing scientists, John Wiley & Sons, New Jersey, (2006).
- [19]. Phenomenex; Part 3. The Role of the Mobile Phase in Selectivity (online) slide 1-56.
- [20]. Philip J. Koerner, Ph.D. Thailand September 2013; General Principles of HPLC Method Development; Part 1. General Chromatographic Theory slide 1-53.
- [21]. Sowjanya P., Subashini D., Rakesh S., A Review on Method Development and validation by HPLC, RRJPPS, Jan-Mar 2015; 4(1).
- [22]. P. D. Sethi; High Performance Liquid Chromatography, Quantitative Analysis of Pharmaceutical Formulations; 1st edition, New Delhi: CBS Publishers And Distributors, 2001; 1, 3-11, 116-120.
- [23]. Ashwini B. Sambherao, Bhushan A. Bhairav and Dr. R. B. Saudagar "Analytical method development and validation by RP-HPLC and UV spectrophotometric methods" ; European Journal of Biomedical and Pharmaceutical sciences; Vol. 4(10);1-6.
- [24]. M. A. Potdar; Pharmaceutical Quality Assurance; Nirali Prakashan; 8.28-8.31.
- [25]. Willard H. H., Merritt L. L., Dean J. A., Settle F. A., Instrumental Methods of Analysis, Seventh Edition, CBS Publishers and Distributors Pvt. Ltd., 118-148, 598-607.
- [26]. Hearn Perkin Elmer RA. www.standardbase.com., A Guide to Validation in HPLC Based on the Work of G. M. Holland.
- [27]. M.W. Dong, Modern HPLC for practicing scientists, John Wiley & Sons, New Jersey,(2006).
- [28]. B. Nigovic, A. Mornar, M. Sertic, Chromatography-The Most Versatile Method of Chemical Analysis, Intech. 385-425 (2012).
- [29]. Lindner W, Wainer IW, Requirements for initial assay validation and publication in J Chromatography B. J Chromatogr. 2006; 707:1.2.
- [30]. Validation of Compendial Procedures, United State Pharmacopeia, USP 36 NF, 27 (2)(2010).
- [31]. Validation of Analytical Procedures: Text and Methodology, International Conferences on Harmonization, Draft Revised (2005), Q2 (R1).
- [32]. Boulanger B, Chiap P, Dewe W, Crommen J, Hubert P. An analysis of the SFSTP guide on validation of chromatographic bioanalytical methods: progress and limitations. J Pharm Biomed Anal. 2003; 32:753-65.
- [33]. Christain GD, Analytical Chemistry, 6th Edn. USA: John Wiley & Sons Inc, (2001). Mrs. S. Durga Mounika, RP-HPLC Method Development and Validation for simultaneous Estimation of paracetamol and alprazolam in bulk and pharmaceutical