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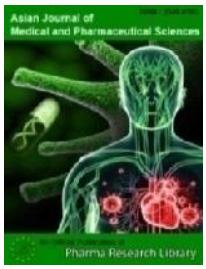

R E S E A R C H A R T I C L E

Analytical Method Development for Sitagliptin and Simvastatin by RP-HPLC Method

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A B S T R A C T

Analytical chemistry is the qualitative and quantitative analysis of drug substances in biological fluids (mainly plasma and urine) or tissue. It plays a significant role in the evaluation and interpretation of pharmacokinetic data. Method development involves evaluation and optimization of the various stages of sample preparation, chromatographic separation, detection and quantification. An accurate and precise HPLC method was developed for the simultaneous determination of Sitagliptin and Simvastatin. Separation of the drug was achieved on reverse phase ODS C18 column using a mobile phase consisting of Phosphate buffer and Acetonitrile in the ratio of 55:45 v/v. The flow rate was 1ml/min and the detection wavelength was 252nm and peaks were eluted at 2.260 min and 3.683 min.

Keywords: Sitagliptin, Simvastatin, RP-HPLC.

A R T I C L E I N F O

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C O N T E N T S

1. Introduction

"Chromatography is a separation process that is achieved by distributing the components of a mixture between two phases, a stationary phase and a mobile phase. Those components held preferentially in the stationary phase are retained longer in the system than those that are distributed selectively in the mobile phase. As a consequence, solutes are eluted from the system as local concentrations in the mobile phase in the order of their increasing distribution

coefficients with respect to the stationary phase; ipso facto a separation is achieved" (1).

Various Types of Chromatography:

Chromatography can be classified by various ways

(I) On the basis of interaction of solute to the stationary phase,

(II) On the basis of chromatographic bed shape,

(III) Techniques by physical state of mobile phase.

On the Basis of Interaction of Solute to Stationary Phase

Adsorption Chromatography: 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 (2).

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

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 onto it. Solute ions of the opposite charge in the mobile liquid phase are attracted to the resin by electrostatic forces.

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.

On The Basis of Chromatographic Bed Shape

Column Chromatography: Column chromatography is a separation technique in which the stationary bed is within a tube. The particles of the solid stationary phase or the support coated with a liquid stationary phase may fill the whole inside volume of the tube (packed column) or be concentrated on or along the inside tube wall leaving an open, unrestricted path for the mobile phase in the middle part of the tube (open tubular column) (3).

Planar Chromatography: Planar chromatography is a separation technique in which the stationary phase is present as or on a plane. The plane can be a paper, serving as such or impregnated by a substance as the stationary bed (paper chromatography) or a layer of solid particles spread on a support such as a glass plate (thin layer chromatography). Different compounds in the sample mixture travel different distances according to how strongly they interact with the stationary phase as compared to the mobile phase.

Paper Chromatography:

Paper chromatography is a technique that involves placing a small dot or line of sample solution onto a strip of chromatography paper. The paper is placed in a jar containing a shallow layer of solvent and sealed. As the solvent rises through the paper, it meets the sample mixture which starts to travel up the paper with the solvent.

Thin Layer Chromatography: Thin layer chromatography (TLC) is a widely employed laboratory technique and is similar to paper chromatography. However, instead of using a stationary phase of paper, it involves a stationary phase of a thin layer of adsorbent like silica gel, alumina, or cellulose on a flat, inert substrate.

Displacement Chromatography: The basic principle of displacement chromatography is, "A molecule with a high affinity for the chromatography matrix (the displacer) will compete effectively for binding sites, and thus displace all molecules with lesser affinities". There are distinct differences between displacement and elution chromatography. In elution mode, substances typically emerge from a column in narrow, Gaussian peaks. Wide separation of peaks, preferably to baseline, is desired in order to achieve maximum purification.

Techniques by Physical State of Mobile Phase

Gas Chromatography: Gas chromatography (GC), also sometimes known as Gas-Liquid chromatography, (GLC), is a separation technique in which the mobile phase is a gas. Gas chromatography is always carried out in a column, which is typically "packed" or "capillary". Gas chromatography (GC) is based on a partition equilibrium of analyte between a solid stationary phase (often a liquid silicone-based material) and a mobile gas (most often Helium). The stationary phase is adhered to the inside of a small-diameter glass tube (a capillary column) or a solid matrix inside a larger metal tube (a packed column) (4).

Liquid Chromatography: Liquid chromatography (LC) is a separation technique in which the mobile phase is a liquid. Liquid chromatography can be carried out either in a column or a plane. Present day liquid chromatography that generally utilizes very small packing particles and a relatively high pressure is referred as high performance liquid chromatography (HPLC). In the HPLC technique, the sample is forced through a column that is packed with irregularly or spherically shaped particles or a porous monolithic layer (stationary phase) by a liquid (mobile phase) at high pressure. HPLC is historically divided into two different sub-classes based on the polarity of the mobile and stationary phases. Technique in which the stationary phase is more polar than the mobile phase (e.g. toluene as the mobile phase, silica as the stationary phase) is called normal phase liquid chromatography (NPLC) and the opposite (e.g. water-methanol mixture as the mobile phase and C18 = octadecylsilyl as the stationary phase) is called reversed phase liquid chromatography (RPLC).

Affinity Chromatography: Affinity chromatography is based on selective non-covalent interaction between an analyte and specific molecules. It is very specific, but not very robust. It is often used in biochemistry in the purification of proteins bound to tags. These fusion proteins are labeled with compounds such as His-tags, biotin or antigens, which bind to the stationary phase specifically. After purification, some of these tags are usually removed and the pure protein is obtained. Affinity chromatography often utilizes a biomolecule's affinity for a metal (Zn, Cu, Fe, etc.).

Supercritical Fluid Chromatography: Supercritical fluid chromatography is a separation technique in which the

mobile phase is a fluid above and relatively close to its critical temperature and pressure.

Introduction to High Performance LC (5, 6)

The acronym HPLC, coined by the late Prof. Csaba Horvath for his 1970 Pittcon paper, originally indicated the fact that high pressure was used to generate the flow required for liquid chromatography in packed columns. In the beginning, pumps only had a pressure capability of 500 psi. This was called high pressure liquid chromatography, or HPLC. The early 1970s saw a tremendous leap in technology. These new HPLC instruments could develop up to 6,000 psi of pressure, and incorporated improved injectors, detectors, and columns. HPLC really began to take hold in the mid-to late-1970s. With continued advances in performance during this time (smaller particles, even higher pressure), the acronym HPLC remained the same, but the name was changed to high performance liquid chromatography.

High performance liquid chromatography is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantitate the compounds that are present in any sample that can be dissolved in a liquid. Today, compounds in trace concentrations as low as parts per trillion (ppt) may easily be identified. HPLC can be, and has been, applied to just about any sample, such as pharmaceuticals, food, nutraceuticals, cosmetics, environmental matrices, forensic samples, and industrial chemicals. A reservoir (Solvent Delivery) holds the solvent (called the mobile phase, because it moves). A high-pressure pump solvent manager is used to generate and meter a specified flow rate of mobile phase, typically milliliters per minute.

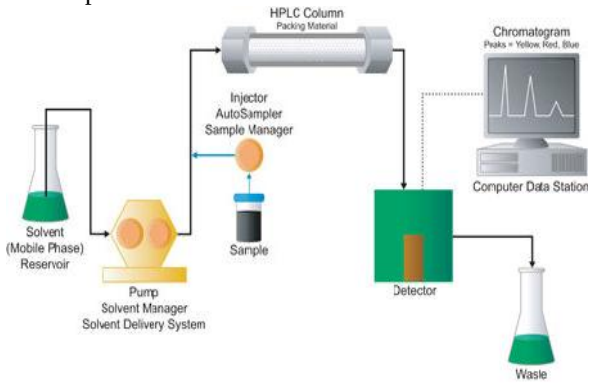

Figure 1: Block Diagram of HPLC

Isocratic and Gradient LC System Operation

Two basic elution modes are used in HPLC:

- ✓ The first is called isocratic elution. In this mode, the mobile phase, either a pure solvent or a mixture, remains the same throughout the run.
- ✓ The second type is called gradient elution, wherein, as its name implies, the mobile phase composition changes during the separation. This mode is useful for samples that contain compounds that span a wide range of chromatographic polarity. As the separation proceeds, the elution strength of the mobile phase is increased to elute the more strongly retained sample components.

HPLC Gradient Mixtures (7):

HPLC gradient mixers must provide a very precise control of solvent composition to maintain a reproducible gradient profile. This can be complicated in HPLC by the small elution volumes required by many systems. It is much more difficult to produce a constant gradient when mixing small volumes then when mixing large volumes. For low pressure systems this requires great precision in the operation of the miniature mixing General Introduction valves used and low dispersion flows throughout the mixer. For multi-pump high pressure systems it requires a very precise control of the flow rate while making very small changes of the flow rate.

HPLC Pumps:

Because of the small particles used in modern HPLC, modern LC pumps need to operate reliably and precisely at pressures of 10,000 psi or at least 6,000 psi. To operate at these pressures and remain sensibly inert to the wide variety of solvents used HPLC pumps usually have sapphire pistons, stainless steel cylinders and return valves fitted with sapphire balls and stainless steel seats. For analytical purposes HPLC pumps should have flow rates that range from 0 to 10 ml/min., but for preparative HPLC, flow rates in excess of 100 ml/min may be required. It is extremely difficult to provide a very constant flow rate at very low flow rates. If 1% is considered acceptable then for 1ml/min a flow variation of less than 10µl/min is required. This level of constancy is required because most HPLC detectors are flow sensitive and errors in quantization will result from change in flow rate.

HPLC Sample Valves:

Since sample valves come between the pump and the column it follows that HPLC sample valves must also tolerate pressures up to 10,000 psi. For analytical HPLC, the sample volume should be selectable from sub micro liter to a few micro liters, whereas in preparative HPLC the sample volume may be even greater than 10 ml. To maintain system efficiency the sample valve must be designed to have very low dispersion characteristics, this is true not only for flow dispersion but also for the less obvious problems of dispersion caused by sample adsorption/desorption on valve surfaces and diffusion of sample into and out of the mating surfaces between valve moving parts. It goes without saying that the valves must deliver a very constant sample size but this is usually attained by the use of a constant size sample loop.

HPLC Columns:

HPLC columns are packed with very fine particles (usually a few microns in diameter). The very fine particles are required to attain the low dispersion that give the high plate counts expected of modern HPLC. Plate counts in excess of 25,000 plates per column are possible with modern columns, however, these very high efficiencies are very rarely found with real samples because of the dispersion associated with injection valves, detectors, data acquisition systems and the dispersion due to the higher molecular weight of real samples as opposed to the common test samples. Packing these small particles into the column is a difficult technical problem but even with good packing a great amount of care must be given to the column end fittings and the inlet and outlet connection to keep

dispersion to a minimum. The main consideration with HPLC is the much wider variety of solvents and packing materials that can be utilized because of the much lower quantities of both which are required. In particular very expensive optically pure compounds can be used to make Chiral HPLC stationary phases and may even be used as (disposable) HPLC solvents.

Guard Column: A 0.5 to 2.0µm Guard columns are placed anterior to the separating column. This serves as a protective factor that prolongs the life and usefulness of the separation column. It also acts as a prefilter to remove particulate matter, if any, and other material.

Analytical Columns: Analytical column is the most important part of the HPLC technique which decides the efficiency of separation. There are several stationary phases available depending upon the technique or mode of separation. These are generally made out of stainless steel tubes with diameter of 3-5mm and a length ranging from 10-30cm. Normally columns are filled with silica gel (2-10µm in diameter) as stationary phase.

HPLC Detectors:

UV/Vis spectrophotometers, including diode array detectors, are the most commonly employed detectors. Fluorescence spectrophotometers, differential refractometers, electrochemical detectors, mass spectrometers, light scattering detectors, radioactivity detectors or other special detectors may also be used. Detector consists of a flow-through cell mounted at the end of the column. A beam of UV radiation passes through the flow cell and into the detector. As compounds elute from the column, they pass through the cell and absorb the radiation, resulting in measurable energy level changes. Fixed (mercury lamp), variable (deuterium or high-pressure xenon lamp), and multi-wavelength detectors are widely available. Modern variable wavelength detectors can be programmed to change wavelength while an analysis is in progress. Multi-wavelength detectors measure absorbance at two or more wavelengths simultaneously. In diode array multi-wavelength detectors, continuous radiation is passed through the sample cell, and then resolved into its constituent wavelengths, which are individually detected by the photodiode array.

HPLC Data Acquisition:

In HPLC data acquisition system the higher sampling rate needed for the rapidly eluting narrow peaks of the HPLC chromatogram. Although the theoretical number of samples needed for good quantization are actually quite small, for real systems a hundred samples or more per peak is recommended; thus, for a 4 sec wide peak, a rate of 25 samples per second may be required. The same data analysis and reporting software can be used as in ordinary LC.

Reverse-Phase (RP) Chromatography (8, 9):

Since 1960's chromatographers started modifying the polar nature of silanol group by chemically reacting silica with organic silanes. The objective was to make less polar or non-polar so that polar solvents can be used to separate water-soluble polar compounds. Since the ionic nature of the chemically modified silica is now reversed i.e. it is non-polar or the nature of the phase is reversed. The chromatographic separation carried out with such silica is referred to as reversed- phase chromatography.

In reversed phase systems the strong attractive forces between water molecules arising from the 3-dimentional inter molecular hydrogen bonded network, from a structure of water that must be distorted or disrupted when a solute is dissolved. Only higher polar or ionic solutes can interact with the water structure. Non- polar solutes are squeezed out of the mobile phase and are relatively insoluble in it but with the hydrocarbon moieties of the stationary phase.

Chemically bonded octadecyl silane (ODS) an alkaline with 18 carbon atoms is the most popular stationary phase used in pharmaceutical industry. Since most pharmaceutical compounds are polar and water soluble, the majority of HPLC methods used for quality assurance, decomposition studies, quantitative analysis of both bulk drugs and their formulations use ODS HPLC columns. The solvent strength in reversed phase chromatography is reversed from that of adsorption chromatography (silica gel) as stated earlier. Water interacts strongly with silanol groups, so that, adsorption of sample molecules become highly restricted and they are rapidly eluted as a result. Exactly opposite applies in reversed phase system; water cannot wet the non-polar (hydrophobic) alkyl groups such as C18 of ODS phase and therefore does not interact with the bonded moiety. Hence water is the weakest solvent of all and gives slowest elution rate. The elution time (retention time) in reversed phase chromatography increases with increasing amount of water in the mobile phase.

2. Materials and Methods

Sitagliptin and Simvastatin, were obtained as a gift sample from Hetero Drugs Ltd., Hyderabad, Telangana, India. Water HPLC grade, Acetonitrile HPLC grade, Potassium phosphate Buffer and Ortho phosphoric acid purchased from Rankem Ltd., Mumbai, India.

Instrument Specifications:

HPLC Instrument (Shimadzu LC 10 AT pump Spinchrome software, UV detector SPD 10A), Injector (Rheodyne), Column (ODS 256×4.6mm,5µ).

Preparation of Mobile Phase:

[Potassium Phosphate Buffer (PH 5.8): Acetonitrile, 55:45]: 1.625g of potassium dihydrogen phosphate and 0.3g of dipotassium hydrogen phosphate was dissolved in sufficient amount of distilled water and the volume was made upto 550ml. pH was adjusted to 5.8. To this, 450ml acetonitrile was added and sonicated for a few minutes. The solution was filtered using 0.45µm membrane filter paper and degassed (10).

Preparation of standard stock solution of Sitagliptin and Simvastatin:

Accurately weigh 10mg of Sitagliptin and 10mg of Simvastatin and transfer into a clean and dry 10ml volumetric flask, dissolve with sufficient volume of mobile phase and make up to 10ml with mobile phase to obtain the concentration of 1000µg/ml of Sitagliptin and 1000 µg/ml of Simvastatin.

Preparation of working standard solution of Sitagliptin and Simvastatin:

1ml of stock solution was further diluted in a 10ml volumetric flask with mobile phase to get a concentration of 100µ g/ml of Sitagliptin and 100µg/ml of Simvastatin.

3. Results and Discussion

Optimized Chromatographic Conditions:
Instrument: Shimadzu LC 10 AT pump, Spinchrome software, UV detector SPD 10A
Column: Inertsil ODS 250 X 4.6mm, 5 µ Column Oven
Temperature: Ambient
Wave length: 252nm
Flow rate: 1ml/min
Injection volume: 20µl
Runtime: 5mins
Mobile phase: Solvent A- Potassium Phosphate Buffer pH 5.8 Solvent B-Acetonitrile
Solvent ratio: 55:45 v/v of A: B

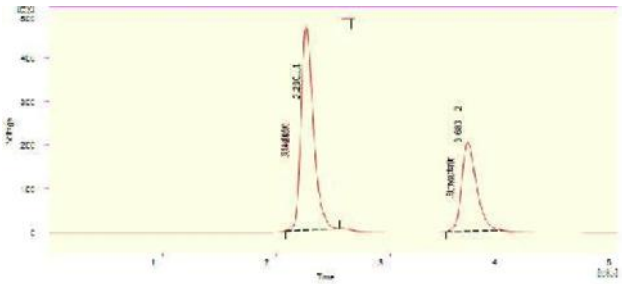

Figure 2: Chromatogram of Sitagliptin and Simvastatin

Observation: In this, the retention time of Sitagliptin was found to be 2.260 and Simvastatin was 3.683. Sharp peaks are obtained with good resolution and no tailing effect.

4. Conclusion

An efficient, rapid, reproducible and sensitive RP- HPLC method was developed and validated for the simultaneous estimation of Sitagliptin and Simvastatin in bulk and pharmaceutical dosage forms. The developed method was validated as per ICH guidelines. In the present investigation was to develop HPLC conditions for the separation of sitagliptin and simvastatin using Potassium phosphate buffer (pH5.8) as aqueous mobile phase and acetonitrile as organic phase. The HPLC method was developed using Inertsil ODS column, 250 X 4.6mm, 5 µ with Mobile phase consisting Potassium phosphate buffer (pH5.8): ACN (55:45) at 1mL/min flow rate Detection was carried out at 252nm and injection volume was 20µL.

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