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Review Article

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Method development and validation of RP-HPLC method in tablet dosage form: A Review

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Abstract HPLC is the dominant separation technique for drug detection, separation and quantification. To optimize the method, a number of chromatographic parameters were analyzed, such as sample pre-treatment, selection of mobile phase, columns, detector selection. The aim of this article is to review the development, optimization and validation of the method. HPLC method development depends on the chemical structure of molecules, synthetic route, solubility, polarity, pH and pKa values and activity of functional groups, etc. Validation of HPLC method according to ICH guidelines provides information on various phases and known characteristics such as accuracy, specificity, linearity limit of detection, limit of quantification.

Keywords: Chromatography, HPLC, Reversed Phase, Validation

Introduction

Basically, HPLC is a liquid chromatographic technique. It involves separation of complex mixtures and quantifications of the resolved components. HPLC provides reliable quantitative precision and accuracy for the determination of the active pharmaceutical ingredients and related substances in the same run. HPLC has excellent reproducibility. By the judicious choice of the column, HPLC is applicable to a wide range of compound types. Chiral molecules can be separated into their respective enantiomers by HPLC.HPLC, particularly reversed-phase HPLC is currently the most suitable method for meeting most of the criteria for quantitative analysis of many drugs. Depending on the type of the stationary phase packed in the column the separation process may be by adsorption, partition, exclusion or ion- exchange principles [1-2].

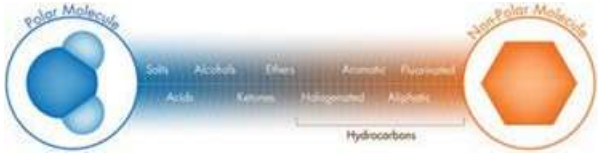
Method Development

There are several valid reasons for developing new methods of analysis for determination of a compound or drug. A suitable method may not be available for the analyte in the specific sample matrix. Existing methods may be erroneous, contamination–prone, or they may be unreliable due to poor accuracy or precision. Existing methods may not provide adequate sensitivity or analyte selectivity in samples of interest. Newer techniques and instrumentation may have evolved that provide scope for improved methods including improved analyte identification or detection limits and greater accuracy and precision.

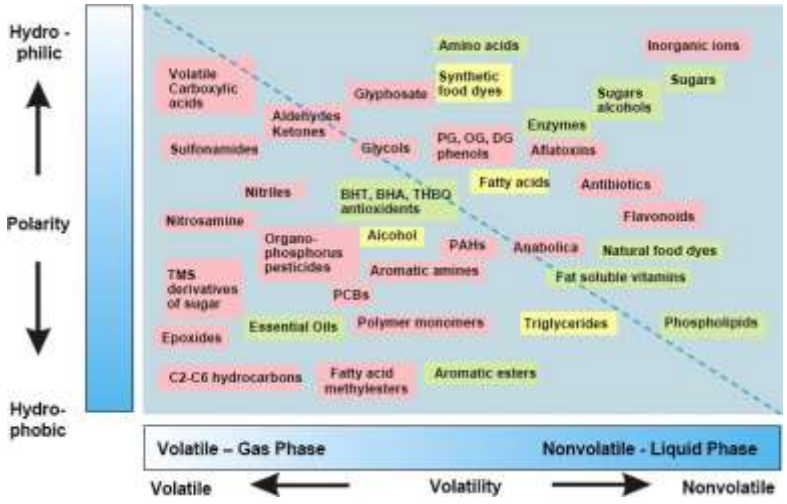
There are three characteristics that can be used to separate compounds using-

Polarity

Functional groups in the chemical structure often determine whether the molecule is polar or non-polar. These chemical structures determine if the compound is polar or non-polar. Figures below represent polarity of compounds based on their chemical structure.



Chromatographic Polarity Spectrum by Analyte Functional Group



Chromatographic Polarity Spectrum by Analyte class

The basis for the separation based on polarity is affinity or attraction between the compound of interest and the stationary phase. Compound with similar chromatographic polarity has more affinity towards each other. For example, polar – polar or nonpolar – nonpolar have more affinity. Compound with different chromatographic polarity has less affinity or even repulsion towards each other. For example, polar - nonpolar interactions. Compounds in the sample that have similar polarity to the stationary phase will elute later as they have strong affinity whereas compounds that have the same polarity to that of the mobile phase will elute faster. Following figures represents typical chromatographic polarity ranges for mobile phases, stationary phases, and sample analytes, respectively [3,4,5].

Mobile Phase Chromatographic Polarity Spectrum

Mobile phase molecules that compete effectively with analyte molecules for the attractive stationary phase sites displace these analytes, causing them to move faster through the column. Water is at the polar end of the mobile-phase-solvent scale, while hexane, an aliphatic hydrocarbon, is at the non-polar end. In between, single solvents, as well as miscible-solvent mixtures, can be placed in order of elution strength. Which end of the scale represents the 'strongest' mobile phase depends upon the nature of the stationary phase surface where the competition for the analyte molecules occurs.

Stationary Phase Particle Chromatographic Polarity Spectrum

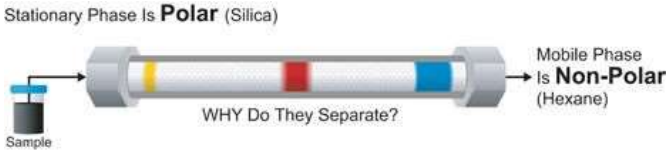
Silica has an active, hydrophilic [water-loving] surface containing acidic silanol [silicon-containing analog of alcohol] functional groups. Consequently, it falls at the polar end of the stationary-phase scale shown in Figure below. The activity or polarity of the silica surface may be modified selectively by chemically bonding to it less polar functional groups [bonded phase]. Examples shown here include, in order of decreasing polarity, cyanopropylsilyl- [CN], n-octylsilyl- [C8], and n- octadecylsilyl- [C18, ODS] moieties on silica. The latter is a hydrophobic [water- hating], very non-polar packing [6,7,8].

Compound/Analyte Chromatographic Polarity Spectrum

Among solvents of similar strength, the chromatographer considers which phase combination may best exploit the subtler differences in analyte polarity and solubility to maximize the selectivity of the chromatographic system. Like attracts like, but, as you probably can imagine from the discussion so far, creating a separation based upon polarity involves knowledge of the sample and experience with various kinds of analytes and retention modes. To summarize, the chromatographer will choose the best combination of a mobile phase and particle stationary phase with appropriately opposite polarities. Then, as the sample analytes move through the column, the rule like attracts like will determine which analytes slow down and which proceed at a faster speed [9].

Normal-Phase HPLC

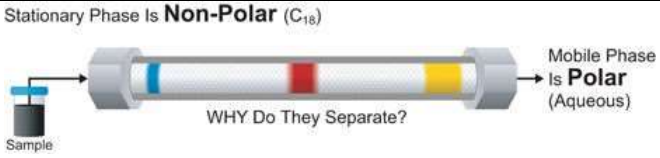
A polar stationary phase with a non-polar mobile phase. The figure below represents a normal-phase chromatographic separation of three compounds. The stationary phase is polar and retains the yellow polar compound most strongly because of strongest polar-polar interactions. The relatively non-polar blue compound elutes quickly since the blue compound has the strongest nonpolar-nonpolar interactions with the stationary phase [10].



Normal-Phase Chromatography

Reversed-Phase HPLC

Reversed-Phase HPLC is just the opposite of the normal phase, namely the use of a polar mobile phase and a non-polar stationary phase. The figure below illustrates the three-compound mixture being separated. The most strongly retained is the more non-polar blue compound, as its attraction to the non-polar stationary phase is greatest. The polar yellow compound is weakly retained because of its strong affinity aqueous mobile phase or weak affinity towards the stationary phase.



Reversed-Phase Chromatography

The table below presents a summary of the phase characteristics for the two principal HPLC separation modes based upon polarity.

Phase Characteristics for Separations Based on Polarity

Separation Mode	Stationary Phase [particle]	Mobile Phase [solvent]
Normal phase	Polar	Non-polar
Reversed phase	Non-polar	Polar

Hydrophilic-Interaction Chromatography [HILIC]

HILIC is a variant of normal phase chromatography. Unlike normal phase chromatography gradient elution used for HILIC separations. Buffer salts can be used to the mobile phase to keep ionizable analytes in a single form. Analytes are eluted in order of increasing hydrophilicity. With HILIC chromatography polar compounds receive the highest degree of retention [11-12].

Hydrophobic-Interaction Chromatography [HIC]

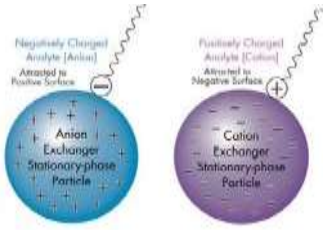
Chromatography maintains the structure of large molecules in an aqueous solution. Organic solvents which will denature the large molecules are not used in HIC. HIC works by hydrophobic interaction of large molecules with a hydrophobic stationary phase. The HIC chromatography starts with higher salt concentrations in water which encourage the proteins to be salted out on the packing. Gradient separations are typically run by decreasing salt concentration. Analytes are eluted in order of increasing hydrophilicity.

Electrical Charge [Ion-Exchange Chromatography [IEC]]

IEC Separations are based on polarity. Cation exchange is used to retain and separate positively charged ions on a negative surface. Anion exchange is used to retain and separate negatively charged ions on a positive surface. With each type of ion exchange, there are at least two general approaches for separation and elution.

Strong ion exchangers bear functional groups [e.g., quaternary amines orsulfonic acids] that are always ionized. They are used to retain and separate weakions. These weak ions may be eluted by displacement with a mobile phase containing ions that are more strongly attracted to the stationary phase sites. Alternately, weak ions may be retained on the column, then neutralized by in situ changing the pH of the mobile phase, causing them to lose their attraction and elute.

Weak ion exchangers [e.g., with secondary-amine or carboxylic-acid functions] may be neutralized above or below a certain pH value and lose their ability to retain ions by the charge. When charged, they are used to retain and separate strongions. If these ions cannot be eluted by displacement, then the stationary phase exchange sites may be neutralized, shutting off the ionic attraction, and permitting elution of the charged analytes. Figure and Table below outlines the separationmechanism and guidelines for the principal categories of ion exchange [13,14,15].

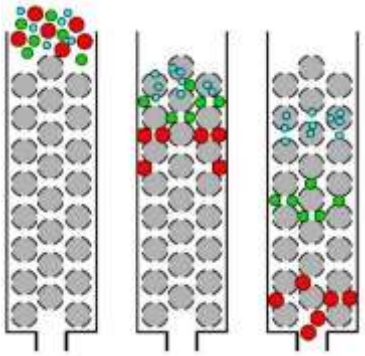


Analyte Type	*Weak* ACID e.g., pK_a ~ 5		*Strong* ACID	*Weak* BASE e.g., pK_a ~ 10		*Strong* BASE
Charge State vs. pH*	No charge at pH < 3	− [anion] at pH > 7	− [anion] Always Charged	+ [cation] at pH < 8	No Charge at pH > 12	+ [cation] Always Charged

Stationary Phase Particle	*Strong* Anion Exchanger	*Weak* Anion Exchanger e.g., pK_a ~ 10		*Strong* Cation Exchanger	*Weak* Cation Exchanger e.g., pK_a ~ 5	
Charge State vs. pH*	+ Always Charged	+ at pH < 8	No Charge at pH > 12	− Always Charged	No Charge at pH < 3	− at pH > 7
Mobile Phase pH Range						
to Retain analyte [capture]	pH > 7	pH < 8		pH < 8	pH > 7	
to Release analyte [elute]	pH < 3	pH > 12		pH > 12	pH < 3	

Molecular Size

Chromatography based on molecular size is performed on stationary phases that have a pore-size distribution over a range. Smaller molecules penetrate more of the pores on their passage through the bed. Larger molecules may only penetrate pores above a certain size so they spend less time in bed. The biggest molecules may be totally excluded from pores and pass only between the particles, elute very quickly in a small volume.



Size-Exclusion Chromatography

Method Optimization

During the optimization stage, the initial sets of conditions evolved from the first stages of development are improved or maximized in terms of capacity, resolution and peak shape, detection limits, plate counts asymmetry, limit of Quantitation, elution time, and overall ability to quantify the specific analyte of interest.

The parameters that are to be optimized during method development are:

a. Mode of separation
b. Selection of stationary phase
c. Selection of mobile phase
d. Selection of detector

Selection of mode of separation

In the reverse phase mode, the mobile phase is more polar than the stationary phase. For the separation of polar or moderately polar compounds, the most preferred mode is a reverse phase. The nature of the analyte is the primary factor in the selection of the mode of separation. Another factor is the nature of the matrix.

Selection of stationary phases / column

Selection of the column is the first and the most crucial step in method development. Some of the important parameters considered while selecting chromatographic column are

a. Length and diameter of the column
b. Packing material.
c. Shape of the particles.
d. Size of the particles.
e. Percent of Carbon loading
f. Pore volume.
g. Surface area.
h. End-capping

The column is selected depending on the nature of the solute and the information about the analyte. Reversed-phase mode of chromatography facilitates a wide range of column like ethyl silane (C2), butylsilane (C4), octylsilane (C8), octadecylsilane (C18), base deactivated silane (C18), BDS phenyl, cyanopropyl (cn), nitro amino etc. C18 was chosen for this study since it is most retentive. Usually, longer columns provide better separation due to higher theoretical plate numbers. As the particle size decreases the surface area available for coating increases. Column with 5 µ particle size gives the best compromise of efficiency, reproducibility and reliability. In this case, the column selected had a particle size of 5 µ and an internal diameter of 4.6 mm [16,17].

Peak shape is equally important in method development. Columns that provide symmetrical peaks are always preferred. A useful and practical measurement of peak shape is the peak asymmetry factor and peak tailing factor. Peak asymmetry is measured at 10% of full peak height and peak tailing factor at 5%. Reproducibility of retention times and capacity factor is important for developing a rugged and repeatable method.

A column which gives separation of all the impurities and degraded products from one another and from the analyte and which is rugged for variations in mobile phase shall be selected. Selection of the mobile phase Selection and optimization of the mobile phase is carried out to achieve optimum separation of all the individual impurities and degraded products from one another and from the analyte. In liquid chromatography, retention of the solute is governed by the solute distribution factor,

This reflects the interactions of the solute – stationary phase, solute – mobile phase and the mobile phase – stationary phase. The parameters following, to be taken into consideration while selecting and optimizing the mobile phase.

a. Buffer
b. pH of the buffer
c. Mobile phase composition

Buffer

Buffer and its strength decide the peak symmetries and separations. Some of the most commonly employed buffers are:

- Phosphate buffersprepared using salts like KH_2PO_4, K_2HPO_4, NaH_2PO_4, Na_2HPO_4, etc
- Phosphoric acid buffers prepared using H_3PO_4.
- Acetate buffers – Ammonium acetate, Sodium acetate, etc.
- Acetic acid buffers prepared using CH_3COOH.

The retention times also depend on the molar strengths of the buffer – Molar strength is increasingly proportional to retention times. The strength of the buffer can be increased, if necessary, to achieve the required separations. The solvent strength is a measure of its ability to pull analytes from the column. It is generally controlled by the concentration of the solvent with the highest strength [18].

pH of the buffer

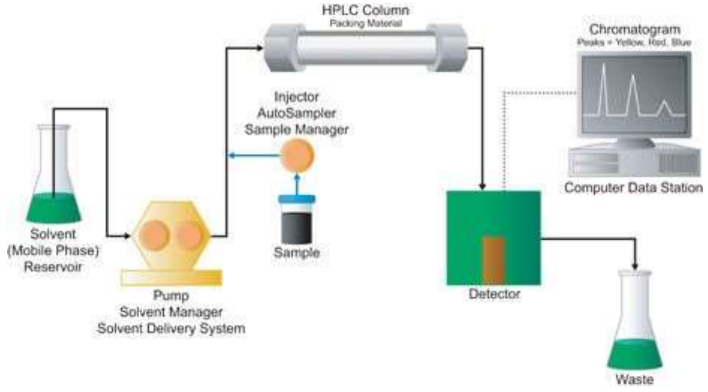
The pH influences the elution properties by controlling the ionization Characteristics and so plays a key role in achieving the chromatographic separations. Experiments were conducted using buffers having different pH to obtain the required separations. It is important to maintain the pH of the mobile phase in the range of 2.0 to 8.0 as most columns do not withstand pH outside this range. The siloxane linkage area is cleaved below pH 2.0 while at pH value above 8.0 silica may dissolve.

Mobile phase composition

Most chromatographic separations can be achieved by choosing the optimum mobile phase composition. Good selectivity can be achieved by choosing the qualitative and quantitative composition of aqueous and organic portions. Most widely used solvents in reverse phase chromatography are methanol and Acetonitrile. Experiments were conducted with mobile phases having buffers with different pH and different organic phases to check for the best separation between the impurities. A mobile phase which gives separation of all the impurities and degradants from each other and from analyte peak and which is rugged for variation of both aqueous and organic phase by at least +0.2% of the selected mobile phase composition.

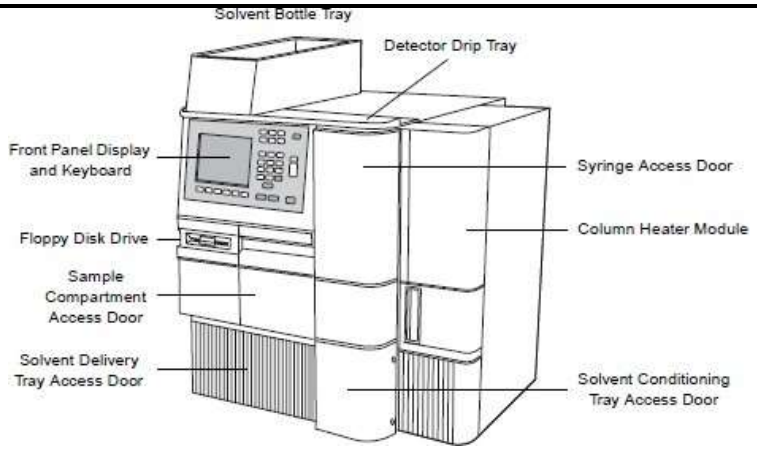
HPLC Instrumentation

The components of a basic high-performance liquid chromatography [HPLC] system is shown in the simple diagram in Figure below.



High-Performance Liquid Chromatography [HPLC] System

The author used a waters Alliance 2695 HPLC system through the study. The system was equipped with a C18 column, a quaternary pump, a waters 2996 PDA detector, a column heater/cooler and an auto-injector. The following is the description of the different components of the system. Empower 2 was the data acquisition software employed [19-20].

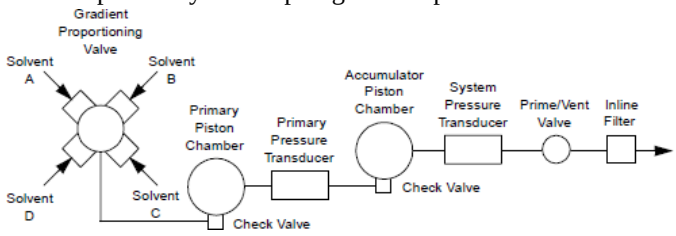


Waters Alliance 2695 Separations Module

Different components of the waters alliance HPLC system were given below.

Solvent Management System

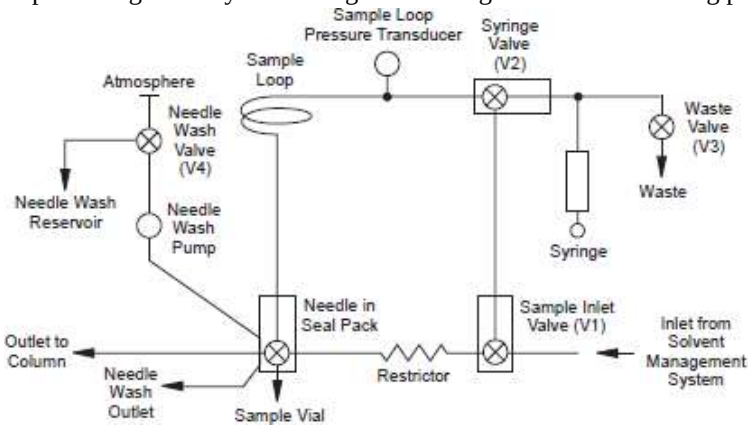
The solvent management system blends and delivers solvents from the reservoir bottles to the sample management system in a pulse-free, low-dispersion flow path. The solvent management system incorporates a fluidic design that uses a serial flow path and two independently driven plungers for optimal flow control.



Fluidic Path through the Solvent Management System

Sample Management System

The sample management system holds and positions the sample vials and injects the samples into the solvent flow. Four valves in the sample management system control the flow of solvent, sample, and needle washes solvent. The flow path through the sample management system changes according to the function being performed.



Fluidic Path through the Sample Management System

Column Heater

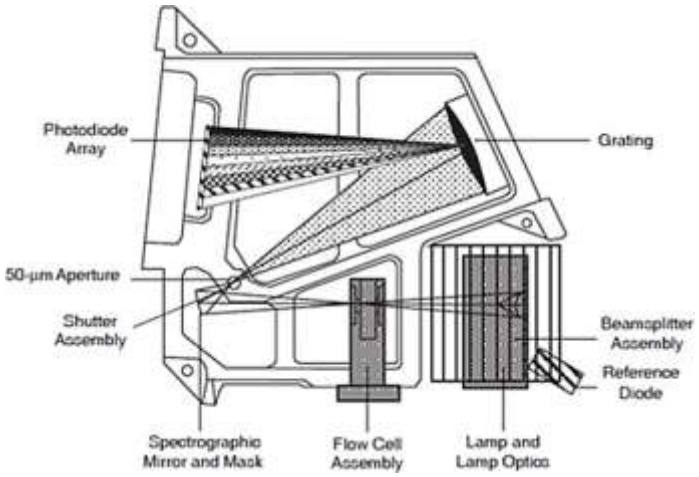
The column heater allows you to maintain the column at temperatures from 5 °C above ambient (minimum of 20 °C) to 60 °C.

Sample Heater/Cooler

The sample heater/cooler allows you to maintain the sample compartment at temperatures from 4 to 40 °C.

Waters 2996 PDA Detector

The 2996 Detector is an ultraviolet/visible light (UV/Vis) spectrophotometer with 512 photodiodes, optical resolution of 1.2 nm per diode, operating wavelength range from 190 to 800 nm. The light path through the optics assembly of the 2996 Detector is shown in Figure below. Optics Assembly Components are given in Table below [21-22].



Optics Assembly Light Path

Optics Assembly Components

Component	Function
Lamp and lamp optics	Focuses light from the deuterium source lamp via a mirror through a beam splitter to the flow cell.
Beam splitterand reference diode	Reflects part of the light back to a reference diode, which measuresthe intensity of the light emitted by the lamp. The detector uses this measurement to keep the lamp output constant.
Flow cell assembly	Houses the segment of the flow path (containing eluent and sample) through which the polychromatic light beam passes. Thisarrangement of optical components, with the flow cell positioned between the lamp and the grating, is commonly called reversedoptics [23]

Reference

[1]. Waters 2695 Separations Module, Operator's Guide, 71500269502, Revision B.
[2]. Waters 2996 PDA Detector, Operator's Guide71500023202, Revision C.
[3]. Lloyd.R.Synder, Joseph J. Kirkland, Joseph L. Glajch , Practical HPLC Method Development, Second Edition, John Wiley & Sons Inc. 1997.
[4]. Validation of analytical procedures: Text and Methodology, ICH Harmonized Tripartite Guideline Q2 (R1), Commission of the European Communities (2005).

[5]. Ashutoshkar, Pharmaceutical Drug Analysis; 2nd ed; New AGF International Pvt. Ltd. Publisher, New Delhi, 2009, pp 1-4
[6]. Verma KM, Analytical Chemistry Theory and Practice; 3rd ed; CBS, Publisher and distributors, New Delhi, 2007, pp 1-8
[7]. Skoog DA., Holler FJ., and Crouch SR., Instrumental Analysis; 8th ed; Cengage Learning India Pvt. Ltd., New Delhi, 2010, pp 1-4
[8]. Vidhyasagar G. Basic of Drug Analysis; Pharmamed Press; Hyderabad, 2009, pp 1-7
[9]. S. Naazneen and A. Sridevi. Development and validation of stability indicating RP-HPLC method for simultaneous estimation of empagliflozine and linagliptin in tablet formulation. Der Pharmacia Lettre, 2016, 8 (17):57-65
[10]. Sun Z. Determination of the related substances of linagliptin by HPLC. Zhongguo Xiandai Yingyong Yaoxue, 2016, 33(4):451-455.
[11]. FDA Guidance for Industry (2000)-Analytical Procedures and Method Validation, Chemistry, Manufacturing, and Controls Documentation, Center for Drug Evaluation and Research (CDER) and Center for Biologics Evaluation and Research (CBER).
[12]. Shirisha S, Haque M A, Sireesha D, Bakshi V and Harshini S. Development and validation of RP-HPLC method for simultaneous estimation of metformin and linagliptin in combined pharmaceutical dosage form. International Journal of Pharma Research and Health Sciences, 2014, 2, 491-95.
[13]. www.en.wikipedia.org/wiki/Ultraviolet%E2%80%93visiblespectroscopy
[14]. Conners KA. A Textbook of Pharmaceutical Analysis; 3rd ed; Wiley India Pvt. Ltd, New Delhi, 2007, pp 1-6
[15]. www.bouman.chem.georgetown.edu/s00/handout/spectrometer.htm
[16]. Sharma BK. Instrumental Method of Chemical Analysis; 25th ed; GOEL Publishing House, New Delhi, 2006, pp 678-682
[17]. Lee DC and Webb ML., Wiley India Pvt. Ltd., Blackwell Publishing, New Delhi,2008, pp 90
[18]. Munson JB.Pharmaceutical Analysis Modern Method; International Medical Book Distributors, Mumbai, 2001, pp 378
[19]. Willard HH., Merritt LL., Dean JA., and Settle FA. Instrumental Method of Analysis;7th ed; CBS Publisher and Distributors,New Delhi, 2001, pp 170
[20]. Belal F et.al. Stability-indicating HPLC Method for the Determination of Atenolol in Pharmaceutical Preparations. J Chromat Separation Techniq. 2013; 4(1): 1-7.
[21]. Chetta N. et.al. Development and validation of a stability indicating high performance liquid chromatographic (HPLC) method for Atenolol and hydrochlorothiazide in bulk drug and tablet formulation. Int J Chem tech res. 2013; 1(3): 654-662.
[22]. Kumar GS. et.al. Development and validation of RP-HPLC method for simultaneous estimation of Atenolol and Chlorthalidone in Bulk and dosage form. Int Res J Pharma 2013; 3(10): 215-19.