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Research Article

ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF ABACAVIR SULPHATE AND LAMIVUDINE

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ABSTRACT

Development of methods to achieve the final goal of ensuring the quantity of drug substances and drug products is not a trivial undertaking. The capabilities of the methods developed were complementary to each other. Hence they can be regarded as simple, specific and sensitive methods for the estimation of Abacavir Sulphate and Lamivudine in single and combined pharmaceutical dosage forms. The developed UV Spectrophotometric methods and RP-HPLC methods were validated according to ICH guidelines and were found to be applicable for the routine analysis of Abacavir Sulphate and Lamivudine in their single and combined dosage forms. The proposed UV methods were simple, sensitive and reliable with good precision and accuracy. This method was specific while estimating the commercial formulations without interference of excipients and other additives.

KEYWORDS: *Abacavir Sulphate, Lamivudine, RP-HPLC, Spectrophotometric, UV methods etc.*

INTRODUCTION

Analytical method development and validation play important roles in the discovery, development, and manufacture of pharmaceuticals. One of the most critical factors in developing pharmaceutical drug substances and drug products today is ensuring that the UV-Visible, HPLC analytical test methods that are used to analyze the products to generate meaningful data.

Analytical Chemistry (B.K. Sharma 2004) is a branch of chemistry that deals with the separation, identification and determination of components in a sample. It is the science of making quantitative measurements, which requires background knowledge of chemical and physical concepts.

Analytical chemistry may be defined as the *"Science and art of determining the composition of materials in terms of the elements or compounds contained".*

Analytical chemistry is important since the early days of chemistry, providing methods for determining which elements and chemicals are present in the world around us. During this period significant analytical contributions to chemistry include the development of systematic elemental analysis by Justis von Liebig and systematized organic analysis based on the specific reactions of functional groups (J.W.Robinson 2009).

To be effective and efficient, analyzing samples requires expertise in:

1. The chemistry that can occur in a sample
2. Analysis and sample handling methods for a wide variety of problems (the tools-of the- trade)
3. Proper data analysis and record keeping.

Pharmaceutical Analysis (www.pubmed.com) plays a major role today, and it can be considered as interdisciplinary subject.

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Analytical instrumentation plays an important role in the production and evaluation of new products and in the protection of consumers and the environment.

Pharmaceutical Analysis Techniques are applied Mainly in Two Areas:

1. Qualitative: Qualitative analysis seeks to establish the presence of a given element or compound in a sample.

2. Quantitative: Quantitative analysis seeks to establish the amount of a given element or compound in a sample.

Quantitative chemical analysis is an important tool to assure that the raw material used and the intermediate products meet the required specifications. Drug analysis is the base for the determination of the product.

Every year number of drugs is introduced into the market. Also quality is important in every product or service but it is vital in medicines as it involves life (K.A. Connors 1994).

Importance of Analytical Methods:

Drug analysis reveals identification characterization & determination of the drugs in mixtures like dosage forms & biological fluids. The number of drugs introduced in to the market has been increasing at very fast rate. These drugs may be either new entities in the market or partial structural modification of the existing drugs (B.K. Sharma 2004). Newer analytical methods are developed for these drugs or drug combination of the below reasons:

1. Official pharmacopoeia may not reveal an analytical procedure for the drugs or its combination.
2. Analytical method may not be available for the drug combination due to interference caused by excipients.

MATERIALS AND INSTRUMENTS

Materials:

Drug Sample:

- Abacavir sulphate (Gift sample procured from Chandra Labs, Hyderabad)

- Lamivudine (Gift sample procured from Chandra Labs, Hyderabad).

Chemicals Required:

For UV-VIS Spectrophotometry:

- Methanol AR grade (S. D. Fine-chem limited, Mumbai).
- Distilled water

For HPLC:

- Methanol (Merck specialties private limited, Mumbai).
- Double distilled water (Merck specialties private limited, Mumbai)

Instruments:

1. Axis Ag N 204-PO digital balance.
2. Elico LI 120 pH meter.
3. 1.5LH Ultrasonic bath sonicator.
4. Elico SL 218 double beam UV-Vis spectrophotometer, with Wide Range Photodiode detection and fixed 10 mm path holders for reference and sample. (Instrument-1).
5. Elico SL 210 double beam UV-Vis spectrophotometer, with Wide Range Photodiode detection and fixed 10 mm path holders for reference and sample. (Instrument-2).
6. Agilent 1120 compact LC system.

Agilent 1120 Compact LC Includes isocratic pump, manual injector, variable wavelength detector, Ezchrome Elite Compact software, LMD software, startup column.

Analytical Method Development and Validation:

Estimation of Abacavir Sulphate by UV-Spectrophotometric Method:

A. Method Development:

1. Solvent Selection: In order to select suitable solvent for determination of Abacavir Sulphate, various solvents were selected for the solubility studies and it was found that Abacavir Sulphate was freely soluble in the following solvents; Water, Methanol, Ethanol etc. In the present investigation Methanol: Water in the ratio of 50:50 was selected as solvent.

2. Preparation of Standard Stock Solution: Standard stock solution was prepared by dissolving accurately weighed 100 mg of Abacavir Sulphate in Methanol: Water (50:50) and the volume was made up to 100 mL with solvent in 100 mL volumetric flask (Stock solution-I, 1000 µg/ mL). 10 mL of stock solution-I was diluted to 100 mL with solvent (Stock solution-II, 100 µg/mL).

3. Determination of λ max:

C. Validation of Spectrophotometric Method:

The following parameters were determined to validate the developed analytical method as per ICH guidelines (ICH Q2B, 1996).

1. Accuracy
2. Precision
   Intra and Inter-day Precision
3. Linearity
4. Ruggedness
5. LOD and LOQ
6. Molar Extinction Coefficient (lit. mol^{-1} cm^{-1})
7. Sandell's Sensitivity (µg/cm^2/0.001 absorbance units)

Estimation of Lamivudine by UV-Spectrophotometric Method:

A. Method Development:

1. Solvent Selection: In order to select suitable solvent for determination of Lamivudine, various solvents were selected for the solubility studies and it was found that Lamivudine was freely soluble in the following solvents; Water, Methanol, Ethanol etc. In the present investigation Methanol: Water in the ratio of 50:50 was selected as solvent.

2. Preparation of Standard Stock Solution: Standard stock solution was prepared by dissolving accurately weighed 100 mg of Lamivudine in Methanol: Water (50:50) and the volume was made up to 100 mL with solvent in 100 mL volumetric flask (Stock solution-I, 1000 µg/ mL). 10 mL of stock solution-I was diluted to 100 mL with solvent (Stock solution-II, 100 µg/ mL).

3. Determination of λ max:

B. Application of Proposed Method for Analysis of Tablet Formulation:

For analysis of commercial formulation, 20 tablets of Lamivudine (Lamivir-HBV) were weighed, powdered in glass mortar and the powder equivalent to 10 mg of Lamivudine was weighed accurately and transferred into a 10 mL standard volumetric flask. The contents were dissolved in solvent and sonicated for few minutes. This solution was filtered through (0.45µ) Whatmann filter paper no. 41. 1 mL of the filtrate was diluted to 10 mL with solvent to get the solution of 100 µg/ mL. An appropriate aliquot of 0.8 mL of test solution was diluted to 10 mL with solvent in 10 mL standard volumetric flask to produce the concentration 8 µg/ mL. The absorbance of the solution was recorded at 272 nm and the concentration of the Lamivudine was determined by linear regression equation.

C. Validation of Spectrophotometric Method:

The following parameters were determined to validate the developed analytical method as per ICH guidelines (ICH Q2B, 1996).

1. Accuracy
2. Precision
   Intra and Inter-day Precision
3. Linearity
4. Ruggedness
5. LOD and LOQ
6. Molar Extinction Coefficient (lit. mol^{-1} cm^{-1})
7. Sandell's Sensitivity (µg/cm^2/0.001 absorbance units)

Simultaneous Estimation of Abacavir Sulphate and Lamivudine by UV- Spectrophotometric Method:

Simultaneous Equation Method (Vierodt's method):

If sample contains two absorbing substances (x and y) and each of which absorbs at the λmax of the other, then it may be possible to determine both the drugs by the technique of simultaneous equation. The information required is:

λ_1: Wavelength maxima for drug x
λ_2: Wavelength maxima for drug y
ax_1 and ax_2: Absorptivity of X at λ_1 and λ_2
ay_1 and ay_2: Absorptivity of Y at λ_1 and λ_2
A_1: Absorbance of sample at λ_1
A_2: Absorbance of sample at λ_2

Let Cx and Cy be the concentration of X and Y respectively in the diluted sample: Two equations are constructed based upon the fact that at λ1 and λ2, the absorbance of the mixture is the sum of the individual absorbances of X and Y.

At λ_1

$$A_1 = ax_1bCy + ay_1bCy \qquad \textbf{Equation 22}$$

At λ_2

$$A_2 = ax_2bCy + ay_2bCy \qquad \textbf{Equation 23}$$

For measurements in 1 cm cells, b = 1
Concentrations of X and Y can be determined by the equations

$$Cx = \frac{A2ay1 - A1ay2}{ax2ay1 - ax1ay2} \qquad \textbf{Equation 24}$$

$$Cy = \frac{A1ax2 - A2ax1}{ax2ay1 - ax1ay2} \qquad \textbf{Equation 25}$$

A. Selection of Sampling Wavelength for Analysis and Preparation of Standard:

1. Solvent Used: Distilled water

2. Preparation of Standard Stock Solution: 10 mg each of standard Abacavir sulphate and Lamivudine were weighed accurately and transferred in to two separate 10mL flasks, dissolved in 5mL of solvent and made up to the mark with distilled water to obtain a final concentration of 1000 µg/mL of each Abacavir sulphate and Lamivudine (standard stock solutions A1 and A2 respectively). From the above stock solution 'A1' and 'A2' 1 mL aliquots were pipetted in to two separate volumetric flasks and dissolved in 5mL of solvent and made up to the

mark with distilled water to obtain a final concentration of 100µg/mL. (Standard stock solutions 'B1' and 'B2' respectively).

3. Selection of Analytical Wavelengths: Appropriate dilution of the standard stock solutions 'A1' and 'A2' were scanned separately in the entire ultraviolet range. The λmax of each standard was selected in such a way that at each absorption maxima the difference in absorption of the two components should be as large as possible. The two wavelengths were 285nm and 271nm for Abacavir sulphate and Lamivudine respectively. At 285nm Abacavir sulphate has higher absorbance than Lamivudine and at 271 Lamivudine has higher absorbance than Abacavir sulpahte.

4. Selection of Analytical Concentration Range and Construction of Calibration Graph

B. Analysis of Tablet Formulation:

Twenty tablets of Abacavir sulphate and Lamivudine combination dosage forms (Abamune-L) were weighed and their average weight was determined. The tablets were crushed in to fine powder. From the tablet triturate a tablet mass equivalent to 10mg of Abacavir sulpahte or 5 mg of Lamivudine was transferred in to a 10mL volumetric flask , dissolved in a small quantity of methanol by sonication for 10min and finally the volume was made up to the mark with methanol. The resultant solution was filtered through a Whatmann filter paper no. 41 and used as sample stock solution 'A' (1000µg/mL Abacavir sulphate and 500µg/mL Lamivudine).

From the above stock solution 1mL aliquot was transferred in to a 10 mL volumetric flask, dissolved in a small quantity of distilled water and the volume was made up to the mark with distilled water to obtain a final concentration of 100µg/mL Abacavir sulphate and 50µg/mL Lamivudine. This solution was used as the sample stock solution 'B'.

0.8mL of the sample stock solution 'B' was transferred in to a 10 mL volumetric flask, dissolved in a small quantity of distilled water and the volume was made up to the mark with distilled water. The absorbance of the resultant solution was measured at the two absorption maxima 285nm and 271nm. This absorbance was noted as A_1 and A_2respectively and amount of the drugs present was calculated using simultaneous equation method and the results were given.

C. Method Validation:

The following parameters were determined to validate the developed analytical method as per ICH guidelines (ICH Q2B, 1996).

1. Accuracy:
2. Precision:
3. Linearity and Range:
4. Ruggedness:
5. Determination of Molar Extinction Coefficient:
6. Sandell's Sensitivity (µg/cm^2/0.001 absorbance units):

A. Selection of Sampling Wavelength for Analysis and Preparation of Standard Calibration Curves.

1. Selection of Mobile Phase: The standard solutions containing Abacavir sulphate and Lamivudine were injected into the HPLC system and run in different solvent systems. By studying literature survey, different mobile phases in different proportions and different pH were tried in order to find the best conditions for the separation.

It was found that methanol and water gives satisfactory results as compared to other mobile phases. This mobile phase system was tried with different proportions and using different flow rates. Finally, the optimal composition of the mobile phase was determined to be Methanol: Water (50:50).

2. Preparation of Mobile Phase: Mobile phase was prepared by mixing methanol and water in the ratio of 50:50 and was initially filtered through 0.45µm millipore membrane filter and sonicated for 15 min before use.

3. Preparation of Standard Stock Solution: The separate stock solutions of ABA and LAM were prepared by accurately weighing 10 mg each into a separate 10 mL volumetric flasks A and B and made up to the volume with mobile phase to get 1000µg/mL respectively. From the above standard stock solutions 1mL from volumetric flask A and 0.5 mL from volumetric flask B was transferred to a 10 mL volumetric flask and made up to the volume with same mobile phase to get 100µg/mL and 50µg/mL of ABA and LAM respectively (Working stock solution).

4. Selection of Analytical Wavelength: By appropriate dilution of each standard stock solution with mobile phase, various concentrations of Abacavir sulphate and Lamivudine were prepared separately. Each solution was scanned using double beam UV visible spectrophotometer between the range of 200 nm to 400 nm and their spectra was overlaid. Abacavir sulphate and Lamivudine, 277 nm was selected as analytical wavelength for Multi component analysis using HPLC method.

5. Optimized Chromatographic Conditions: Mobile phase consisting of Methanol: Water (50:50 v/v) was used in isocratic mode. The mobile phase was initially filtered through 0.45µm millipore membrane filter and sonicated for 15 min before use. The flow rate was maintained at 1 mL/min and the injection volume was 20µL. UV detection was performed at 277 nm and the separation was achieved at ambient temperature.

6. Selection of Analytical Concentration Range and Construction of Calibration Curve for Abacavir Sulphate and Lamivudine: Appropriate aliquots ranging from 0.5 mL to 2.5 mL were pipetted out from the working stock solution (100 µg/mL of Abacavir sulphate and 50 µg/mL of Lamivudine) in to a series of 10 mL volumetric flasks. The volume was made up to the mark with the mobile phase to get a set of solutions having the concentration range, ranging from 5-25 µg/mL of Abacavir sulphate and 2.5-12.5 µg/mL of Lamivudine respectively. Triplicate dilutions of each of the above mentioned concentrations were prepared separately and from these triplicate solutions, 20 µL of each concentration of the drug were injected into the HPLC system three times separately and their chromatograms were recorded under the same chromatographic conditions as described above.

Peak areas were recorded for all the peaks and a standard calibration curve of area against concentration was plotted as concentration of the drug Vs peak area The results were shown in Both the drugs follow the Beer's Lambert's law in the concentration range of 5-25 µg/mL of Abacavir sulphate and 2.5-12.5 µg/mL of Lamivudine.

The linearity of calibration curves and adherence of the system to Beer's Lambert's law was validated by high value of correlation coefficient and less than 2% percent relative standard deviation (%RSD) for the intercept value which were shown in

B. Analysis of Tablet Formulation:

The tablets (Abamune-L) were initially powdered and an amount equivalent to 100 mg of Abacavir sulphate and 50 mg of Lamivudine was accurately weighed into a 100 mL volumetric flask, mixed with 50 mL of mobile phase. The solution was made up to the volume with mobile phase and sonicated for 5 minutes. The solution was then filtered through 0.45µm millipore membrane filter. Final stock containing 20µg/mL and 10µg/mL of Abacavir sulphate and Lamivudine respectively was prepared by subsequent dilution with the same mobile phase. 20µL of sample solution was injected into chromatographic system and the peak responses were measured. The solution was injected three times into the column. The amount present in each tablet was calculated by comparing the areas of test with that of the standard.

C. Method Validation:

The method was validated according to ICH Q2 B guidelines for validation of analytical procedures in order to determine system suitability, linearity, sensitivity, precision, accuracy and robustness for the analytes(ICH Q2B, 1996).

1. System Suitability:
2. Accuracy:
3. Linearity and Range:
4. Precision:
   - *Intraday Precision*
   - *Interday Precision*
5. Specificity and Selectivity:
6. Robustness:
7. Ruggedness:
8. LOD and LOQ:

RESULTS AND DISCUSSION

Drug combinations are commonly used clinically and analyst is required to develop suitable methods of their analysis. For routine analytical purposes it is always of interest to establish methods capable of analyzing a large number of samples in a short time period with good accuracy and precision. The commonly used tests of pharmaceutical analysis generally entail compendia testing method development, setting specifications, and method validation.

Analytical testing is one of the more interesting ways for scientists to take part in quality process by providing actual data on the identity, content and purity of the drug products. New methods are now being developed with a great deal of consideration to worldwide harmonization. As a result, new products can be assured to have comparable quality and can be brought to international markets faster.

A liquid chromatographic technique coupled with spectrophotometric analysis is a versatile tool that can generate extensive analytical data that is highly useful in the routine drug analysis. For routine analytical purposes it is always of interest to establish methods capable of analyzing a large number of samples in a short time period with good accuracy and precision.

In the present work, an attempt was made to provide a newer, simple, accurate and low cost spectrophotometric and HPLC methods for the effective quantitative determination of Abacavir sulphate and Lamivudine as an active pharmaceutical ingredients as well as in pharmaceutical preparations in their single and combined dosage forms, without the interferences of other constituent in combined formulations. Hence it is planned to develop both HPLC and Spectrophotometric methods.

The results were summarized as follows:

Estimation of Abacavir Sulphate and Lamivudine by UV-Spectrophotometric Method:

Table No. 1: Summarized Results of UV- Spectrophotometric Methods

Parameter	Results	
	Abacavir Sulphate	Lamivudine
λ max	285	272
Beer's Law Range (µg/mL)	2-10	2-10
Regression Equation	Y= 0.0619x+0.0061	Y= 0.0563x+0.0093
Correlation Coefficient (r^2)	0.9993	0.9991
Molar Extinction Coefficient (lit.mol^{-1}cm^{-1})	637.94	592.34
Sandell's Senstivity(µg. cm^{-2}/0.001 abs units)	0.0151	0.0158
% Recovery (w/w)	99.21-100.7%	99.48-100.45%
LOD (µg/mL)	0.91	0.70
LOQ (µg/mL)	2.76	2.14
Assay (% Purity) w/w	99.18%	99.63
Precision (%RSD)		
Intraday Precision	0.99	1.21
Inter day Precision	1.36	1.85
Ruggedness (%RSD)		
Analyst 1	0.31	0.46
Analyst 2	0.20	0.13
Instrument 1	0.11	0.14
Instrument 2	0.61	0.55

Simultaneous Estimation of Abacavir Sulphate and Lamivudine by UV- Spectrophotometric Method:

Table No. 2: Summarized Results of Simultaneous UV-Spectrophotometric Method

Parameter	Results	
	Abacavir Sulphate	Lamivudine
λ max	285	271
Beer's Law Range (µg/mL)	2-10	2-10
Regression Equation	Y= 0.0675x+0.0077	Y= 0.0564x+0.0003
Correlation Coefficient (r^2)	0.9992	0.9997
Molar Extinction Coefficient (lit.mol^{-1}cm^{-1})	699.46	565.5
Sandell's Senstivity (µg. cm^{-2}/0.001 abs units)	0.0137	0.0174
% Recovery	99.69-100.56%	98.53-100.36%
LOD (µg/mL)	0.12	0.11
LOQ (µg/mL)	0.38	0.34
Assay (% Purity) w/w	99.79%	99.57%
Precision (%RSD)		
Intraday Precision	0.55	0.94
Inter day Precision	0.18	1.00
Ruggedness (%RSD)		
Analyst 1	0.19	1.08
Analyst 2	0.11	0.21
Instrument 1	0.57	0.81
Instrument 2	0.19	0.91

Simultaneous Estimation of Abacavir Sulphate and Lamivudine by RP-HPLC Method:

Table No. 3: Summarized Results of RP-HPLC Method

Parameter	Results	
	Abacavir Sulphate	Lamivudine
Detection Wavelength	277	
R_t (min)	3.37	6.40
Beer's Law Range (µg/mL)	5-25	2.5-12.5
Regression Equation	Y= 644751x+192617	Y= 694801x+69996
8888888Correlation Coefficient (r^2)	0.9993	0.9997
% Recovery (w/w)	99.3-100.2%	98.4-101.2%
LOD (µg/mL)	0.16	0.33
LOQ (µg/mL)	0.49	1.01
Assay (% purity) w/w	99.83%	100.53%
Precision		
Intraday Precision	0.83	0.62
Inter day Precision	1.24	0.89
Robustness		
Flow Rate 0.9 mL/min	1.53	1.52
Flow Rate 1.1 mL/min	1.96	0.98
Detection Wavelength 275 nm	0.42	0.43
Detection Wavelength 279 nm	0.71	0.59
Ruggedness		
Analyst 1	1.51	1.11
Analyst 2	0.72	0.61

CONCLUSION

Development of methods to achieve the final goal of ensuring the quantity of drug substances and drug products is not a trivial undertaking. The capabilities of the methods developed were complementary to each other. Hence they can be regarded as simple, specific and sensitive methods for the estimation of Abacavir sulphate and Lamivudine in single and combined pharmaceutical dosage forms. The developed UV Spectrophotometric methods and RP-HPLC methods were validated according to ICH guidelines and were found to be applicable for the routine analysis of Abacavir sulphate and Lamivudine in their single and combined dosage forms. The proposed UV methods were simple, sensitive and reliable with good precision and accuracy. This method was specific while estimating the commercial formulations without interference of excipients and other additives. Hence, this method can be used for the estimation of Abacavir sulphate and Lamivudine in bulk samples and their pharmaceutical formulations individually and in combination by simultaneous equation method. The developed and validated RP-HPLC method was found to be economical due to the use of higher percentage of water as a solvent in mobile phase. The low solvent consumption (1mL/min), along with short analytical run time of less than 10.0 minutes lead to an environmental friendly chromatographic procedure that allows the analysis of a large number of samples in a short period of time. This method has been found to be better than previously reported methods, due to its wider range of linearity, use of readily available mobile phase, lack of extraction procedures. Hence above method can be used in quality control for routine analysis of finished products of Abacavir sulphate and Lamivudine simultaneously without any interference.

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