ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF CHLORAMPHENICOL AND POLYMYXIN-B FORMULATION BY USING RP -HPLC

1 S. Padmavathi* 2 G. Kavyasri

Department of Pharmaceutical chemistry & Phytochemistry, Department of pharmaceutical analysis

Nirmala college of Pharmacy, Atmakur, Mangalagiri , Guntur, AP- 522503.

Phone no: 9966511567

ABSTRACT:

A simple, rapid, precise, sensitive and reproducible reverse phase high performance liquid chromatography (RP-HPLC) method has been developed for the quantitative analysis of Chloramphenicol and Polymyxin B in pharmaceutical dosage form. Chromatographic separation of Chloramphenicol and Polymyxin B was achieved on Waters Alliance-e2695 by using Hyperclone 5µ BDS C18 130°A (250x 4.6mm, 5µ) column and the mobile phase containing Acetonitrile: HSA pH-2.5/OPA in the ratio of 40:60% v/v. The flow rate was 1.0 ml/min; detection was carried out by absorption at 294nm using a photodiode array detector at ambient temperature. The retension times are 2.3 & 4.8minutes respectively. The number of theoretical plates and tailing factor for Chloramphenicol and Polymyxin B were NLT2000 and should not more than 2 respectively. % Relative standard deviation of peak areas of all measurements always less than 2.0. The proposed method was validated according to ICH guidelines. The method was found to be simple, economical, suitable, precise, accurate & robust method for quantitative analysis of Chloramphenicol and Polymyxin B study of its stability.

Key words: HPLC Chloramphenicol and Polymyxin B

INTRODUCTION:

Chromatography: Chromatography is belongs to the family of analytical chemistry techniques for the partition of mixtures [1]. It requires to introduces the sample, and composition that carry the analyte, in the "mobile phase", frequently in a stream of solvent across the "stationary phase." The stationary phase delays the passing of the components of the sample. When components move through the system at different rates they become split in time, like runners in a marathon. Rather, each component has a characteristic time of passage through the system. This is called its "retention time."

Types of Chromatography:

- ❖ Based on modes of chromatography
  - Normal phase chromatography
  - Reverse phase chromatography
- ❖ Based on principles of separation
  - Adsorption chromatography
  - Ion exchange chromatography
  - Ion pair chromatography
  - Size exclusion chromatography
  - Affinity chromatography
  - Chiral phase chromatography
- ❖ Based on elution technique

- Isocratic separation
- Gradient separation

❖ Based on the scale of operation

- Analytical HPLC
- Preparative HPLC

Normal Phase High Performance Liquid Chromatography (NP-HPLC):

The NP-HPLC uses high polar stationary phase and low polar mobile phase. To choose the ideal mobile phase it is good to start with clear hydrocarbon mobile phase such as heptane's. If the sample is strongly retained, the polarity of the mobile phase should be increased, may be by adding small amounts of methanol or dioxin.

The separation of oil-soluble vitamins, essential oils, nitro phenols, or more polar corresponding series have been performed by using alcohol/heptane as the mobile phase in normal phase mode. The column is used for the chiral separation: Chiracel OJ and Chiracel OD in normal phase chromatography.

Reverse Phase High Performance Liquid Chromatography (RP-HPLC):

The RP-HPLC uses hydrophobic bonded packing, generally with an octadecyl or octyl functional group and a polar mobile phase, usually it is a partially or fully aqueous mobile phase. Polar substances propose the mobile phase and shun first. If the hydrophobic character of the solutes increases, automatically the retention time is increases. Overall, the polarity of the mobile phase is low , the strength is high. The elution order of the classes of compounds in table is reversed (thus the name reverse-phase chromatography).

Introduction to Method Development:

Every year the number of new drugs are increased in the market. The new drugs in the market also have new units or partial structural moderation of the current one. Frequently a time fall of exists from the date of launch of a new drug into the market to the date of its incorporation in pharmacopoeias. This is occur because of the viable ambiguity in the stable and higher usage of these drugs, description of new toxicities (resulting in their departure from the market), development of patient defiance and establishment of better drugs by opponent. In process of these conditions, standards and analytical method for these drugs may not be vacant in the pharmacopoeias. It develop into crucial, therefore to develop newer analytical methods for such drugs.

Introduction to Method Validation:

Validation is one of the elemental part of quality assurance; Validation in itself does not improve processes but confirms that the processes have been appropriately developed and are under control.Validation method is a process of proving (through scientific studies) that an analytical method is supportable for its intended use.

DRUG PROFILE:

CHLORAMPHENICOL:

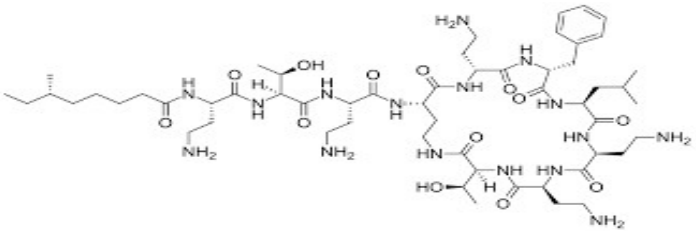
CHLORAMPHENICOL

IUPAC name	2,2-dichloro-N-[(1R,2R)-1,3-dihydroxy-1-(4-nitrophenyl)propan-2-yl]acetamide
Molecular Formula	$C_{11}H_{12}Cl_2N_2O_5$
Molecular Weight	323.13 g/mol
Description	Chloramphenicol is a medication used in the management and treatment of superficial eye infections such as bacterial conjunctivitis, and otitis externa. It has also been used for the treatment of typhoid and cholera. Chloramphenicol is an antibiotic and is in the class of antimicrobials that inhibits protein synthesis.
Solubility	Slightly soluble in water (2.5 mg/ml). Very soluble in methanol, ethanol, ethyl acetate, acetone.
pKa	8.69

Mechanism of action: Chloramphenicol is lipid-soluble, allowing it to diffuse through the bacterial cell membrane. It then reversibly binds to the L16 protein of the 50S subunit of bacterial ribosomes, where transfer of amino acids to growing peptide chains is prevented (perhaps by suppression of peptidyl transferase activity), thus inhibiting peptide bond formation and subsequent protein synthesis.

Absorption: Rapidly and completely absorbed from gastrointestinal tract following oral administration (bioavailability 80%). Well absorbed following intramuscular administration (bioavailability 70%). Intraocular and some systemic absorption also occurs after topical application to the eye.

POLYMYXIN B :



IUPAC name	N-[4-amino-1-[[1-[[4-amino-1-oxo-1-[[6,9,18-tris(2-aminoethyl)-15-benzyl-3-(1hydroxyethyl)-12-(2-methylpropyl)-2,5,8,11,14,17,20-heptaoxo-1,4,7,10,13,16,19-heptazacyclotricos-21-yl]amino]butan-2-yl]amino]-3-hydroxy-1-oxobutan-2-yl]amino]-1-oxobutan-2-yl]-6-methyloctanamide
Molecular Formula	$C_{56}H_{100}N_{16}O_{17}S$
Molecular Weight	1301.57 g/mol^{-1}
Description	Polymyxin B is an antibiotic that disrupts the outer cell membrane of Gram negative bacteria, binds and neutralizes lipopolysaccharide, and inhibits respiration

	of Gram-negative bacterial cells 4 . Polymyxin B can be given by a number of routes to treat susceptible Gram negative bacterial infections 4 .
Absorption	Administration by the oral route does not lead to absorption 4 .
Solubility	Soluble in water
pKa	10.23

Mechanism of Action: The alpha and gamma diaminobutyric acid of a positively charged polymyxin B forms an electrostatic interaction with the phosphate groups of a negatively charged lipid A on the outermembrane of a Gram negative bacterium 4 . Calcium and Magnesium ions are displaced fromphosphates of the membrane lipids, destabalising the lipopolysaccharide (LPS), increasingmembrane permeability, causing cytoplasmic leaking, and killing the cell 4 . Po lymyxin B can also bind and neutralize LPS released during bacterial lysis, preventing reactions to endotoxin 4. A third activity of polymyxin B is the inhibition of type II NADH-quinone oxidoreductases in the bacterial inner membrane, which are essential for respiration 4 . Polymyxin is active against common Gram negative bacteria but not Gram negative cocci, Gram positive bacteria, or anaerobic bacteria 4 .

MATERIALS AND METHODS: HPLC, PH meter, Weighing balance, Pipettes, beakers and Burettes, Ultra sonicator, Pump. Chemicals used are Acetonitrile, Water(Milli Q), Hexane sulfonic acid, Ortho Phosphoric acid.

General preparations:

HSA Buffer Preparation: 1.8 gms of Hexane sulfonic acid is dissolved in 1 litre of HPLC water adjust pH-2.5 with OPA and filter through 0.45µ membrane filter paper.

Preparation of Mobile Phase: Mobile phase was prepared by mixing Acetonitrile and HSA pH-2.5/OPA taken in the ratio 40:60. It was filtered through 0.45µ membrane filter to remove the impurities which may interfere in the final chromatogram.

Chromatographic condition:

Use suitable High Performance Liquid Chromatographic equipped with PDA detector.

Column : Hyperclone 5µ BDS C18 130°A (250x4.6 mm, 5µ)

Mobile phase ratio : Acetonitrile: HSA pH-2.5/OPA (40:60)

Detection wavelength : 294 nm

Flow rate : 1ml/min

Injection volume : 10µl

Run time : 6min

Preparation of Diluent: Methanol and Water (50+50) is used as a diluent.

Preparation of standard solution: Accurately weigh and transfer 4 mg of Chloramphenicol working standard into a 10 ml clean dry volumetric flask and add 1 ml of Polymyxin B parent stock solution, diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1 ml of the above stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluent. (40ppm of Chloramphenicol, 5ppm of Polymyxin B).

Sample Solution Preparation: Accurately weighed and transfer 1ml of Chloramphenicol and Polymyxin B sample into a 10Ml clean dry volumetric flask add diluent and sonicate it up to 30 mins to dissolve it completely and make volume up to the mark with the same solvent. Then it is filtered through 0.45 micron Injection filter (Stock solution).

Further pipette 1 ml of the above stock solutions into 10 ml volumetric flask and dilute up to the mark with diluents. (40ppm of Chloramphenicol, 5ppm of Polymyxin B).

RESULTS AND DISCUSSION : Six trails were conducted on Chloramphenicol and Polymyxin B drug by using different solvent systems ,columns and experimental conditions.The optimized chromatographic conditions used in the solvent system were acetonitrile : water and other conditions are represented .

OPTIMIZED CHROMATOGRAPHIC CONDITION:

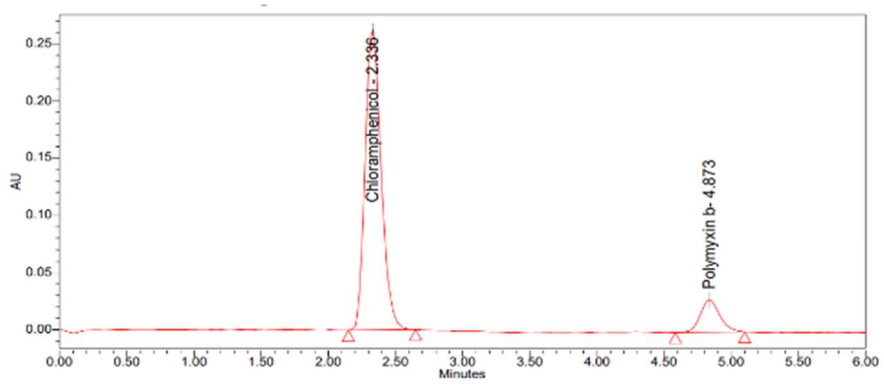
PARAMETERS	OBSERVATION
Instrument used	Waters HPLC with auto sampler and PDA detector.
Injection volume	10µl
Mobile Phase	Acetonitrile: HSA pH-2.5/OPA (40:60)
Column	Hyperclone 5µ BDS C18 130°A (250x4.6 mm, 5µ)
Detection Wave Length	294nm
Flow Rate	1 mL/min
Runtime	7min
Temperature	Ambient(25° C)
Mode of separation	Isocratic mode

Optimized chromatogram:

All the parameters are within the limit this method is suitable for validation

Results for (Optimized trail)

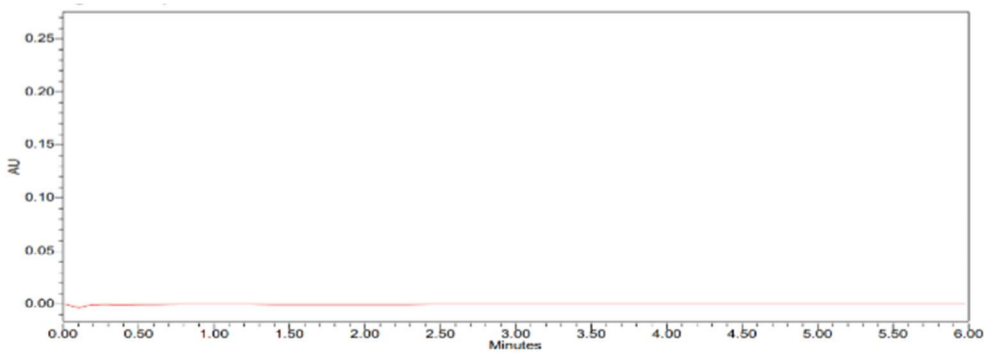
S.No	Name	RT	Area	USP Plate Count	USP Tailing	USP Resolution
1	Chloramphenicol	2.336	2537472	5482	1.14	
2	Polymyxin B	4.873	317184	6615	1.05	10.26



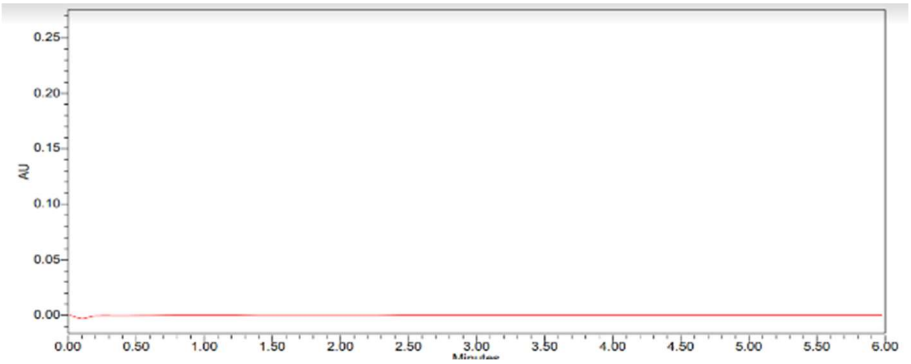
Optimized chromatogram

ANALYTICAL METHOD VALIDATION:

Specificity: Retention times of Chloramphenicol and Polymyxin B were 2.336 min and 4.873 min respectively. We did not found and interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.



Chromatogram of blank



Chromatogram of placebo

System suitability: All the system suitability parameters were within the range and satisfactory as per ICH guidelines

System suitability parameters for Chloramphenicol & Polymyxin B

S.no	Parameter	Chloramphenicol	Polymyxin B
1	Retention time	2.336	4.873
2	Plate count	5482	6615
3	Tailing factor	1.14	1.05
4	Resolution	----	10.26
5	%RSD	0.61	0.80

LINEARITY:

Preparation of Polymyxin B parent stock solution: Accurately weigh and transfer 5 mg of Polymyxin B working standard into a 10 ml clean dry volumetric flask add diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. This is called Polymyxin B parent stock solution.

Preparation of standard solution: Accurately weigh and transfer 4 mg of Chloramphenicol working standard into a 10 ml clean dry volumetric flask and add 1 ml of Polymyxin B parent stock solution, diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Preparation of Level – I (10.00ppm of Chloramphenicol, 1.25ppm of Polymyxin B): 0.25 ml of above stock solutions has taken in different 10 ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – II (20.00ppm of Chloramphenicol, 2.50ppm of Polymyxin B): 0.5 ml of above stock solutions has taken in different 10 ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – III (30.00ppm of Chloramphenicol, 3.75ppm of Polymyxin B): 0.75 ml of above stock solutions has taken in different 10 ml of volumetric flasks, dilute up to

the mark with diluents.

Preparation of Level – IV (40.00ppm of Chloramphenicol, 5.00ppm of Polymyxin B): 1.0 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

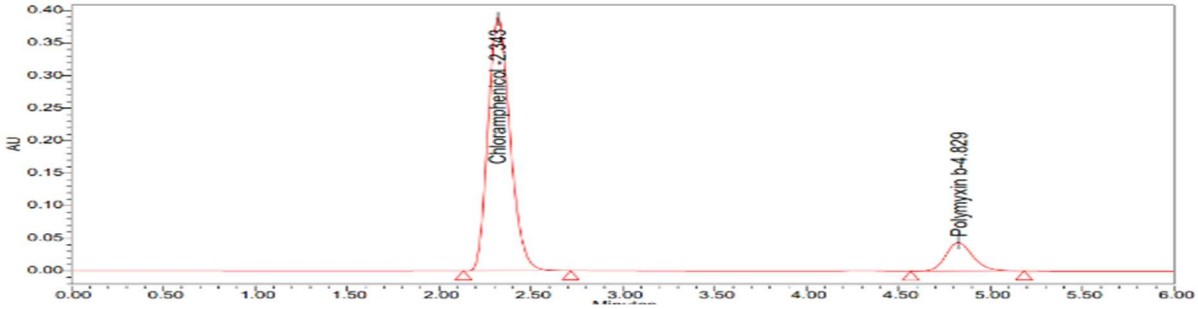
Preparation of Level – V (50.00ppm of Chloramphenicol, 6.25ppm of Polymyxin B): 1.25 ml of above stock solutions has taken in different 10 ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – VI (60.00ppm of Chloramphenicol, 7.50ppm of Polymyxin B): 1.5 ml of above stock solutions has taken in different 10 ml of volumetric flasks, dilute up to the mark with diluent.

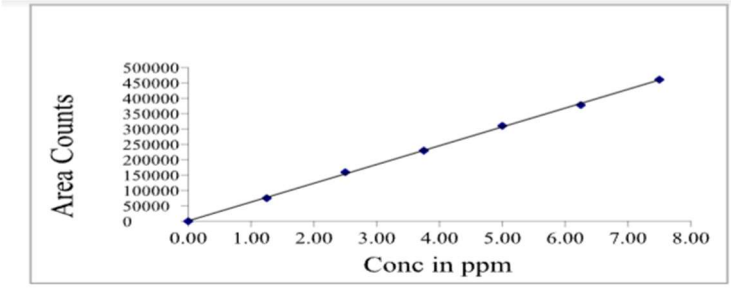
Results of linearity for Chloramphenicol & Polymyxin B

S.NO	Chloramphenicol		Polymyxin B	
	Conc.(µg/ml)	Peak area	Conc.(µg/ml)	Peak area
1	10.00	639653	1.25	75123
2	20.00	1295299	2.50	159437
3	30.00	1963147	3.75	229737

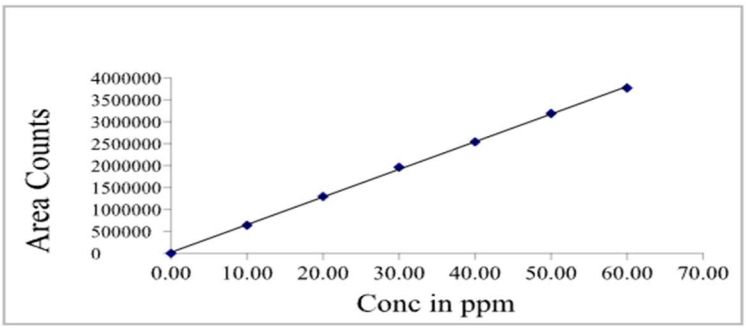
4	40.00	2541563	5.00	310524
5	50.00	3188539	6.25	378132
6	60.00	3770639	7.50	378132
Regression equation	y = 63056.98x +22410.75		y =61140.03x + 1279.89	
Slope	63056.98		61140.03	
Intercept	22410.75		1279.89	
R2	0.99979		0.99976	



Chromatogram of Linearity-150%



Calibration curve for Polymyxin B



Calibration curve for Chloramphenicol

Assay:

Formula for Assay:

$$\%\ Assay = \frac{AT}{AS} * \frac{WS}{DS} * \frac{DT}{WT} * \frac{Average\ weight}{Label\ Claim} * \frac{P}{100} * 100$$

Where: AT = average area counts of test (sample) preparation.

AS = average area counts of standard preparation.

WS = Weight of working standard taken in mg.

DS = Dilution of working standard in ml.

DT = Dilution of test (sample) in ml.

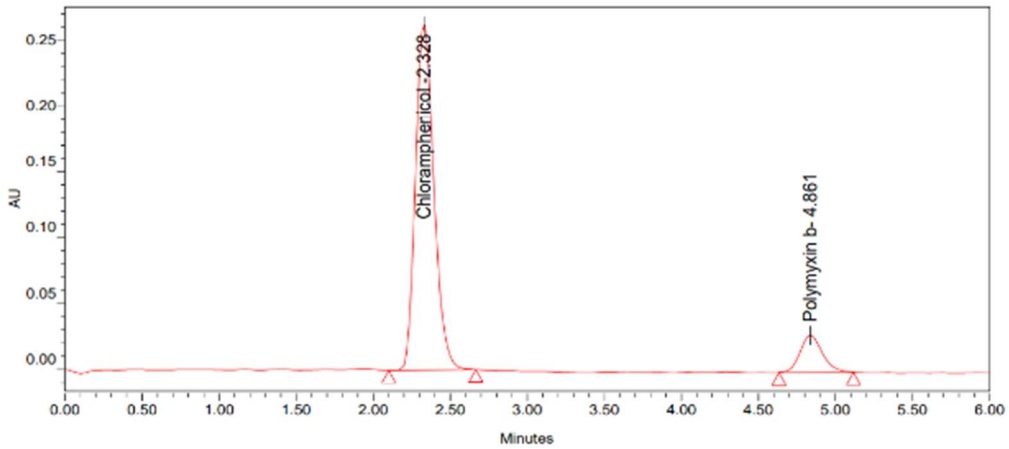
WT = Weight of test (sample) taken in mg.

P = Percentage purity of working standard

LC = Label Claim mg/ml.

Assay of Chloramphenicol & Polymyxin B

Brand	Drug	Area	Avg sample area (n=5)	Std. Conc. (µg/ml)	Sample Conc. (µg/ml)	Label amount (mg)	Std purity	Amount found (µg/ml)	% assay
-	Chloramphenicol	2517462 2530814	2524138	40	40	4	99.7	4.00	100.0
	Polymyxin B	313247 313247	314214	5	5	0.5	99.9	0.49	99.5



Chromatogram of Assay

Accuracy:

Accuracy results of Chloramphenicol by RP-HPLC method

%Concentration(at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	1267441	2.00	2.01	100.5	99.7
	1263234	2.00	2.00	100.0	
	1246126	2.00	1.97	98.5	
100%	2525921	4.00	4.00	100.0	100.1
	2536023	4.00	4.02	100.5	
	2517308	4.00	3.99	99.8	
150%	3772308	6.00	5.98	99.7	99.4
	3761124	6.00	5.96	99.3	
	3753564	6.00	5.95	99.2	

The Accuracy results for by RP-HPLC method

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	159153	0.25	0.2520	100.8	99.9
	156336	0.25	0.2475	99.0	
	157724	0.25	0.2497	99.9	
100%	313996	0.50	0.4971	99.4	100.0
	318384	0.50	0.5040	100.8	
	315567	0.50	0.4996	99.9	
150%	479997	0.75	0.7599	101.3	100.2
	469699	0.75	0.7436	99.1	
	469699	0.75	0.7500	100.0	

Three levels of Accuracy samples were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 99.7% and 100.00% for Chloramphenicol and Polymyxin B respectively.

Robustness:

Robustness results of Chloramphenicol by RP-HPLC

Parameter	Chloramphenicol						
	Condition	Retention time(min)	Peak area	Resolution	Tailing	Plate count	% RSD

Flow rate Change (mL/min)	Less flow (0.9ml)	2.545	2342709		1.15	5527	0.72
	Actual (1ml)	2.336	2537472		1.14	5482	0.61
	More flow (1.1ml)	2.088	2741736		1.10	5366	0.81
Organic Phase change	Less Org (36:64)	2.832	2191340		1.19	5582	0.57
	Actual (40:60)	2.337	2545965		1.17	5478	0.61
	More Org (44:56)	1.926	2836811		1.14	5324	0.20

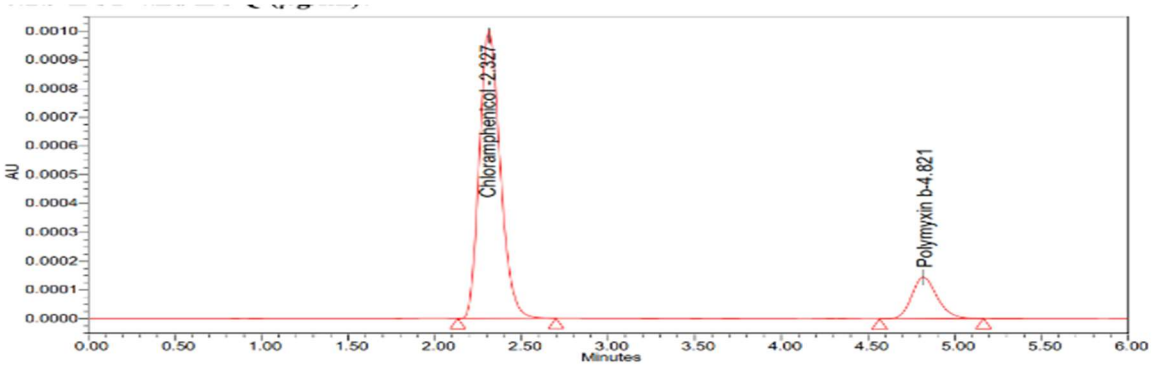
Robustness results of Polymyxin B by RP-HPLC

Parameter	Polymyxin B						
	Condition	Retention time(min)	Peak area	Resolution	Tailing	Plate count	%RSD
Flow rate Change (mL/min)	Less flow (0.9ml)	5.056	284210	9.89	1.14	6711	1.10
	Actual (1ml)	4.873	317184	10.26	1.05	6615	0.80
	More flow (1.1ml)	4.605	321542	10.05	1.02	6513	1.05
Organic Phase change	Less Org (36:64)	5.336	294417	9.92	1.11	6770	0.65
	Actual (40:60)	4.874	315095	10.21	1.06	6610	0.80
	More Org (44:56)	4.324	346259	9.86	1.00	6566	0.66

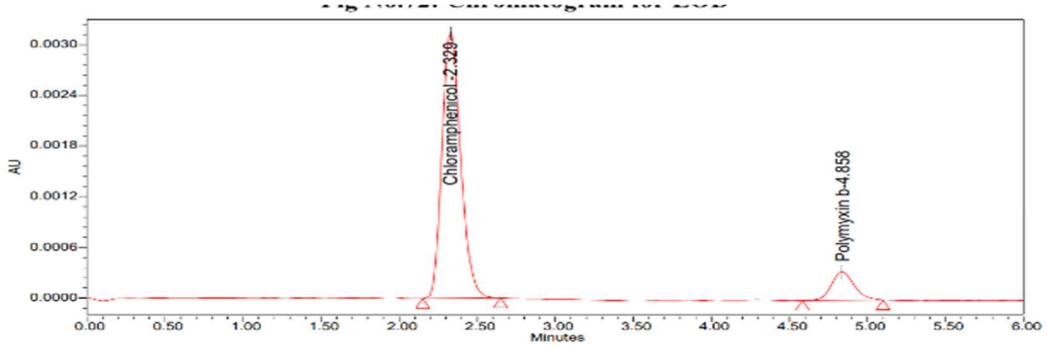
LOD and LOQ (µg/ml):

Sensitivity parameters (LOD & LOQ) by RP-HPLC

Name of drug	LOD(µg/ml)	s/n	LOQ(µg/ml)	s/n
Chloramphenicol	0.160	3	0.020	10
Polymyxin B	0.520	3	0.065	10



Chromatogram for LOD

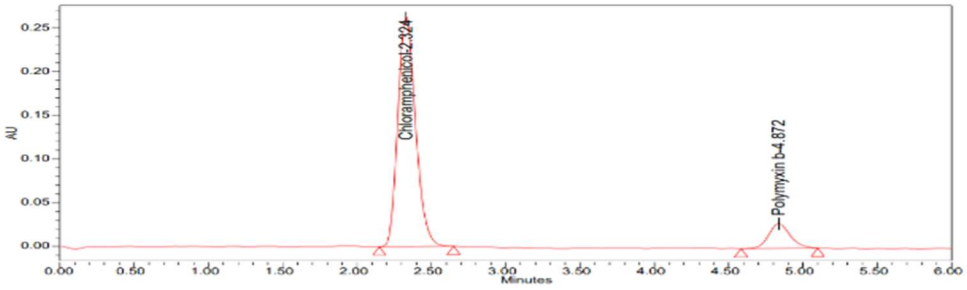


Chromatogram for LOQ

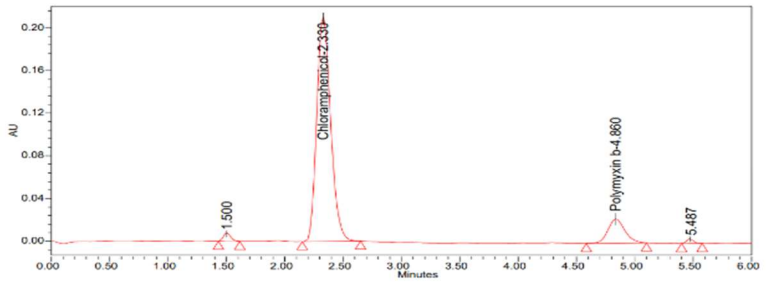
Degradation Studies:

Forced Degradation results for Chloramphenicol and Polymyxin B

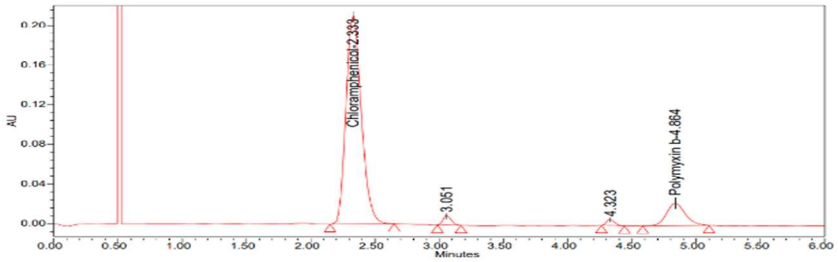
Results: % Degradation results	Chloramphenicol					Polymyxin B				
	Area	% Assay	% Deg	Purity Angle	Purity Threshold	Area	% Assay	% Deg	Purity Angle	Purity Threshold
Control	2525530	100	0	0.293	4.061	316106	100	0	1.253	6.225
Acid	2204763	87.3	12.7	0.207	4.035	277670	87.8	12.2	1.230	6.241
Alkali	2172274	86.0	14.0	0.201	4.055	275863	87.2	12.8	1.242	6.295
Peroxide	2132715	84.4	15.6	0.273	4.054	270513	85.5	14.5	1.265	6.242
Reduction	2263289	89.6	10.4	0.222	4.065	289184	91.4	8.6	1.298	6.258
Thermal	2496377	98.8	1.2	0.222	4.057	311412	98.5	1.5	1.254	6.223
Photolytic	2439561	96.6	3.4	0.254	4.021	305854	96.7	3.3	1.253	6.264
Hydrolysis	2464172	97.5	2.5	0.236	4.079	309746	97.9	2.1	1.283	6.257



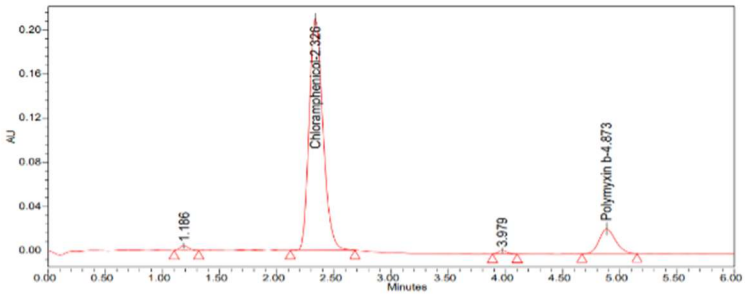
Chromatogram of Acid degradation



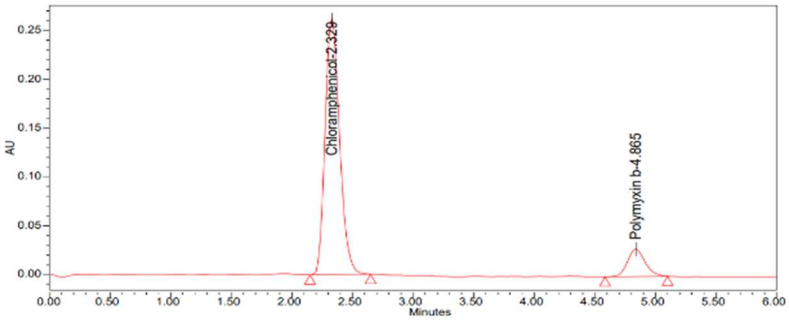
Chromatogram of Alkali degradation



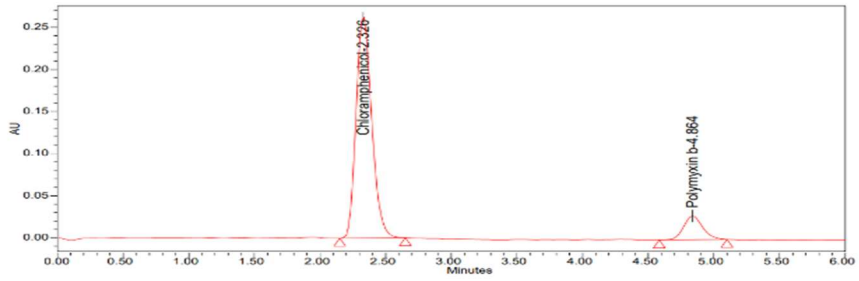
Chromatogram of Peroxide degradation



Chromatogram of Reduction degradation



Chromatogram of Thermal degradation



Chromatogram of Photolytic degradation

CONCLUSION:

The developed HPLC method for the estimation of selected drugs is simple, rapid, accurate, precise, robust and economical. The mobile phase and solvents are simple to prepare and economical, reliable, sensitive and less time consuming.

The sample recoveries were in good agreement with their respective label claims and they suggested non interference of formulation recipients in the estimation and can be used in laboratories for the routine analysis of selected drugs.

Since the system validation parameters of HPLC method used for estimation of selected drugs in pure and have shown satisfactory, accurate and reproducible results (without any interference of recipients) as well, it is deduced that the simple and short proposed methods be most useful for analysis purpose.

The present work concluded that stability indicating assay method by RP-HPLC was simple, accurate, precise, and specific and has no interference with the placebo and degradation products. Hence these can be used for routine analysis of Chloramphenicol and Polymyxin B.

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REFERENCES:

1. Drug Dictionary.com Unbridge Vol.1.1, Random house 20 September 2007.

2. Similer R, Walsh G, Mattaliano RJ, Guziewicz N and Perez-Ramirez B (2008). Maximizing data collection and analysis during formulation of Biotherapeutic Proteins, Bioprocess International 6(10), 38-45.

3. Journals Ranked by Impact: Toxicology 2014. Journal Citation Reports. Web Sciences (Sciences ed.). Thomson Reuters 2015.

4. Van Tellingen C, “Pliny’s pharmacopoeia or the Roman treat, Netherlands heart journal 15(3) : 118-20, March 2007.

5. Merriam Webster dictionary, 1828.

6. World Health Organization. Working document 2011 : Defination of Active Pharmaceutical Ingredient. Geneva, Switzerland: World Health Organization;2011.

7. Bhattacharyya, Lokesh, Schuder, Stefan, Sheehan, Catherine, William, Exipinets Background/Introduction in Katdare Ashok, Chaubal Mahesh. Excipents Development for Pharmaceutical, Biotechnology and Drug Delivery Systems 2006.

8. Juran, Joseph M, A history of Managing for Quality. The evalution, trends and future directions of managing quality. Milwaukee, Wisconsin. The American society for quality control, ed.1995.

9. Managing Quality Across the Enterprise; Enterprise Quality Management Solution for medical device companies. Sparta systems 2015-02-02.

10. Skoog Douglas A, West Donald M, Holler F, James Crouch, Stanley R. Fundamentals of Analytical chemistry, Belmont, Brokes/cole, Cengage Learning.p-1 (2014).

11. Wolf, Jakob, Schnellkurs HGB-Jahresabschluss, Das neue Bilanzrecht, Richtig vorgehen-erfolreich umstellen. Walhalla Fachverlag.p.90. 15 January 2010.

12. Chromatography Hand Book of HPLC, Katz(Wiley & Sons); page no.14-16.2002.

13. Henry Richard L, “The early days of HPLC at Dupont” chromatography online. Avanstar communications Inc. 1 February 2009.

14. IUPAC, Compendium of Chemical Terminology, 2 nd ed. (the Gold Book) 1997.

15. W.John Lough, Irving W.Wainer, High performance Liquid ChromatographyFundamental principles and practice. Blackie Academic & Professional pp.120.

16. Practical HPLC method development and validation second edition Lloyd R. Synder, Jpseph J. Kirkland and Joseph L.Glaich pg no: 1-3.

17. Emer Joachim, John H, McB Miller, Method Validation in Pharmaceutical Analysis. A Guide to best practice Wiley-VCH page no. 418.

18. IUPAC, Compendium of Chemical Terminology, 2 nd edition The gold book,1997.

19. Mac Dougall, Daniel, Crummett, Warren B et.al., “Guidelines for data acquisition and data quality evalution in environmental chemistry. Anal.chem.52:2242-49.

20. Method Validation; "Archived copy". Archived from the original on 11 September 2011.

21. Health Sciences Authority. "Guidance Notes on Analytical Method Validation: Methodology".

22. Heyden, Y. Vander; S.W. Smith; et al. (2001). "Guidance for robustness/ruggedness tests in method validation". Journal of Pharmaceutical and Biomedical Analysis. Elsevier. 24 (5–6): 723–753.

23. Subcommittee E11.20 on Test Method Evaluation and Quality Control (2014), Standard Practice for Use of the Terms Precision and Bias in ASTM Test Methods,

24. Lukacs, E. (1970) Characteristic Functions. Griffin, London.

25. National Council on measutement in Education.
http://www.ncme.org/ncme/NCME/Resource_Center/Glossary/NCME/Resource_Center/Glossary.

26. Bland, J.M.; Altman, D.G. (1996). Statistics notes: measurement error. BMJ. 312 (7047): 1654.

27. FDA Issues Dietary Supplements Final Rule" (Press release). U.S. Food and Drug Administration. 2007-06-22. Retrieved 2010-06-04.

28. Kevin Robinson for BioPharm International, Aug 1, 2003. GLPs and the Importance of Standard Operating Procedures.

29. ICH Harmonised Tripartite Guideline Q2(R 1), Current Step 4 version Parent Guideline; 27 October 1994.

30. Validation definition and FDA, Regulatory agencies guidelines requirement Accessed 27 Feb 2014.

31. sakinala padmavathi, Gudivada Lavanya priya , RP-HPLC method development and validation of metformin, dopagliflozin, saxagliptin in tablet dosage forms, Journal of cardiovascular drugs, vol-4, page no 1011-24, 2021

32 S.Padmavathi, GSS.Lakshmi, KSD. Bhavani, SA. Rahaman, RP-HPLC Method development, validation and stability studies of oliazapine and semidorphan in combination dosage forms, journal of negative results, vol -13, issue-9, page no- 8691-8713

33. Development and validation of stability indicating assay for simultaneous determination of pentaprazole, diclofinac, , chloroxazone in pharmaceutical dosage form by using RP-HPLC, International journal of pharmaceutical science and research 11 (4) 1757-67 2020