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Development and Validate RP –HPLC Method for Estimation of Levosalbutamol Sulphate and Ipratropium Bromide in Bulk and Nebulizer Dosage Form

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ABSTRACT:
Chromatography is a physicochemical method for separation of complex mixtures was discovered at the very beginning of the twentieth century by Russian Italian botanist M. S. Tswett. .
In his paper "On the new form of adsorption phenomena and its application in biochemical analysis" presented on March 21, 1903 at the regular meeting of the biology section of the Warsaw Society of Natural Sciences,
Tswett gave a very detailed description of the newly discovered phenomena of adsorption-based separation of complex mixtures, which he later called "chromatography" as a transliteration from Greek "color writing" .
Serendipitously, the meaning of the Russian word "tswett" actually means color. Although in all his publications Tswett mentioned that the origin of the name for his new method was based on the colorful picture of his first separation of plant pigments , he involuntarily incorporated his own name in the name of the method he invented.
KYE WORLD:DEVELOPMENT,VALIDATION, RP –HPLC, LEVOSALBUTAMOL SULPHATE, IPRATROPIUM BROMIDE, NEBULIZER DOSAGE FORM

I. INTRODUCTION:

The chromatographic method was not appreciated among the scientists at the time of the discovery, as well as after almost 10 years when L. S. Palmer
In the United States and C. Dhere in Europe independently published the description of a similar separation processes. Twenty-five years later in 1931,
Lederer read the book of L. S. Palmer and later found an original publications of M. S. Tswett, and in 1931 he (together with Kuhn and Winterstein) published a paper
On purification of xantophylls on $CaCO_3$ adsorption column following the procedure described by M. S. Tswett. In 1941 A. J. P. Martin and R. L. M. Synge at Cambridge University, in UK discovered partition chromatography

For which they were awarded the Noble Prize in 1952. In the same year, Martin and Synge published a seminal paper which, together with the paper of A.T. James and A. J. P. Martin. , Laid a solid foundation for the fast growth of chromatographic techniques that soon followed. Prior to the 1970's, few reliable chromatographic methods were commercially available to the laboratory scientist. During 1970's, most chemical separations were carried out using a variety of techniques including open-column chromatography, paper chromatography, and thin-layer chromatography. However, these chromatographic techniques were inadequate for quantification of compounds and resolution between similar compounds.

During this time, pressure liquid chromatography began to be used to decrease flow through time, thus reducing purification times of compounds being isolated by column chromatography.

However, flow rates were inconsistent, and the question of whether it was better to have constant flow rate or constant pressure was debated .

High pressure liquid chromatography was developed in the mid-1970's and quickly improved with the development of column packing materials and the additional convenience of online detectors.

In the late 1970's, new methods including reverse phase liquid chromatography allowed for improved separation between very similar compounds. By the 1980's HPLC was commonly used for the separation of chemical compounds. Because of these differences, some components of the mixture stay longer in the stationary phase, and they move slowly in the chromatography system,

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while others pass rapidly into mobile phase, and leave the system faster .

Based on this approach three components form the basis of the chromatography technique.

- Stationary phase:

This phase is always composed of a "solid" phase or "a layer of a liquid adsorbed on the surface a solid support".

- Mobile phase: This phase is always composed of "liquid" or a "gaseous component." Separated molecules The type of interaction between stationary phase, mobile phase, and substances contained in the mixture is the basic component effective on separation of molecules from each other.

Chromatography methods based on partition are very effective on separation, and identification of small molecules as amino acids, carbohydrates, and fatty acids. However, affinity chromatographies (ie. ion-exchange chromatography) are more effective in the separation of macromolecules as nucleic acids, and proteins.

Paper chromatography is used in the separation of proteins, and in studies related to protein synthesis; gasliquid chromatography is utilized in the separation of alcohol, esther, lipid, and amino groups, and observation of enzymatic interactions,

While molecular-sieve chromatography is employed especially for the determination of molecular weights of proteins. Agarose-gel chromatography is used for the purification of RNA, DNA particles, and viruses .

Stationary phase in chromatography, is a solid phase or a liquid phase coated on the surface of a solid phase. Mobile phase flowing over the stationary phase is a gaseous or liquid phase.

If mobile phase is liquid it is termed as liquid chromatography (LC), and if it is gas then it is called gas chromatography (GC). Gas chromatography is applied for gases, and mixtures of volatile liquids, and solid material.

Liquid chromatography is used especially for thermal unstable, and non-volatile samples .

The purpose of applying chromatography which is used as a method of quantitative analysis apart from its separation, is to achive a satisfactory separation within a suitable timeinterval.

New techniques improved separation, identification, purification and quantification far above the previous techniques. Computers and automation added to the convenience of HPLC. Improvements in type of columns and thus reproducibility were made as such terms as micro-column, affinity columns, and Fast HPLC began to immerge.

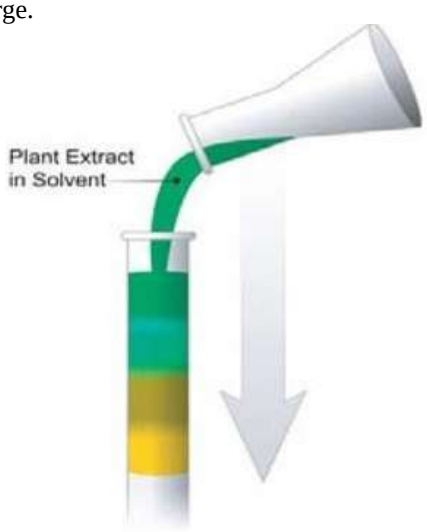

Figure 1: Tswett's Experiment

By the 2000 very fast development was undertaken in the area of column material with small particle size technology and other specialized columns.

The dimensions of the General Introduction typical HPLC column are 100-300 mm in length with an internal diameter between 3-5 mm. The usual diameter of micro-columns, or capillary columns, ranges from 3 μm to 200 μm .

In this decade, sub 2 micron particle size technology (column material packed with silica particles of < 2μm size) with modified or improved HPLC instrumentation becomes popular with different instrument brand name like UPLC (Ultra Performance Liquid Chromatography) of Waters and RRLC (Rapid Resolution Liquid Chromatography) of Agilent.Today,

Chromatography is an extremely versatile technique; it can separate gases, and volatile substances by GC, in-volatile chemicals and materials of extremely high molecular weight (including biopolymers) by LC and if necessary very inexpensively by TLC.

All three techniques, (GC), (LC) and TLC have common features that classify them as chromatography systems. Chromatography has been defined as follows,

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

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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" .

Chromatography is based on the principle where molecules in mixture applied onto the surface or into the solid, and fluid stationary phase (stable phase) is separating from each other while moving with the aid of a mobile phase. The factors effective on this separation process include molecular characteristics related to adsorption (liquid-solid), partition (liquid-solid), and affinity or differences among their molecular weights , Various chromatography methods have been developed to that end. Some of them include column chromatography, thin-layer chromatography (TLC),

Paper chromatography, gas chromatography, ion exchange chromatography, gelpermeation chromatography,
High-pressure liquid chromatography, and affinity chromatography [16].

High Performance Liquid Chromatography

There are different modes of separation in HPLC. They are normal phase mode, reversed phase mode, reverse phase ion pair chromatography, affinity chromatography and size exclusion chromatography (gel permeation and gel filtration chromatography).

Normal phase mode:

The stationary phase is polar and the mobile phase is nonpolar in nature. In this technique, nonpolar compounds travel faster and are eluted first. This is because of the lower affinity between the nonpolar compounds and the stationary phase.

Polar compounds are retained for longer times because of their higher affinity with the stationary phase. These compounds, therefore take more times to elute.
Normal phase mode of separation is therefore, not generally used for pharmaceutical applications because most of the drug molecules are polar in nature and hencetake longer time to elute.

Reversed phase mode:

The most popular mode for analytical and preparative separations of compound of interest in chemical, biological, pharmaceutical, food and biomedical sciences.

In this mode, the stationary phase is nonpolar hydrophobic packing with octyl or octa decyl functional group bonded to silica gel and the mobile phase is polar solvent.

An aqueous mobile phase allows the use of secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pairing and complexation) to control retentionand selectivity.

The polar compound gets eluted first in this mode and nonpolar compounds are retained for longer time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer times and hence elute faster. The different columns used are octa decyl silane (ODS) or C_{18}, $C_{8,}$ C_4, etc., (in the order of increasing polarity of the stationaryphase).

4.Aims & Objectives:

The main aim of present work is to develop and validate RP-HPLC method for the simultaneous estimation of Levosalbutamol sulphate and Ipratropium Bromide in Bulk and Nebulizer Dosage Form"

5.Objective:

1. To develop the RP-HPLC method for the simultaneous estimation of Levosalbutamol sulphate and Ipratropium Bromide in Bulk and Nebulizer Dosage Form.
2. To validate the developed method as per ICH guidelines.

A. Steps in developing the method and optimization of chromatographic condition.

- Literature survey
- Selection of drugs.
- Selection of detection wavelength
- Selection of chromatographic conditions
- Selection of Mobile Phase (Selection of Organic solvent and aqueous solvent).
- Selection of suitable pH.
- Selection of Column and Column temperature.
- Optimization of Mobile phase, Column and Solvent system.

B. Stability indicating analytical method validation using RP-HPLC as per ICH Q2 R1,

- Specificity,
- Linearity,
- Precision,

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- Accuracy,
- Limit of Quantification (LOQ) and
- Limit of Detection (LOD)

C. Application of develop method for (Estimation of Drug in Nebulizer solution)

6.Review of Literature

1) **Panda Sagar Suman, et al. (2013)** have accurate and precise RP-HPLC method for simultaneous determination of levosalbutamol sulfate and theophylline has been developed and validated.(31)

Mobile Phase: methanol: 10 mM TBAHS(tetrabutyl ammonium hydrogen sulfate) (50:50,v/v)
Column: C18 column (250 mm × 4.6 mm i.d., 5 µm)Wavelength: 274 nm.
Detector: UV-visible
Flow rate: 1.0 mL.min-1.
Injection flow: 20 µL

2) **P. Nagaraju et al(2014)** have rapid and sensitive reverse phase high performance liquid chromatography (RP-HPLC) method has been developed for simultaneous estimation of Levosalbutamol and Ipratropium bromide.(32)

Mobile phase: Méthanol: 0.01M potassium dihydrogen phosphate (pH was adjusted to 3.0 with O-phosphoric acid in the ratio of 50:50 v/v). Column: C18 column (250 X 4.6 mm, 5 µm)Wavelenght: 245 nm.
Detecror: UV-visible
Flow rate: 1.0 mL/min.

3) **Ravi Vankudoth et al(2013)** have accurate, sensitive, precise, rapid and isocratic Reversed-Phase HPLC, (RP-HPLC) method for simultaneous estimation of Ipratropium bromide and Levosalbutamol in the bulk drug and in the pharmaceutical metered dose inhalershas been developed and validated.(33)

Mobile phase: acetonitrile as the organic modifier and Di-Potassium Hydrogen Phosphate[0.03M] in water with pH 3.2 adjusted with Ortho-Phosphoric acid (0.1% v/v) in theproportion of [30:70 v/v.].

4) **Sowjanya G,et al (2018)** have RP-HPLC method was developed and validated for the simultaneous assay of albuterol sulphate and ipratropium bromide in nasal inhalations.(34)

Mobile Phase:
Mobile Phase A: anhydrous potassium dihydrogen orthophosphate, 1-pentane sulphonic acid sodium salt monohydrate (pH 4.0) and acetonitrile (95:5 v/v)
Mobile Phase B: anhydrous potassium dihydrogen orthophosphate, 1-pentane sulphonic acid sodium salt monohydrate (pH 4.0) and acetonitrile (70:30 v/v)
Column; Peerless basic C8 column (150 x 4.6 mm, 5µ)

Wavelenght: 276-220 nm

Detector: VU/PDA

5) **Anees Begum et al (2016)** have RP-HPLC technique is depicted for the determination of Albuterol and Ipratropium Bromide in dosage forms.(35)

Mobile Phase:Phosphate buffer pH: 3.4 Methanol (30:70 v/v),Column: Inertsil ODS C18 (250 x 4.6 mm, 5 µm).
Detector: PDA

Wavelenght: 239 nm.

Flow rate: 1.2 mL/min.

6) **Elvis A.M, et al.(2011)**have rapid and sensitive reverse phase isocratic HPLC simultaneous estimation of Salbutamol Sulphate and Beclomethasone Dipropionate in Rotacaps formulation has been developed.(36)

Mobile Phase: Water:Acetonitrile(40:60 v/v)

Column: octadecylsilane column.

Wavelength: 230 nm

Detectore: UV-Visible

7) **Narendra Nyola,et al (2012)** have rapid and accurate RP-HPLC method was developed for the determination of levosalbutamol in pure and tablet dosage form.(37)

Mobile phase: Acetonitrile and buffer in the ratio of 20:80 (v/v) was used and maintain the pH 3 with the help of ortho phosphoric acid.

7.Ion pair chromatography:

May be used for the separation of ionic compounds and this method can also substitute for ion exchange chromatography.Strong acidic and basic compounds may be separated by reversed phase mode by forming ion pairs (columbic association species formed between two ions of opposite electric charge) with suitable counter ions.
This technique is referred to as reversed phase ion

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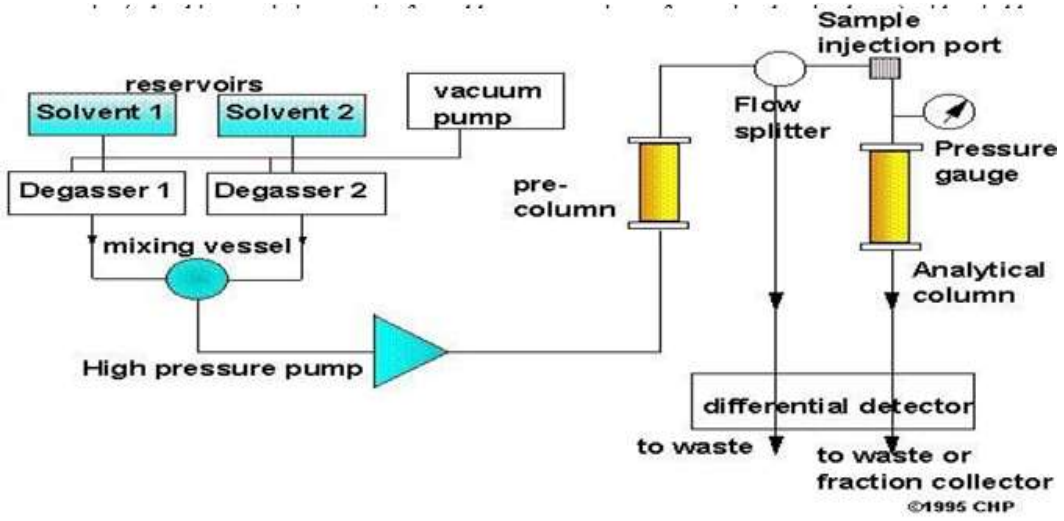
pair chromatography or soap chromatography.

Affinity chromatography:

The uses highly specific biochemical interactions for separation. The stationary phase contains specific groups of molecules which can absorb the sample if certain steric and charge related conditions are satisfied.

This technique can be used to isolate proteins, enzymes as well as antibodies from complex mixtures.



Size exclusion chromatography:

Separates molecules according to their molecular mass. Largest molecules are eluted first and the smallest molecules last.

This method is generally used when a mixture contains compounds with a molecular mass difference of at least 10%.This mode can be further subdivided into gel permeation chromatography (with organic solvents) and gel filtration chromatography (with aqueous solvents).Schematic diagram of HPLC equipment.

Various components of HPLC are:

- A solvent delivery system, including,
- Pump
- Sample injection system,
- A chromatographic column,
- A detector,
- A strip chart recorder,
- Data handling device and microprocessor control.

Solvent delivery system:

A mobile phase is pumped under pressure from one or several reservoir and flows through the column at a constant rate. For normal phase separation eluting power increases with increasing polarity of the solvent but for reversed phase separation, eluting power decreases with increasing polarity.

A degasser is needed to remove dissolved air and other gases from the solvent. Special grades of solvents are available for HPLC and these have been purified carefully in order to remove absorbing impurities and particulate matter to prevent these particles from damaging the pumping or injection system or clogging the column.

Pumps:

The pump is one of the most important component of HPLC, since its performance directly affects retention time, reproducibility and detector sensitivity.

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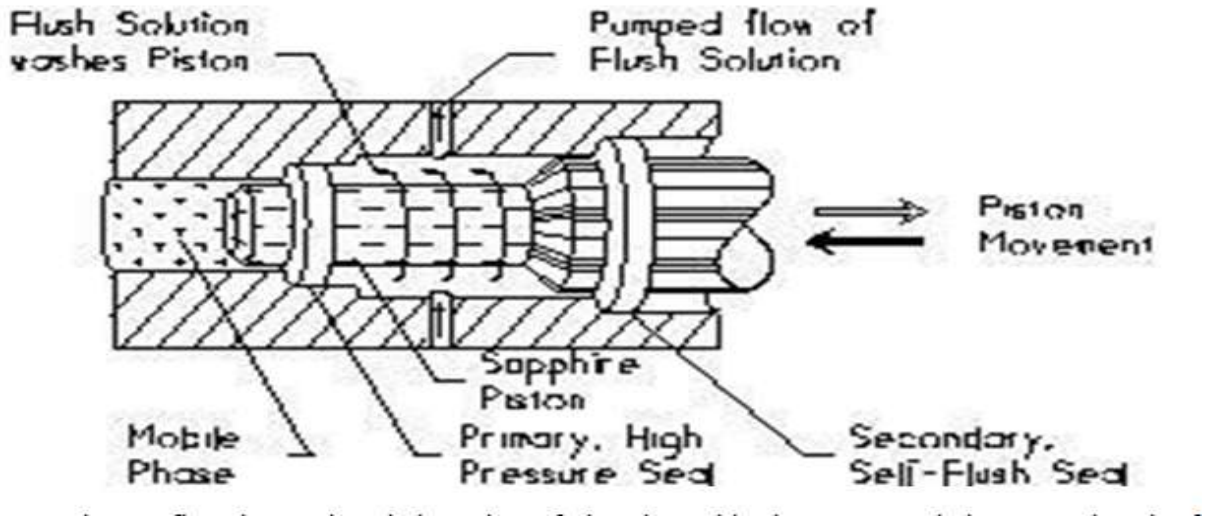

Three main types of pumps are used in HPLC to propel the liquid mobile phase through thesystem.

✓ **Displacement pump:**

It produces a flow that tends to independent of viscosity and back pressure and also output is pulse free. But it possesses limited capacity (250 ml).

✓ **Reciprocating pump:**

It has small internal volume (35 to 400 µl), their high output pressure (up to 10,000 psi) and their constant flow rates. But it produces a pulsed flow.

Pneumatic or constant pressure pump: They are pulse free; suffer from limited capacity as well as a dependence of flow rate on solvent viscosity and column back pressure. They are limited to pressure less than 2000 psi.

Sample injection system:

Insertion of the sample onto the pressurized column must be as a narrow plug so that the peak broaening attributable to this step is negligible.The injection system itself should have no dead (void) volume.

There are three important ways of introducing the sample into injection port.

✓ **Loop injection:**

In which, a fixed amount of volume is introduced by making use of fixedvolume loop injector.

✓ **Valve injection:**

In which, a variable volume is introduced by making use of an injection valve.

✓ **On column injection:**

In which, a variable volume is introduced by means of a syringe through a sesptum.

Chromatographic column:

The column is usually made up of heavy glass or stainless steel tubing to withstand high pressure.

The columns are usually 10-30 cm long and 4-10 mm inside diameter containing stationary phase at particle diameter of 25 µm or less.

Columns with an internal diameter of 5 mm give good results because of compromise between efficiency, sample capacity, and the amount of packing and solvent required.

The packing used in modern HPLC consist of small, rigid particles having a narrow particle size distribution. There are three main types of column packing in HPLC.

✓ **Porous, polymeric beds:**

Porous, polymeric beds based on styrene divinyl benzene co-polymers are used in ion exchange and size exclusion chromatography.

For analytical purpose these have now been replaced by silica based, packing which are more efficient and more stable.

✓ **Porous layer beds:**

Consisting of a thin shell (1-3 µm) of silica or modified silica on an spherical inert core (e.g. Glass).

After the development of totally porous micro particulate packings, these have not been used in HPLC.

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✓ **Totally Porous silica particles (dia. <10 µm):**

These packing have widely been used for analytical HPLC in recent years.

Particles of diameter >20 µm are usually dry packed.

While particles of diameter <20 µm are slurry packed in which particles are suspended on a suitable solvent and the slurry so obtained is driven into the column under pressure.[17-19]

Detectors:

Detector is the eye of LC system and measures the compounds after their separation on the column. Before the first sample is injected during method development,

The chromatographer must ensure that the detector so selected is capable or responding to changes in concentration of all components in the sample with adequate sensitivity even to measure trace substance.

Detector may not sense every component so selected before commencing the analysis depending on the nature of the sample.

✓ There are basically two types of detectors: **Bulk property detectors:**

It compares overall changes in a physical property of the mobile phase with and without an eluting solute. e.g. refractive index, dielectric constant or density.

✓ **Solute property detectors:**

It responds to a physical property of the solute which is not exhibited by the pure mobile phase. e.g. UV absorbance, fluorescence or diffusion current.

Such detectors are about 1000 times more sensitive giving a detectable signal for a few nanograms of sample.

Quantitative Analysis in HPLC:

Three methods are generally used for quantitative analysis. They are the external standard method, the internal standard method and the standard addition method.

External standard method:

The external standard method involves the use of a single standard or up to three solutions. The peak area or the height of the sample and the standard use are compared directly.

One can also use the slope of the calibration curve based on standard that contain known concentrations of the compound of interest.

8.Internal standard method:

A widely used technique of quantitation involves the addition of an internal standard to compensate for various analytical errors.

In this approach, a known compound of a fixed concentration is added to the known amount of samples to give separate peaks in the chromatograms to compensate for the losses of the compounds of interest during sample pretreatment steps.

Any loss of the component of interest will be accompanied by the loss of an equivalent fraction of the internal standard.

The accuracy of this approach obviously dependence on the structural equivalence of the compounds of interest and the internal standard.

The requirements for an internal standardare:

- ✓ It must have a completely resolved peak with no interferences,
- ✓ It must elute close to the compound of interest,
- ✓ It must behave equivalent to the compound of interest for analysis like pretreatments, derivative formations, etc.,
- ✓ It must be added at a concentration that will produce a peak area or peak height ratio of about unity with the compound,
- ✓ It must not be present in the original sample,
- ✓ It must be stable, non-reactive with sample components, column packing and the mobile phase and
- ✓ It is desirable that this compound is commercially available in high purity.

The internal standard should be added to the sample prior to sample preparation procedure and homogenized with it. To be able to recalculate the concentration of a sample component in the original sample,

We have to demonstrate first the response factor. The response factor (RF) is the ratio of peak areas of sample component (A_x) and the internal standard (A_{ISTD}) obtained by injectingthe same quantity.

It can be calculated by using the formula,

$$RF = A_x / A_{ISTD}$$

On the basis of the response factor and strength of the internal standard (N_{ISTD}), the amount of the

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analyte in the original sample can be calculated using the formula,

X =AS / RF * AISTD X NISTD

The calculations described above can be used after proving the linearity of the calibration curve for the internal standard and the analytical reference standard of the compound of interest,When more than one component is to be analyzed from the sample,
The response factor of each component should be determined in the calculations using similar formula.

Standard addition method:

In the standard addition method, a known amount of the standard compound is added to the sample solution to be estimated.

This method is suitable if sufficient amount of the sample is available and is more realistic in the sense that it allows calibration in the presence of excipients or other components.

As gradient elution can be a source of additional error in quantitative analysis. It is also necessary to keep the flow rate and the mobile phase composition constant. The sample should be dissolved in the mobile phase.
If the solvent used in the preparing the sample solution and the mobile phase are not identical, the analysis can become less accurate and it is also possible that the detector response is more dependent on the sample.

9.Method Development and Design of Separation Method:

Methods for analyzing drugs in multicomponent dosage forms can be developed, provided one has knowledge about the nature of the sample, namely, its molecular weight, polarity, ionic character and the solubility parameter.

An exact recipe for HPLC, however, cannot be provided because method development involves considerable trial and error procedures. The most difficult problem usually is where to start, what type of column is worth trying with what kind of mobile phase.

In general one begins with reversed phase chromatography, when the compounds are hydrophilic in nature with many polar groups and are water soluble.The organic phase concentration required for the mobile phase can be estimated by gradient elution method. For aqueous sample mixtures, the best way to start is with gradient reversed phase chromatography. Gradient can be started with 5-10% organic phase in the mobile phase and the organic phase concentration (methanol or acetonitrile) can be increased up to 100% within 30-45min.

Separation can then be optimized by changing the initial mobile phase composition and the slope of the gradient according to the chromatogram obtained from the preliminary run.

The initial mobile phase composition can be estimated on the basis of where the compounds of interest were eluted, namely, at what mobile phase composition.Changing the polarity of mobile phase can alter elution of drug molecules. The elution strength of a mobile phase depends upon its polarity, the stronger the polarity, higher is the elution.

Ionic samples (acidic or basic) can be separated, If they are present in un-dissociated form. Dissociation of ionic samples may be suppressed by the proper selection of pH.

The pH of the mobile phase has to be selected in such a way that the compounds are not ionized. If the retention times are too short,

The decrease of the organic phase concentration in the mobile phase can be in steps of 5%. If the retention times are too long, an increase of the organic phase concentration is needed.

Selection of buffer as a mobile phase in HPLC:

In RP-HPLC the retention of analyte are related to their hydrophobicity. The more hydrophobic the analyte the longer it is retained. When an analyte is ionized it become less hydrophobic and retention decreased .

When pH increases acid loose a proton and base gain a proton when pH decreases and become ionized. Therefore when separating mixture containing acids and/or bases by RP-HPLC it is necessary to control the pH of the mobile phase using an appropriate buffer in order to achieve reproducible result.

For the most robust method it is recommended that separation be develop a mobile phase pH, where a retention of ananlyte is little affected by changes in pH when separating bases.

For example - acid mobile phase is usually shows better reproducibility then neutral mobile phases.

It is surprising how often reverse phase methods are develop using buffer that have little or no buffering capacity at the specified mobile phase pH.

Methods that specify the phosphate buffer

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 4 July-Aug 2023, pp: 913-929 www.ijprajournal.com ISSN: 2249-7781

in the pH range of 4-6 or an acetate buffer in the range of 6-7 are unfortunately not uncommon.

These buffer are not just useless in these pH range they complicate the preparation of the mobile phase unnecessary and give the analyst a false sence of controlling the reproducibility of the separation.

Optimum buffering capacity occurs at a pH equal to the pKa of the Buffer. In general you can expect most buffer to provide adequate buffering capacity for controlling the mobile phase pH only within one unit of their pKa beyond capacity will be inadequate.

Selection of analytical wavelength:

In UV detection, good analytical results are obtained only when the wavelength is selected carefully. This requires knowledge of the UV spectra of the individual components present in the sample.

If analyte standards are available, their UV spectra can be measured prior to HPLC method development.

The molar absorbance at the detection wavelength is also an important parameter. When peaks are not detected in the chromatograms, it is possible that the sample quantity is not enough for the detection.

An injection of volume of 20 µl from a solution of 1 mg/ml concentration normally provides good signals for UV active compounds around 220 nm. Even if the compounds exhibit higher λ_{max}, they absorb strongly at lower wavelength.

It is not always necessary to detect compounds at their maximum absorbance. It is, however, advantageous to avoid the detection at the sloppy part of the UV spectrum for precise quantitation.

When acceptable peaks are detected on the chromatogram, the investigation of the peak shapes can help further method development.

Correcting peak tailing problem in RP-HPLC:

When tailing or fronting is observed, it means that the mobile phase is not totally compatible with the solutes. In most case the pH is not properly selected and hence partial dissociation or protonation takes place.

Peak tailing in RP-HPLC continues to be a common complained .It is particularly prevalent when separating a basic compound and therefore a source of constant problems to those analyzing pharmaceutical compound by HPLC.

It causes a number of problems including poor resolution; reduce sensitivity, poor precision and poor quantization.

✓ **Sample mass overload:**

The cure for peak tailing caused by sample mass over load is to inject fewer samples and the mass to inject depends on the column internal diameter.

Stationary phase silanols interactions with amines:- Operating at a pH below 3, protonate silanols groups on the silica stationary phase support and thereby makes the silanols less available for interacting with solutes. Add a competing amine to the mobilephase.

Adsorption of acidic compounds on to silica: - Increase the salt concentration of the mobile phase to suppress the secondary interaction, reduce the mobile phase pH to protonate silanols and solutes and add a competing acid to the mobile phase.

✓ **Void in the column packing bed:**

The best cure for a column that is giving tailing peaks because of void in the packing bed is to replace it.

The addition of peak modifiers to the mobile phase can affect the separation of ionic samples. For examples, the retention of the basic compounds can be influenced by the addition of small amounts of triethylamine (a peak modifier) to the mobile phase.

Similarly for acidic compounds small amounts of acids such as acetic acid can be used.This can lead to useful changes in selectivity. When the peak shape does not improve by lower (1-2) or higher (8-9) pH, then ion-pair chromatography can be used.

For acidic compounds, cationic ion pair molecules at higher pH and for basic compounds, anionic ion-pair molecules at lower pH can be used.For amphoteric solutes or a mixture of acidic and basic compounds, ion-pair chromatography is the method of choice

The low solubility of the sample in the mobile phase can also cause bad peak shapes. It is always advisable to use the same solvents for the preparation of sample solution as the mobile phase to avoid precipitation of the compounds in the column or injector.

Optimization can be started only after a reasonable chromatogram has been obtained.a reasonable chromatogram means that more or less symmetrical peaks on the chromatogram detect all the compounds.

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 4 July-Aug 2023, pp: 913-929 www.ijprajournal.com ISSN: 2249-7781

By sight change of the mobile phase composition, the position of the peaks can be predicted within the range of investigated changes. An optimized chromatogram is the one in which all the peaks are symmetrical and are well separated in less run time.

The peak resolution can be increased by using a more efficient column (column with higher theoretical plate number, N) which can be achieved by using a column of smaller particle size, or a longer column.

These factors, however, will increase the analysis time. Flow rate does not influence resolution, but it has a strong effect on the analysis time.

System suitability parameters for HPLC

The parameters that are affected by the changes in chromatographic conditions are: [21, 22]

- ✓ Resolution (R_s),
- ✓ Capacity factor (k'),
- ✓ Selectivity ( ),
- ✓ Column efficiency (N) and
- ✓ Peak asymmetry factor (A_s).

Resolution (Rs):

Resolution is the parameter describing the separation power of the complete Chromatographic system relative to the particular components of the mixture The resolution, R_s, of two neighboring peaks is defined as the ratio of the distance between twopeak maxima.

It is the difference between the retention times of two solutes divided by their average peak width. For baseline separation, the ideal value of R_s is 1.5. It is calculated by using the formula,

Rs = Rt2 - Rt1 / 0.5 (W1 + W2)

Where,

Rt_1 and Rt_2 =are the retention times of components 1 and 2 and W_1 and $W_{2=}$ are peak width of components 1 and 2.

There are three fundamental parameters that influence the resolution of a chromatographic separation:

- ✓ Capacity factor
- ✓ Selectivity
- ✓ Column efficiency

Capacity Factor (k'):

Capacity factor is the ratio of the reduced retention volume to the dead volume. Capacity factor, k', is defined as the ratio of the number of molecules of solute in the stationary phase to the number of molecules of the same in the mobile phase.

Capacity factor is a measure of how well the sample molecule is retained by a column during an isocratic separation. The ideal value of k' ranges from 2-10. Capacity factor can be determined by using the formula,

k'= V1 - V0 / V0

Where,

V_1 = retention volume at the apex of the peak (solute) andV_0 = void volume of the system.

The values of k' of individual bands increase or decrease with changes in solvent strength. In reversed phase HPLC, solvent strength increases with the increase in the volume of organic phase in water/ organic mobile phase.

Typically an increase in percentage of the organic phase by 10% by volume will decrease k' of the bands by a factor of 2-3.Adjusting capacity factor (k'):

Good isocratic method usually have a capacity factor (k') in the range of 2 -10 (typically 2 – 5). Lower values may give inadequate resolution. Higher values are usually associated with excessively broad peak and unacceptably long run time. If the shift in k' value is observed with both analyst and the column test solution,

The problem is most likely due to change in column, temperature or mobile phase composition. This is true if the shift occurs gradually over series of run.

Capacity factor (k') values are sensitive to:

- ✓ Solvent strength
- ✓ Composition
- ✓ Purity
- ✓ Temperature
- ✓ Column chemistry
- ✓ Sample

Selectivity ( ):

The selectivity (or separation factor), , is a measure of relative retention of two components in a mixture. Selectivity is the ratio of the capacity factors of both peaks, and the ratio of its adjusted retention times.

Selectivity represents the separation power of particular adsorbent to the mixture of these particular components.

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 4 July-Aug 2023, pp: 913-929 www.ijprajournal.com ISSN: 2249-7781

This parameter is independent of the column efficiency; it only depends on the nature of the components, eluent type, and eluent composition, and adsorbent surface chemistry. In general,
If the selectivity of two components is equal to 1, then there is no way to separate them by improving the column efficiency.
The ideal value of □ is 2. It can be calculated by using formula,

□= V2 – V1 / V1 – V0 = k1'/ k2'

Where,
V_0 = the void volume of the column,

V_1 and V_2 = the retention volumes of the second and the first peak(α)= Adjusting selectivity
When troubleshooting changes in selectivity (α), the approach is similar to the approach used in the capacity factor.

Column Efficiency/ Band broadening:

Efficiency, N, of a column is measured by the number of theoretical plates per meter. It is a measure of band spreading of a peak. Similar the band spread, higher is the number of theoretical plates,
Indicating good column and system performance. Column with N ranging from 5,000 to 1,00,000 plates/meter are ideal for a good system.
Efficiency is calculated using the formula,

$$N = 16\ Rt^2 / W^2$$

Where,

Rt = is the retention time andW = is the peak width.
A decline in measured column efficiency may be due to:

- ✓ Age and history of the column
- ✓ Extra column band broadening (such as due to malfunctioning injector or impropertubing i.d.)
- ✓ Inappropriate detector setting.
- ✓ Change in flow rate and solvent viscosity

You can recognize problems in your separation due to a loss of column efficiency when the width or shapes of all peaks are affected.
Methods of measuring column efficiency (N):
Method used for the measurement and calculation of column include (in order to sensitivity to abnormal peak shape):

- Asymmetry based (most sensitive to tailing or fronting)
- 5 sigma
- 4 sigma
- Tangent
- 3 sigma
- ½ height
- 2 sigma (least sensitive to tailing or fronting).

Chose the method that best suits your operating requirements. It is critical that the same method always be used and executed reproducibly.

Peak asymmetry factor (As):

Peak asymmetry factor, A_s, can be used as a criterion of column performance. The peak half width, b, of a peak at 10% of the peak height, divided by the corresponding front half width, a, gives the asymmetry factor.
As = b / a

For a well-packed column, an asymmetry factor of 0.9 to 1.1 should be achievable.

VALIDATION OF METHOD

Validation is a process of establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics.

Method validation is the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, quality, purity, and potency of the drug substances and drug products.

Simply, method validation is the process of proving that an analytical method is acceptable for its intended purpose.

A successful validation guarantees that both the technical and regulatory objectives of the analytical methods have been fulfilled. The transfer of a method is best accomplished by a systematic method validation process.
The real goal of validation process is to challenge the method and determine limits of allowed variability for the conditions needed to run the method.

Type of analytical procedures to be validated:
Validation of analytical procedures is directed to the four most common types of analytical

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 4 July-Aug 2023, pp: 913-929 www.ijprajournal.com ISSN: 2249-7781

procedures.

1. Identification test.
2. Quantitative test for impurities content.
3. Limit test for the control of impurities.
4. Quantitative test of the active moiety in samples of drug substance on drug product on other selected components in the drug product.

In our method of validation, we are following last type.

Assay procedures are intended to measure the analyst present in given sample, assay represent a quantitative measurement of the major component(s) in the drug sample.

Objective of validation

The primary objective of validation is to form a basis for written procedure for production and process control which are designed to assure that the drug products have the identity, strength, quality and purity they purport or are represented to possess quality, safety and efficacy must be designed to build into the product.

Each step of the manufacturing process must be controlled to maximize the probability that the finished products meet all quality and design specification.

Benefits of validation:

a) Produces quality products.
b) Helps in process improvement technology transfer, related product validation, failure investigation, and increased employee awareness.
c) Cost reduction by increasing efficacy, few reject, longer equipment life, production of cost effective products.
d) Helps in optimization of process or method.
e) Regulatory affairs-produces approved products and increased ability to export.

Validation as defined by different agencies:

1. USFDA:

According to this "Validation is the process of establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its predetermined specifications and quality attributes.

2. WHO:

Defines Validation as an action of providing any procedure, process, equipment, material, activity or system actually leads to the expected results.\

3. EUROPEAN COMMITTEE:

Defines Validation as an action of providing in accordance with the principles of GMP that any procedure. Process, material, activity or systems actually lead to expectedresults.

This process consists of establishment of the performance characteristics and the limitations of the method.

Method validation is required when

1. A new method is been developed
2. Revision established method
3. When established methods are used in different laboratories and different analystsetc.
4. Comparison of methods
5. When quality control indicates method changes.

Performance characteristics examined when carrying out method validation are;

- Selectivity / Specificity
- Linearity / Range
- Precision
- Accuracy
- Limit of detection (LOD)/ Limit of quantification (LOQ)
- Robustness / Ruggedness

Significance of Method Validation:

The quality of analytical data is a key factor in the success of a drug development programme. The process of method development and validation has a direct impact on the quality of these data.

- To trust the method.
- Regulatory requirement.

Analytical validation is a very important feature of any package of information submitted to international regulatory agencies in support of new product marketing or clinical trials applications.a thorough method development can almost rule out all potential problems.

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 4 July-Aug 2023, pp: 913-929 www.ijprajournal.com ISSN: 2249-7781

SR No.	Ingredients	Company	Brand /Batch no.
1	Levosalbutamol Sulphate eq. to Levosalbutamol 2.5 mg+ Water for Injections	CIPLA	Salbair A3003AP
2	Levosalbutamol Sulphate eq. to Levosalbutamol 1.25 mg +Ipratropium bromide 0.50mg + Water for Injections	CIPLA	Salbair-I A2607JAP

1 List of the Instrument used:

- SHIMADZU LC2010CHT HPLC equipped with UV-visible detector.
- Digital Sonicator Transonic from HARRISON'S
- Electronic weight balance CPA225D from SARTORIUS.
- A Shimadzu's double beam UV spectrophotometer (UV-1800).
- Membrane Filter Assembly.
- pH meter from EUTEC.

5.2 List of chemicals and materials

- Water (HPLC Grade)
- Methanol (HPLC Grade)
- 2-tert-butylamino-1-(4-hydroxy-3-methylphenyl) ethanol sulphate RS
- Ammonium acetate (AR Grade)
- Propan-2-ol (AR Grade)
- Glacial acetic acid (AR Grade)
- Levosalbutamol Sulphate WS
- Potassium dihydrogen phosphate (G.R. grade)
- Ortho phosphoric acid (A.R. grade)
- Glasswares and Syringes

II. RESULT AND DISCUSSION:

Analytical method development and simultaneous estimation of levosalbutamol sulphate and ipratropium bromide and its validation by RP-HPLC, **Table: 20 Validation report of summary (HPLC)**

Parameter	Experiment	Discussion	Acceptance criteria
Specificity	Placebo / Blank interference	1) No interference was observed at retention time of Levosalbutamol Sulphate and Ipratropium bromide	1) No interference should be observed at retention time of Levosalbutamol Sulphate and Ipratropium bromide.
	System suitability	2) The relative standard deviation for six replicate measurements of	2) The relative standard deviation for six replicate

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 4 July-Aug 2023, pp: 913-929 www.ijprajournal.com ISSN: 2249-7781

		peak area response of standard preparation was found 0.102% 3) The relative standard deviation for retention time of six replicate injections of standard preparation was found 1.158%.	measurements of peak area response of standard preparation should be not more than 2.0%. 3) The relative standard deviation for retention time of six replicate injections of peak should not be more than 1.0%.
Precision	System Precision Method precision	1) The relative standard deviation for six replicate measurements of peak area response of standard preparation was found 0.102% 2) The relative standard deviation for retention time of six replicate injections of standard preparation was found 1.158%. 3) The individual assay of Levosalbutamol Sulphate and	1) The relative standard deviation for six replicate measurements of peak area response of standard preparation should be not more than 2.0% 2) The relative standard deviation for retention time of six replicate injections of peak should not be more than 1.0%.
		Ipratropium bromide was found in limit 100% ±5.0%	3) The individual assay of Levosalbutamol Sulphate and Ipratropium bromide should be 100% ±5.0%
Linearity	Linearity For Levosabutamol Sulphate.	Coefficient of correlation (r^2) For Levosalbutamol Sulphate 0.999	The regression coefficient (r^2) should be not less than 0.99.

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 4 July-Aug 2023, pp: 913-929 www.ijprajournal.com ISSN: 2249-7781

Reproducibility (Intermediate precision)	Analyst to analyst	The individual assay of Levosalbutamol Sulphate and Ipratropium bromide was found in limit 100% ±5.0%	The individual assay of Levosalbutamol Sulphate and Ipratropium bromide should be 100% ±5.0%
Accuracy	Recovery study	The mean recoveries for Levosalbutamol and Ipratropium bromide were found to be 99.64 %.	All the individual recoveries should be 100±2.0%.

The HPLC method for Levosalbutamol Sulphate assay in Levosalbutamol sulphate and Ipratropium bromide Inhalation Solution has been successfully validated to show specificity, Linearity, Precision, Intermediate Precision and Accuracy.

III. CONCLUSION

Considering the efficiency of HPLC, attempt has been made to develop simple, accurate, precise, rapid and economic method for simultaneous estimation of Levosalbutamol sulphate and Ipratropium bromide in a pressurized meter dose inhaler dosage form. Thus method described enables to the quantification of both API.

The advantages lie in the simplicity of sample preparation and the low costs of reagents used. Experimental results were indicative of satisfactory precision and reproducibility. Hence, this method can be used for analysis of pressurised meter dose form in quality control department. The methods described can successfully be employed for the determination of Levosalbutamol sulphate and Ipratropium pressurised meter dose inhaler for marketed formulations for routine analysis in quality control laboratories.

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