The Pharmaceutical and Chemical Journal, 2024, 11(3):140-148

Available online www.tpcj.org

Review Article

ISSN: 2349-7092
CODEN(USA): PCJHBA

Analytical Method Development and Validation by RP-HPLC: A Review

Aditi Adarsh*[1], Vikas Kumar Singh[2], Pindi Aruna[3], Manish Kumar, Virendra Kumar, Dhiraj Kumar, Binku Kumar

[1]*Associate Professor, Mahadeva Lal Schroff College of Pharmacy, Aurangabad (Bihar)
[2]Associate Professor, Mahadeva Lal Schroff College of Pharmacy, Aurangabad (Bihar)
[3]Joginapally Bhaskar Pharmacy College, Moinabad, Hyderabad, Telangana
Mail ID: aditiadarsh93@gmail.com

Abstract Pharmaceutical analysis plays a very prominent role in quality assurance as well as quality control of bulk drugs and pharmaceutical formulations. Rapid increase in pharmaceutical industries and production of drug in various parts of the world has brought a rise in demand for new analytical techniques in the pharmaceutical industries. As a consequence, analytical method development has become the basic activity of analysis. RP-HPLC is the most, sensitive, universal analytical procedure. It is extremely versatile tech where analytes are separated by passes through column of different sized particles. Analytical method development ensures the specific characteristics of the drug substances against the preconceived acceptance criteria, while method meets objectives required at each stage of development of drug which is a continuous process, and should be robust and accessible. Validation of analytical method demonstrates the scientific firmness of measurement or characterization and is required throughout the regulatory submission process and the main purpose of validation of API is to demonstrate that it maintains the desired level of compliance and also suitable for its intended purpose.

Keywords Quality Assurance, Productions, RP-HPLC, Validation

1. Introduction

A branch of chemistry that deals with the identification of compounds and mixtures (qualitative analysis) or the determination of the proportions of the constituents (quantitative analysis). The techniques commonly used are titration, precipitation, spectroscopy, chromatography, etc.

Analytical chemists work to improve the reliability of existing techniques to meet the demands for better chemical measurements, which arise constantly in our society. They adapt proven methodologies to new kinds of materials or to answer new questions about their composition. They carry out research to discover completely new principles of measurement and are at the forefront of the utilization of major discoveries such as lasers and microchip devices for practical purposes. They make important contributions to many other fields as diverse as forensic chemistry, archaeology, and space science.

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.

1.1 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.

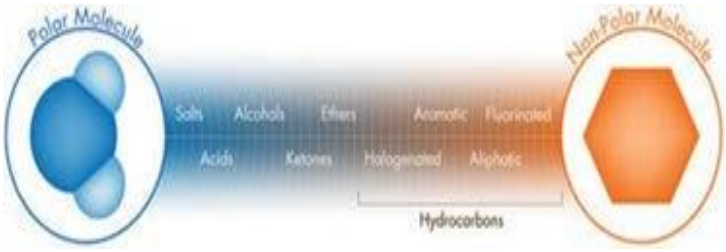

Figure 1: Chromatographic Polarity Spectrum by Analyte Functional Group

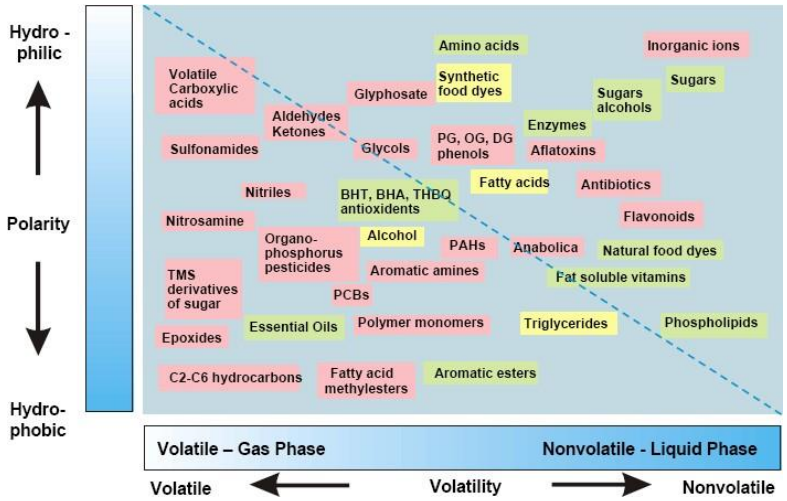

Figure 2: Chromatographic Polarity Spectrum by Analyte class

Figure 3: 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.

Figure 4: 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.

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.

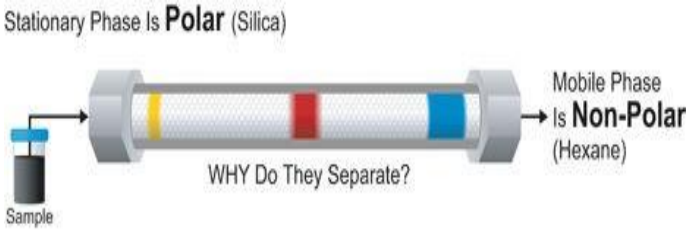

Figure 5: Normal-Phase Chromatography

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.

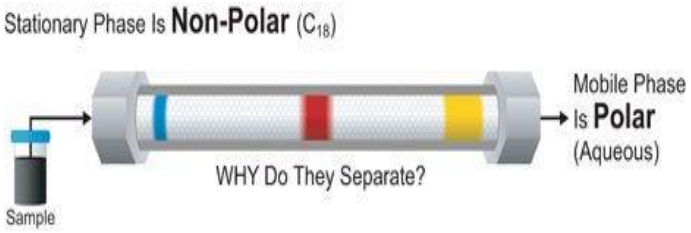

Figure 6: Reversed-Phase Chromatography

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

Table 1: comparison of normal phase and reverse phase HPLC

Properties	Normal Phase	Reversed Phase
Polarity of Stationary Phase	High	Low
Polarity of mobile Phase	Low to medium	Low to high
Sample elution order	Least polar First	Most polar First
Retention will increase by	Increasing surface of stationary phase Increasing of n-alkyl chain length of stationary phase. Decreasing polarity of mobile phase Increasing polarity of sample molecules	Increasing surface of stationary phase. Increasing polarity of mobile phase Decreasing polarity of sample molecules

1.2 Different Types of Chromatography

1.2.1 Adsorption Chromatography

Adsorption chromatography is probably one of the oldest types of chromatography around. It utilizes a mobile liquid or gaseous phase that is adsorbed onto the surface of a stationary solid phase. The equilibration between the mobile and stationary phase accounts for the separation of different solutes.

1.2.2 Partition Chromatography

This form of chromatography is based on a thin film formed on the surface of the solid support by a liquid stationary phase. Solute equilibrates between the mobile phase and the stationary liquid.

1.2.3 Ion Exchange Chromatography

In this type of chromatography, the use of a resin (the stationary solid phase) is used to covalently attach anions or cations on it. Solute ions of the opposite charge in the mobile liquid phase are attracted to the resin by electrostatic forces.

1.2.4 Molecular Exclusion Chromatography

Also known as gel permeation or gel filtration, this type of chromatography lacks an attractive interaction between the stationary phase and solute. The liquid or gaseous phase passes through a porous gel, which separates the molecules according to its size. The pores are normally small and exclude the larger solute molecules, but allow smaller molecules to enter the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through the column at a faster rate than the smaller ones.

1.2.5 Affinity Chromatography

This is the most selective type of chromatography employed. It utilizes the specific interaction between one kind of solute molecule and a second molecule that is immobilized on a stationary phase. For example, the immobilized molecule may be an antibody to some specific protein. When this molecule passes solute containing a mixture of proteins, only the specific protein is reacted to this antibody, binding it to the stationary phase.

1.3 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

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.

Selection of detector

The detector was chosen depending upon characteristic property of the analyte like UV absorbance, oxidation, conductance, fluorescence, reduction etc. Characteristics that are to be fulfilled by a detector used in HPLC analysis are:

• High sensitivity, facilitating trace analysis
• Negligible baseline noise. To facilitate lower detection
• Low dead volume
• Non-destructive to sample
• Inexpensive for purchase and operation

Pharmaceutical ingredients do not absorb UV light equally, so the selection of detection wavelength is important. An understanding of the UV light absorptive properties of the organic impurities and the active pharmaceutical ingredient is very helpful.

For the greatest sensitivity λ max should be used. UV wavelengths below 200 nm should be avoided because of noise increases in this region. Higher wavelengths give greater selectivity.

1.4 HPLC Method Development

International Conference on Harmonisation (ICH) of technical requirements

for registration of pharmaceuticals for human use; ICH harmonised tripartite guideline; Validation of Analytical Procedures: Text and Methodology Q2(R1); Current Step 4 version Parent Guideline dated 27 October 1994 (Complementary Guideline on Methodology dated 6 November 1996 incorporated in November 2005) was used as a guide on how to accomplish validation. The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. As per the ICH guidelines, typical validation characteristics which should be considered are accuracy, precision, specificity, robustness, Detection Limit (DL), Quantitation Limit (QL), linearity, range and system suitability.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Specificity was demonstrated as per the guidelines following below procedures. Representative chromatograms and individual components labeled appropriately.

Resolution of the components which elute closest to each other was established. As per the impurities are not available procedure samples stored under relevant stress conditions: light, heat, humidity, acid/base hydrolysis and oxidation were performed. Peak purity tests were conducted using a photodiode array detector.

Accuracy

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. This is sometimes termed trueness.

To demonstrate accuracy for the drug product point b in ICH Q2 (R1) guidelines was used. As per the guidelines "in cases where it is impossible to obtain samples of all drug product components, it may be acceptable either to add known quantities of the analyte to the drug product".

Acceptance Criteria: The % RSD for percent recovered at each level (n = 3) should be ≤ 2.0 %. The overall %RSD (n = 9) should be ≤ 2.0 %. The % recovered in each sample should be 98.0 – 102.0 % of theoretical.

Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same

homogeneous 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.

The precision of established by analyzing standard six times and performing assay six times on different days. Precision was also demonstrated during accuracy by analyzing nine determinations (3 replicates each at 3 concentrations)

Detection Limit

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

DL was determined based on the Standard Deviation (SD) of the Response and the Slope

$$DL = 3.3\ \sigma / S$$

Where σ = the standard deviation of the response S = the slope of the calibration curve

The slope "S" was estimated from the calibration curve of the analyte. The estimate of "σ" may be carried out based on the Calibration Curve

Quantitation Limit

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is used particularly for the determination of impurities and degradation products.

QL was determined based on the Standard Deviation (SD) of the Response and the Slope.

$$QL = 10\ \sigma / S$$

Where σ = the standard deviation of the response S = the slope of the calibration curve

The slope "S" was estimated from the calibration curve of the analyte. The estimate of "σ" may be carried out based on the calibration curve

Linearity

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

Linearity was demonstrated directly on the drug substance using the proposed method. A linear relationship was established by calculation of a regression line by the method of least squares. A plot of the data was also presented. Linearity was established by seven concentrations of drug substance and peak area.

Range

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.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness was studied by the variations in mobile phase composition; temperature; flow rate.

System Suitability

Current United States Pharmacopeia (USP); General Chapters; General Tests and Assays; Physical Tests and Determinations; <621> Chromatography was used as

a guide. These tests are used to verify that the chromatographic system is adequate for the intended analysis. The tests are based on the concept that the equipment, electronics, analytical operations, and samples analyzed constitute an integral system that can be evaluated as such. System suitability was established and maintained for each analytical run. An acceptance criterion of ≤ 2.0 for the area was selected as system suitability specification as per USP.

1.5 HPLC Instrumentation

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

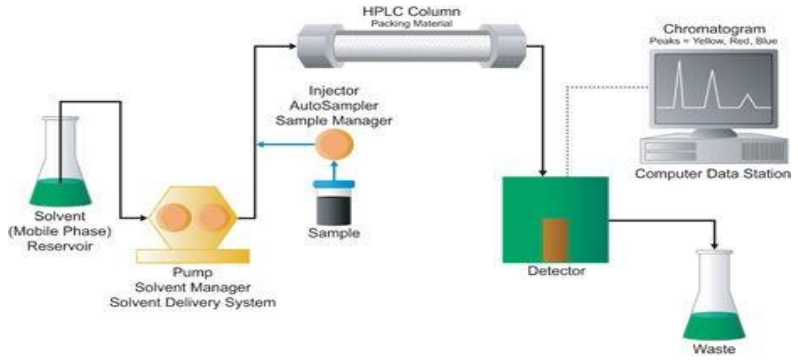

Figure 7: High-Performance Liquid Chromatography [HPLC] System

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.

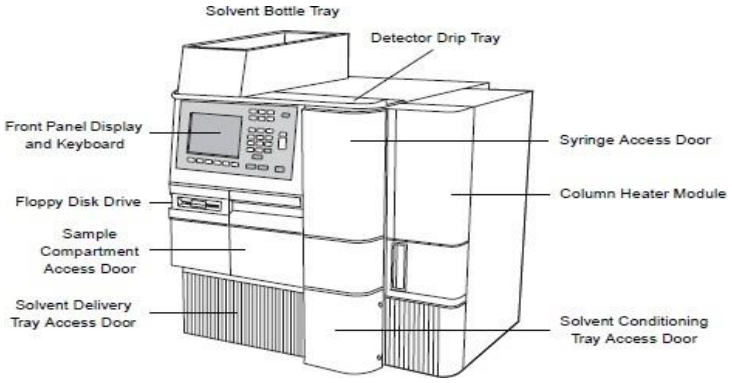

Figure 8: Waters Alliance 2695 Separations Module

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.

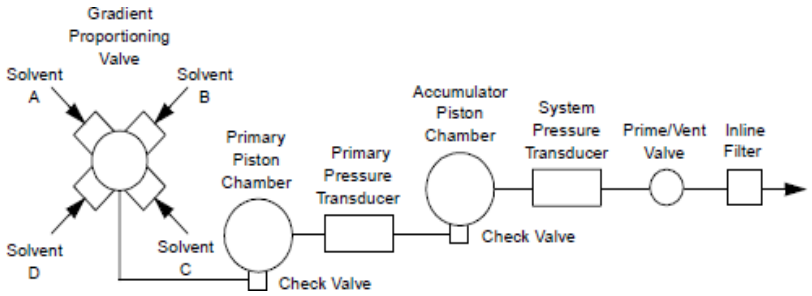

Figure 9: Fluidic Path through the Solvent Management System

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.

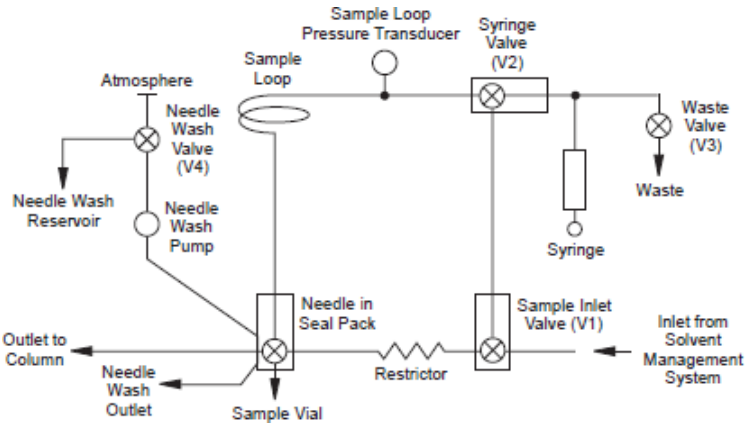

Figure 10: Fluidic Path through the Sample Management System

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.

References

[1]. Sharma B.K, Instrumental Methods of Chemical Analysis, 23rd ed., Goel Publishing House. Meerut, 2004, C10, C11, C68.

[2]. Hobart H. Willard, N. Howell Furman and Egbert. K. Bacon, A Short Course in Quantitative Analysis, 2nd ed., D. Van Nostrand Company, 1968, 4-5.

[3]. Hobart H. Willard, Lynne. L. Merritt, Dean J.J.A and A. S. Frank, Instrumental Method of Analysis, CBS Publishers and Distributors, New Delhi, 1986, 3.

[4]. Skoog D.A, James Holler.F and Nieman T.A, Principles of Instrumental Analysis, 5th ed., Thomson Brooks /Cole Publishers, 2005, 674.

[5]. Hobart H. Willard, Lynne. L. Merrit, Jr. John. A. Dean and Frank. A. Settle, Instrumental Methods of Analysis, CBS Publishers and Distributors, 1986, 514.

[6]. Satinder, Ahuja and Stephen. Scypinski, Handbook of Modern Pharmaceutical Analysis, Harcourt Science and Technology Company, 2001, 423.
[7]. Sethi P.D, HPLC Quantitative Analysis of Pharmaceutical Formulations, CBS Publishers, 2001, 69-70.
[8]. 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
[9]. Sun Z. Determination of the related substances of linagliptin by HPLC. Zhongguo Xiandai Yingyong Yaoxue, 2016, 33(4):451-455.
[10]. Varaprasad C, Asif M and Ramakrishna K. RP-HPLC method for simultaneous estimation of metformin and linagliptin in tablet dosage form. Rasayan Journal of Chemistry, 2015, 8(4), 426-432.
[11]. Ayoub B M. UPLC simultaneous determination of empagliflozin, linagliptin and metformin. RSC Advances, 2015, 5(116), 95703-95709.
[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]. Reddy B S, Rao N B and Saraswathi K. A validated stability indicating HPLC assay method for linagliptin. Der Pharmacia Sinica, 2014, 5(5), 131-137.
[14]. Zubair Md, Balaram V Murali, Gajula Rajesh G. RP-HPLC method development and validation of linagliptin in bulk drug and pharmaceutical dosage form. Pharmacia Sinica, 2014, 5(5):123130, 2014.
[15]. Pednekar S, Lokhande R, Sutar R, Kolhal S, Surve S and Gudekar S. Simultaneous determination of metformin, sitagliptin, saxagliptin, linagliptin and vildagliptin in multicomponent pharmaceutical preparations by RP-HPLC. International Journal of Pharmaceutical Sciences Review and Research, 2014, 28(1):128.
[16]. Dubey N, Singh G N, Tyagi A, Bhardwaj R and Raghav C S. Development and validation of ultraperformance liquid chromatography (UP-LC) method for estimation of a new antidiabetic drug linagliptin in bulk and its tablet formulation. Indian Journal of Chemistry, 2014, 53(B), 1136-1139.
[17]. Pandya R H, Rathod R and Maheswari D G. Bioanalytical method development and validation for simultaneous determination of linagliptin and metformin drugs in human plasma by RP-HPLC method. Pharmacophore, 2014, 5(2), 202-218.
[18]. Swamy A J and Baba K H. Analytical method development and method validation for the simultaneous estimation of metformin HCl and linagliptin in bulk and tablet dosage form by RP-HPLC method. Int. J. Pharm,2013, 3(3), 594-600.
[19]. Lakshmi B and Reddy T V. A novel RP-HPLC method for the quantification of linagliptin in formulations. Journal of Atoms and Molecules, 2012, 2(2), 155.
[20]. Emoto Y, Ishikawa K, Ito H, Hiraide S, Nagata Y and Kanazawa H. Rapid and simultaneous analysis of oral antidiabetic drug by ultra-high-speed HPLC. Bunseki Kagaku, 2013, 62(8), 725-730.
[21]. Sekhar K. A new RP–HPLC method for simultaneous estimation of metformin HCl and linagliptin in tablet dosage form. World Journal of Pharmacy and Pharmaceutical Sciences, 2013, 2(3), 1332-1341.