Asian Journal of Chemical and Pharmaceutical Research

Journal Home Page: www.pharmaresearchlibrary.com/ajcpr

Research Article Open Access

Analytical Method Development and Validation for the Simultaneous Estimation of Acyclovir and Hydrocortisone by RP-HPLC Method in Bulk and Tablet Dosage Form

CH. Shankar*, B. Rajesh

Department of Pharmaceutical Analysis, Vikas College of Pharmaceutical Sciences, Rayanigudem, Suryapet, Nalgonda, Telangana, India-508376

A B S T R A C T

The chromatographic conditions were successfully developed for the separation of Acyclovir and Hydrocortisone by using Agilent C_{18} Column (250mm x 4.6mm)5µm, flow rate was 1ml/min, mobile phase ratio was Phosphate buffer P^H 2.5:Methanol (65:35 v/v), detection wavelength was 254 nm. The Spectroscopic method was done in solvent using methanol and the instrument lab India 3000+ with UV win software. The instrument used was WATERS HPLC Auto Sampler, Separation module 2690, photo diode array detector, Empower-software version 2. The retention times were found to be 2.113 min and 3.560 min. The % purity of Acyclovir and Hydrocortisone was found to be with in the limits. The linearity study of Acyclovir and Hydrocortisone was found in concentration range of 5µg-25µg and 20µg-100µg and correlation coefficient (r^2) was found to be 0.999 and 0.999 respectively, % recovery for Acyclovir and Hydrocortisone was found to be within the limits. %RSD for repeatability and precision was found to be <2. LOD values were 0.001 and 0.005 and LOQ value was 0.004 and 0.015 respectively for Acyclovir and Hydrocortisone.

Keywords: Acyclovir, Hydrocortisone, HPLC.

A R T I C L E I N F O

CONTENTS

1. Introduction . . . 56
2. Materials and Methods . . . 56
3. Results and discussion . . . 58
4. Conclusion . . . 60
5. References . . . 61

Article History: Received 18 December 2015, **Accepted** 25 January 2016, **Available Online** 12 April 2016

***Corresponding Author**
CH. Shankar
Department of Pharmaceutical Analysis,
Vikas College of Pharmaceutical
Sciences, Rayanigudem, Suryapet,
Nalgonda, Telangana, India
Manuscript ID: AJCPR2906

PAPER-QR CODE

Citation: CH. Shankar, *et al.* Analytical Method Development and Validation for the Simultaneous Estimation of Acyclovir and Hydrocortisone by RP-HPLC Method in Bulk and Tablet Dosage Form. *A. J. Chem. Pharm, Res.*, 2016, 4(1): 55-62.

Copyright© 2016 CH. Shankar, *et al.* This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.

1. Introduction

Acyclovir is an anti viral drug. Viral (HSV-1, HSV-2 and VZV) thymidine kinase converts acyclovir to the acyclovir monophosphate, which is then converted to the diphosphate by cellular guanylate kinase, and finally to the triphosphate by phosphoglycerate kinase, phosphoenolpyruvate carboxy-kinase, and pyruvate kinase. Acyclovir triphosphate competitively inhibits viral DNA polymerase and competes with the natural deoxyguanosine triphosphate, for incorporation into viral DNA. Once incorporated, acyclovir triphosphate inhibits DNA synthesis by acting as a chain terminator. One may consider acyclovir to be a prodrug as it is metabolized to more active compounds. Acyclovir is selective and low in cytotoxicity as the cellular thymidine kinase of normal, uninfected cells does not use acyclovir effectively as a substrate. Hydrocortisone binds to the cytosolic glucocorticoid receptor. After binding the receptor the newly formed receptor-ligand complex translocates itself into the cell nucleus, where it binds to many glucocorticoid response elements (GRE) in the promoter region of the target genes. The DNA bound receptor then interacts with basic transcription factors, causing the increase in expression of specific target genes.

The anti-inflammatory actions of corticosteroids are thought to involve lipocortins, phospholipase A2 inhibitory proteins which, through inhibition arachidonic acid, control the biosynthesis of prostaglandins and leukotrienes. Specifically glucocorticoids induce lipocortin-1 (annexin-1) synthesis, which then binds to cell membranes preventing the phospholipase A2 from coming into contact with its substrate arachidonic acid. This leads to diminished eicosanoid production. The cyclo-oxygenase (both COX-1 and COX-2) expression is also suppressed, potentiating the effect. In other words, the two main products in inflammation Prostaglandins and Leukotrienes are inhibited by the action of Glucocorticoids. Glucocorticoids also stimulate the lipocortin-1 escaping to the extracellular space, where it binds to the leukocyte membrane receptors and inhibits various inflammatory events: epithelial adhesion, emigration, chemotaxis, phagocytosis, respiratory burst and the release of various inflammatory mediators (lysosomal enzymes, cytokines, tissue plasminogen activator, chemokines etc.) from neutrophils, macrophages and mastocytes. Additionally the immune system is suppressed by corticosteroids due to a decrease in the function of the lymphatic system, a reduction in immunoglobulin and complement concentrations, the precipitation of lymphocytopenia, and interference with antigen-antibody binding.

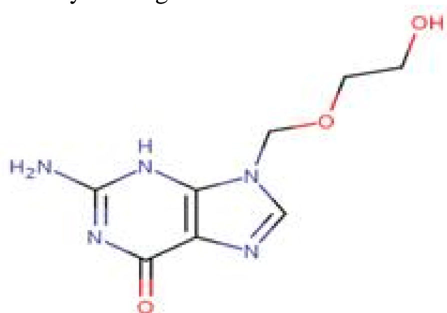

Figure 1: Acyclovir

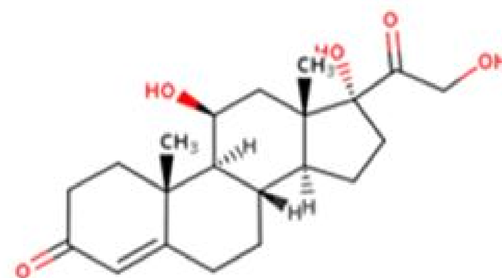

Figure 2: Hydrocortisone

Analytical methods

The technique employed in qualitative and quantitative analysis is based upon the performance of suitable chemical reactions and either measuring the amount of reagent needed to complete the reaction, or ascertaining the amount of reaction product obtained [1]. Quality is important in every product or service but it is vital in medicine as it involves life. Unlike ordinary consumer goods, there can be no "second quality" in drugs. Quality control is a concept, which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stages of production [2]. Physico-chemical methods are used to study the physical phenomenon that occurs as a result of chemical reactions. Among the Physico-chemical methods, the most important are optical (Refractometry, Polarimetry, Emission, Fluorescence methods of analysis, Photometry including Photocolorimetry and Spectrophotometry covering UV-Visible and IR regions and Nephelometry or Turbidimetry) and chromatographic (Column, Paper, TLC, GLC, HPLC) methods[3]. Methods such as Nuclear Magnetic Resonance and Para Magnetic Resonance are becoming more and more popular. The combination of Mass Spectroscopy with Gas Chromatography and Liquid Chromatography are the most powerful tools available.

The number of new drugs is constantly growing. This requires new methods for controlling their quality. Modern pharmaceutical analysis must need the following requirements.

1. The analysis should take a minimal time.
2. The accuracy of the analysis should meet the demands of pharmacopeia
3. The analysis should be economical.
4. The selected method should be precise and selective.

2. Materials and Methods

Apparatus

The instrument used for the study was Alliance Waters HPLC Auto Sampler, Separation module 2690, photo diode array detector with Empower-software version-2.

Reagents and Materials

The solvents used were Methanol, Ortho phosphoric acid, Potassium dihydrogen ortho phosphate, Tri Ethyl Amine of HPLC Grade and HPLC Water.

Selection of chromatographic condition

Proper selection of the method depends upon the nature of the sample, its molecular weight and solubility. The drugs

selected in the present study are polar in nature and hence reversed phase or ion-pair or ion exchange chromatography method may be used. The reversed phase HPLC was selected for the separation because of its simplicity and suitability [4].

Selection of detection wavelength:

The sensitivity of method that uses UV- Vis detector depends upon the proper selection of wavelength. An ideal wavelength is that gives maximum absorbance and good response for both the drugs to be detected [5]. Standard solutions of Acyclovir and Hydrocortisone were scanned in the UV range (200-400nm) and the spectrums obtained were overlaid and the overlain spectrum was recorded. From the overlain spectrum, 254 nm was selected as the detection wavelength for the present study [6].

Selection of mobile phase:

Initially the mobile phase tried was methanol and water, methanol and Methanol, buffer and water in various proportions. Finally, the mobile phase was optimized to Buffer: Methanol in proportion 65:35 v/v respectively [7].

Chromatographic trials for Simultaneous Estimation of Acyclovir and Hydrocortisone by RP- HPLC.

Trial-1 Chromatographic conditions

Parameters Description

Flow rate : 1 ml min-1

Column : Inertsil C18 Column (250mm x 4.6 mm)5µ

Mobile Phase: Buffer P^H (2.5): Methanol (30:70 v/v)

Detector: PDA

Column temperature: Ambient

Wavelength: 254 nm

Type of elution: Isocratic

Injection volume: 20 µl

Run time: 10 min

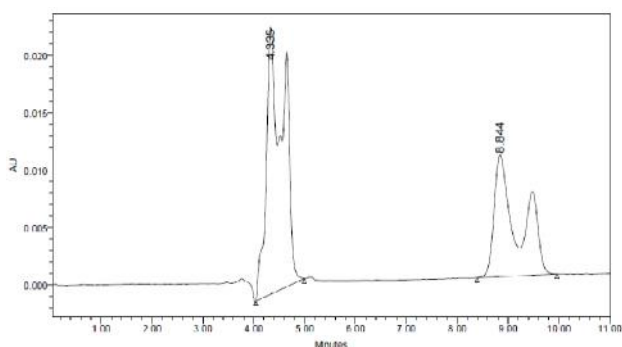

Figure 3: Chromatogram of Trial-1

Observation: The separation of two analytical peaks was not proper, so the mobile phase ratio has been changed for next trial.

Trial-2 Chromatographic condition

Parameters Description

Flow rate: 1ml min-1

Column: Inertsil C18 Column (250 mm x 4.6 mm) 5µg.

Mobile Phase: Buffer: Methanol PH 3.0 (40:60 v/v)

Buffer: Potassium dihydrogen orthophosphate p^H 2.5 adjusted with Orthophosphoric acid

Detector: PDA

Column temperature: Ambient

Type of elution: Isocratic

Wavelength: 254nm

Injection volume: 20µl

Run time: 10min

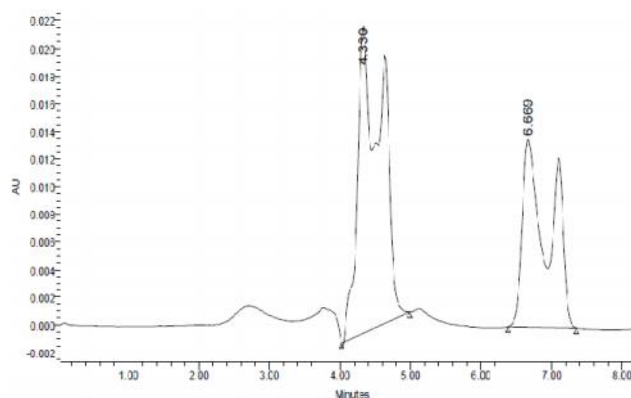

Figure 4: Chromatogram of Trial-2

Observation: The separation of two analytical peaks was not proper, so the mobile phase ratio has been changed for next trial.

Trial-3 Chromatographic condition

Parameters Description

Flow rate: 1 ml min-1

Column: Inertsil C18 Column (250mm x 4.6mm) 5µg.

Mobile Phase: Buffer PH 4.0: ACN (60:40 v/v)

Buffer: Potassium dihydrogen orthophosphate p^H 2.5 adjusted with OPA

Detector: PDA

Column temperature: Ambient

Type of elution: Isocratic

Wavelength: 254 nm

Injection volume: 20µl

Run time: 10min

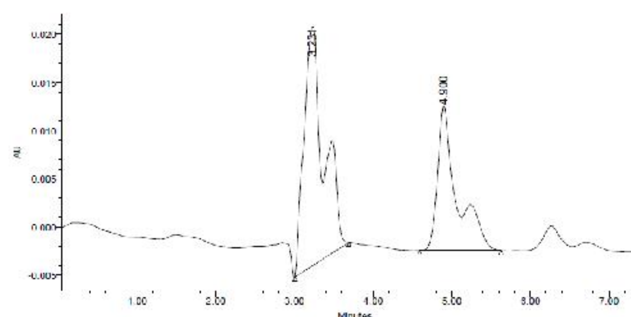

Figure 5: Chromatogram of Trial-3

Observation: The separation of two analytical peaks was not proper, so the mobile phase ratio has been changed for next trial.

Trial-4 Chromatographic condition

Parameters Description

Flow rate: 1ml min-1

Column: Agilent C18 Column (150mm x 4.6 mm) 5µg.

Mobile Phase: Phosphate buffer: Methanol PH 2.5 (20:80 v/v)

Buffer: Potassium dihydrogen orthophosphate PH 2.5 adjust with orthophosphoric acid
Detector: PDA
Column temperature: Ambient
Type of elution: Isocratic
Wavelength: 254 nm
Injection volume: 20µl
Run time: 10min

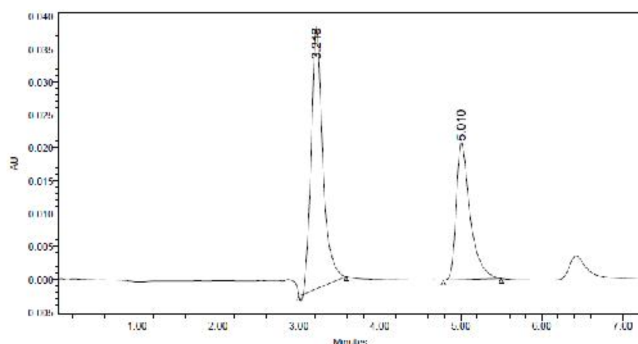

Figure 6: Chromatogram of Trial-4

Observation: The separation of two analytical peaks is occurred but fronting occurs in Hydrocortisone peak.

Trial-5 Chromatographic condition
Parameters Description
Flow rate: 1ml min-1
Column: Agilent C18 Column (250mm x 4.6mm) 5µm.
Mobile Phase: Phosphate buffer: Methanol P^H 2.5(55:45 v/v)
Buffer: Potassium dihydrogen orthophosphate P^H 4.0 adjust with Orthophosphoric acid
Detector : PDA
Column temperature: Ambient
Type of elution: Isocratic
Wavelength: 254 nm
Injection volume: 10µl
Run time: 10min

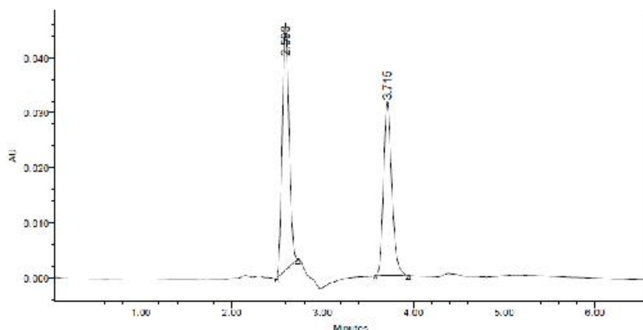

Figure 7: Chromatogram of Trial-5

Observation: The separation of two analytical peaks was good but base line noise is occurred. So the mobile phase ratio has been changed for next trial.

Trial-6 Chromatographic condition (Optimized Method)
Parameters Description
Flow rate: 1ml min-1
Column: Inertsil C18 Column (250mm x 4.6mm) 5µm.
Mobile Phase: Phosphate buffer: Methanol PH 2.5 (65:35 v/v)
Buffer: Potassium dihydrogen ortho-phosphate PH 2.5 adjust with Ortho-phosphoric acid
Detector: PDA
Column temperature: Ambient
Type of elution: Isocratic
Wavelength: 254 nm
Injection volume: 20µl
Run time: 10min

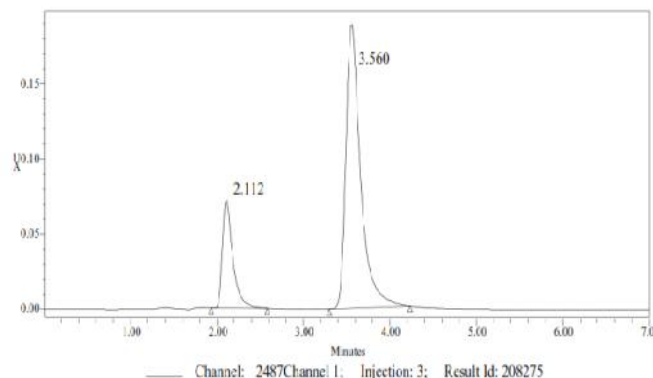

Figure 8: Chromatogram of Trial-5(optimized method)

Procedure
Preparation of Buffer:
About 7.0 g of potassium dihydrogen orthophosphate was dissolved in 1000 ml of HPLC grade water and pH 2.5 was adjusted with orthophosphoric acid. It was filtered through 0.45 µm nylon membrane filter and degassed with sonicator. It was used as a diluent for the preparation of sample and standard solution [8].
Preparation of mobile phase:
Mobile phase consist of buffer: Methanol of P^H 2.5 (35:65) was taken sonicated and degassed for 10min and filtered through 0.45 µm nylon membrane filter
Standard Preparation:
Weigh accurately 10 mg Hydrocortisone Working Reference Standard and 15 mg of Acyclovir Working Reference Standard is taken in to 100 ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark. After that 50 ml of the above solution was taken into 100 ml standard flask and made up with mobile phase. (Stock solution) Further pipette 0.5 ml of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluents [9].

3. Results and discussion

Method Validation Parameters
1. Specificity
The system suitability for specificity was carried out to determine whether there is any interference of any impurities in retention time of analytical peak. The specificity was performed by Injecting blank

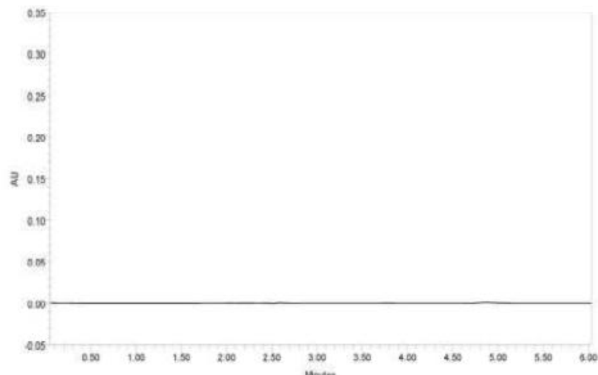

Figure 9: Chromatogram of Blank

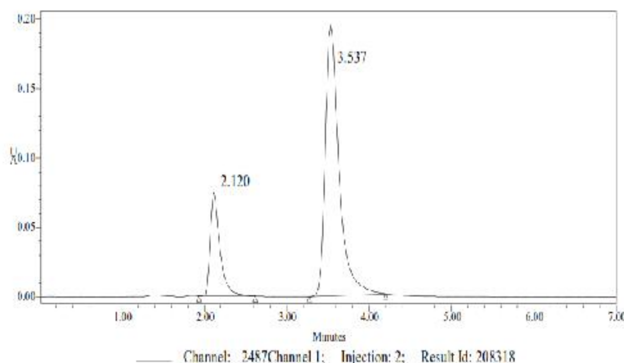

Figure 10: Chromatogram of Sample

2. Linearity

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range.Serial dilutions of Acyclovir and Hydrocortisone (5-25 µg/ml and 20-100 µg/ml) were injected into the column and detected at a wavelength set at 254 nm. The calibration curve was obtained by plotting the concentration vs. peak area [10].

Acceptance criteria: Correlation coefficient should be not less than 0.999.

3. Range: Based on precision, linearity and accuracy data it can be concluded that the assay method is precise, linear and accurate in the range of 5-25µg/ml and 20-100 µg/ml for Acyclovir and Hydrocortisone respectively

4. Accuracy

Accuracy of the method was determined by recovery experiments. There are mainly 2 types of recovery studies are there.

a) Standard addition method: To the formulation, the reference standard of the respective drug of known concentration was added, analyzed by HPLC and compared with the standard drug concentration [11].
b) Percentage method: For these assay method samples are prepared in three concentrations of 50%, 100%, and 150% respectively.

Acceptance criteria: The mean % recovery of the Acyclovir and Hydrocortisone at each level should be not less than 95.0% and not more than 105.0%.

Assay procedure: 20µL of the standard and sample solutions of Acyclovir and Hydrocortisone were injected into the HPLC system and the chromatograms were recorded. Amount of drug present in the Tablets were calculated using the peak areas [12].

5. Precision

The precision of the method was demonstrated by intra-day and inter-day precision studies. Intra-day studies were performed by injecting three (3) repeated injections within a day. Peak area and %RSD were calculated and reported [13]. The chromatograms of intra-day precision studies were shown. Inter-day precision studies, was done by injecting three (3) repeated injections for three consecutive days. Peak area and %RSD were calculated and reported [14].

Acceptance criteria: The % RSD for the area of six sample injections results should not be more than 2.

Selection of solvent

Solutions of Acyclovir and Hydrocortisone were prepared in different solvents like methanol, ethanol, acetonitrile and UV spectrum of each were recorded by scanning between 200-400 nm.

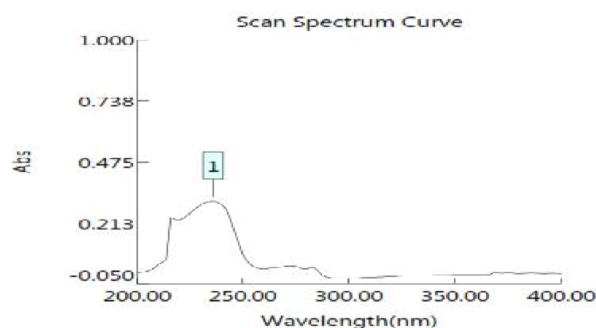

Figure 11: Overlain Spectra of Acyclovir and Hydrocortisone

Validation of the method

Linearity

Acyclovir and Hydrocortisone: The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. Serial dilutions of Acyclovir and Hydrocortisone (5-25 µg/ml and 20-100 µg/ml) were injected into the column and detected at a wavelength set at 254 nm. The calibration curve was obtained by plotting the concentration vs. peak area.

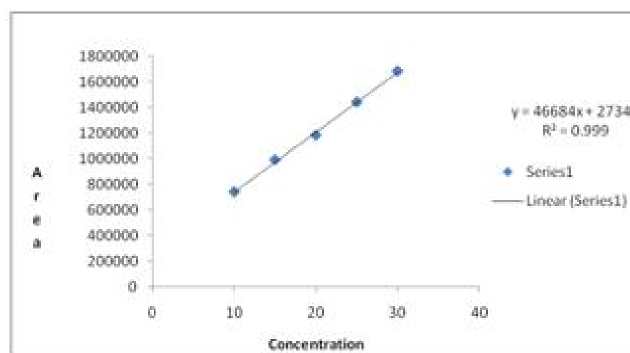

Figure 12: Calibration graph of Acyclovir

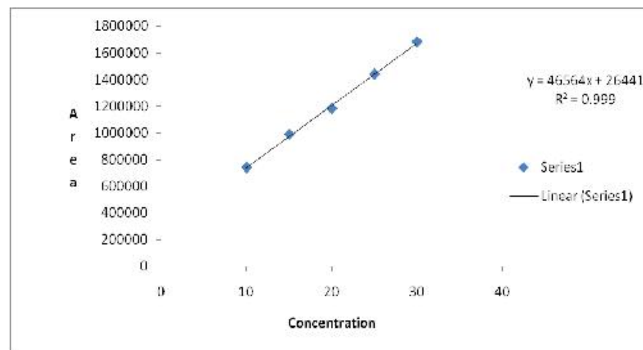

Figure 13: Calibration graph of Hydrocortisone

Recovery studies

In order to ensure the suitability and reliability of proposed method, recovery studies were carried out. To an equivalent quantity of formulation powder a known quantity of standard Acyclovir and Hydrocortisone were added at 50%, 100% and 150% level and the contents were re-analyzed by the proposed method

4. Conclusion

The proposed HPLC method was found to be simple, specific, precise, accurate, rapid and economical for simultaneous estimation of Acyclovir and Hydrocortisone in tablet dosage form. The developed method was validated in terms of accuracy, precision, linearity, robustness and ruggedness, and results will be validated statistically according to ICH guidelines. The Sample recoveries in all formulations were in good agreement with their respective label claims. From literature review and solubility analysis initial chromatographic conditions Mobile phase phosphate buffer: Methanol 65:35 were set (Buffer P^H 2.45 adjusted with ortho phosphoric acid), Agilent C18 (250×4.6 mm, 5μ) Column, Flow rate 1.0 ml/min and temperature was ambient, eluent was scanned with PDA detector in system and it showed maximum absorbance at 254 nm. As the methanol content was increased Acyclovir and Hydrocortisone got eluted with good peak symmetric properties. The retention times for Acyclovir and Hydrocortisone was found to be 2.113 min and 3.560 min respectively. System suitability parameters were studied by injecting the standard five times and results were well under the acceptance criteria. Linearity study was carried out between 50% to 150 % levels, R^2 value was found to be as 0.999. By using above method assay of marketed formulation was carried out, 100.7% was present. Full length method was not performed; if it is done this method can be used for routine analysis of Acyclovir and Hydrocortisone.

Table 1: Calibration data of Brinzolamide and Timolol Maleate

Sample ID	Acyclovir		Hydrocortisone	
	Conc(mcg/ml)	Area	Conc (mcg/ml)	Area
20% of operating conc	5	740046	20	1224140
40% of operating conc	10	990204	40	1595681
60% of operating conc	15	1183023	60	1992966
80% of operating conc	20	1439886	80	2356546
100% of operatingconc	25	1682302	100	2797214
Correlation Coefficient	0.999		0.999	

Table 2: Showing accuracy results for Acyclovir

Sample Id	Conc. Obtained(μg/ml)	% Recovery of drug	Mean accuracy	%RSD
50%	4.92	98.0		
50%	4.96	99.2		
50%	5.02	100.4	**99.2**	**1.2**
100%	9.95	99.5		
100%	9.94	99.4		
100%	9.98	99.8	**99.5**	**0.2**
150%	14.78	98.6		
150%	14.94	99.6	**99.0**	**0.530**
150%	14.83	98.8		

Table 3: Showing accuracy results for Hydrocortisone

Sample Id	Conc found (μg/ml)	Concn Obtained (μg/ml)	%Recovery	Mean recovery	Statistical Analysis
50%	5	5.01	100.2		
50%	5	4.96	99.2	99.73	
50%	5	4.99	99.8		%RSD= 0.505
100%	10	9.95	99.5		
100%	10	9.87	98.7	98.8	
100%	10	9.82	98.2		%RSD=0.66

150%	15	14.64	97.6		
150%	15	14.76	98.4	98.8	
150%	15	15.06	100.4		%RSD=1.45

Table 4: Robustness

	Name	Retention Time (min)	Area (V*sec)	Height (V)	USP Plate Count	USP Tailing	USP Resolution
1	Hydrocartisone	2.290	606093	67217	2642.0	1.4	
2	Acyclovir	4.435	2239255	149462	2310.5	1.2	6.9

System Suitability Results at flow rate 0.8 ml/min

Table 5: Precision

Name: Hydrocartisone

	Name	RT	Area
1	Hydro	2.108	602223
2	Hydro	2.105	607748
3	Hydro	2.113	607302
4	Hydro	2.109	608674
5	Hydro	2.109	607376
Mean			606665
Std. Dev.			2542.3
% RSD			0.42

Name: Acyclovir

	Name	RT	Area
1	Acyclovir	3.552	2220333
2	Acyclovir	3.550	2221573
3	Acyclovir	3.564	2215483
4	Acyclovir	3.564	2217379
5	Acyclovir	3.565	2211255
Mean			2217205
Std. Dev.			4100.8
% RSD			0.18

System Suitability Results at flow rate 1.2 ml/min

Table 6: LOD and LOQ

Hydrocortisone			Acyclovir		
Conc.(x) (µg/ml)	**Peak Areas (y)**	**Statistical Analysis**	**Conc.(x) (µg/ml)**	**Peak Areas (y)**	**Statistical Analysis**
5	1196	S = 39092 c = 618048 LOD: 0.001µg/ml LOQ: 0.004µg/ml	20	1661	S = 39092 c =369381 LOD:0.005 µg/ml LOQ: 0.015µg/ml
5	4026		20	5528	

5. References

[1] Douglas A.Skoog, F. James Holler & Stanley R. Crouch. Instrumental analysis, India edition, **2007**, pg: 13-14.

[2] Gurdeep R. Chatwal & Sham K. Anand. Instrumental Methods of Chemical Analysis (Analytical Chemistry), pg: 2.566-2.567.

[3] Ahuja S & Dong MW. Handbook of Pharmaceutical Analysis by HPLC. 1st edition, Academic Press Publisher. UK **2005**.

[4] Modern Instrumental Analysis 47 (Comprehensive Analytical Chemistry) volume-47, pg-7-8.

[5] Willard HH, Merrit LL, D. an JA, Settle FA. Instrumental methods of analysis, CBS Publishers and Distributors, New Delhi, 6th edition, **1986**, 1-15.

[6] Douglas A. Skoog, F. James Holler, Timothy A. Nieman. Principles of instrumental analysis, Saunders Golden Sun burst Series, Philadelphia, 2nd edition, **1980**, 725-760.

[7] David G.Watson. Pharmaceutical Analysis, A text book for Pharmacy students and Pharmaceutical Chemists, Harcourt Publishers Limited, 2nd Edition, **1999**, 221-232, 267-311.

[8] Snyder LR, Kirkland JJ, Joseph LG. Practical

HPLC Method Development, Wiley Inter Science, New York, 2nd Edition, **1997**, 1-56, 234-289,685-712.

[9] Stulzer HK, Tagliari MP, Murakami FS, Silva MA, Laranjeira MC et al, Development and validation of an RP-HPLC method to quantitate acyclovir in cross-linked chitosan microspheres produced by spray drying. J Chromatogr Sci. **2008** Jul; 46(6):496-500.

[10] Ramalingam Peraman, Bhadra Kalva, Siva Shanka & Y. Padmanabha Reddy et al, Analytical Quality by Design (AQbD) Approach to Liquid Chromatographic Method for Quantification of Acyclovir and Hydrocortisone in dosage forms. Analytical Chemistry Letters, Volume 4, Issue 5-6, **2014**

[11] Selvadurai Muralidharan et al, Development and Validation of Acyclovir HPLC External Standard Method in Human Plasma: Application to Pharmacokinetic Studies. Advances in Pharmaceutics, Volume 2014 (**2014**), Article ID 284652, 5 pages

[12] P. Ravisankar, A. Niharika, S. Sireesha, O. Sai Koushik, V.Himaja et al, Development And Validation Of Rp-Hplc Method For Quantitative Estimation Of Acyclovir In Bulk Drug And Tablets. Journal of Chemical and Pharmaceutical Sciences, Volume 8 Issue 1

[13] Vilas Chaudhari and Milind Ubale et al, A Validated Stability-Indicating Hplc Assay Method For Acyclovir In Bulk Drug. International Journal of Analytical, Pharmaceutical and Biomedical Sciences, Volume: 1: Issue-2: April-June -**2012.**

[14] G.S. Holkar, M.D. Rokade, R.R. Yadav and V.N. Daphal et al, Determination of Assay and Peak Purity Evaluation of Acyclovir and Valacyclovir by RP-HPLC Method. International Journal of Theoretical & Applied Sciences, 4(2): 56-65 (**2012**).