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A VALIDATED STABILITY INDICATING RP-UPLC METHOD FOR DETERMINATION OF ERYTHROMYCIN ESTOLATE IN PHARMACEUTICAL FORMULATION

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ABSTRACT: A novel, sensitive and selective stability-representing RP-UPLC method was developed and validated for the quantitative determination of Erythromycin estolate in Erythromycin 250 mg capsules. The chromatographic separation was achieved on BEH C18; 50 × 2.1 mm; 1.7 µm column by using mobile phase containing a mixture of 0.002M di-potassium hydrogen phosphate and acetonitrile 53:47 v/v at a flow rate of 0.6 ml/min. The column temperature was maintained at 40 °C and detection was carried out at 210 nm. To ascertain the stability-signifying ability of the method, drug product was subjected to strain conditions of acid, base, oxidative, hydrolytic, thermal and photolytic degradation. The drug undergoes degradation at oxidative and thermal / humidity stress conditions. The resultant degrading peaks were well resolved from the drug peak. The drug was found to be stable in thermal and photolytic conditions. The proposed method was validated as per ICH guidelines with respect to specificity, linearity, accuracy, precision and robustness and the method show excellent linearity and a correlation coefficient of more than 0.99. Therefore, the projected method can be employed for the determination of Erythromycin estolate in various pharmaceutical formulations during regular and quality-control analysis.

INTRODUCTION: Erythromycin is a classic representative of the macrolide group of antibiotics and is produced by *Streptomyces erythreus*. It is extensively used in the treatment and prevention of diseases. Current indications for the drug includes respiratory infections, whooping cough, legionnaires disease and campylobacter enteritis. The side-effects are comparatively low [1]. Erythromycin is effective against penicillin - challenging *Staphylococcus*, *Chlamydia* and *Mycoplasma* bacteria.

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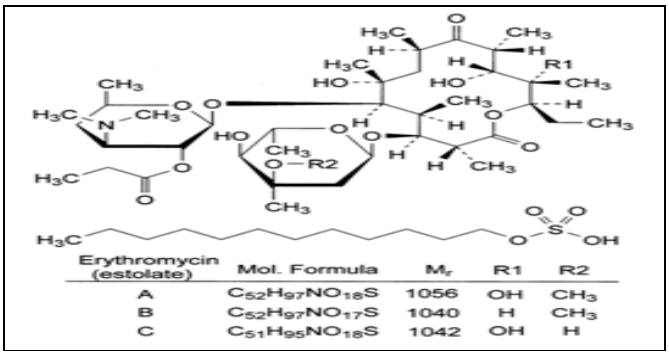
Chemically Erythromycin estolate is (3R, 4S, 5S, 6R, 7R, 9R, 11R, 12R, 13S, 14R) -4-[(2, 6-Dideoxy -3- C-methyl -3 -O- methyl -α- L- ribo- hexo pyranosyl) Oxy] -14- ethyl -7, 12, 13-trihydroxy-3, 5, 7, 9, 11, 13- hexamethyl-6-[[3, 4, 6-trideoxy- 3-(dimethylamino) -2- O- propionyl -β- D- Xylo-hexopyranosyl] Oxy] oxacyclo tetra decane-2, 10-Dione dodecyl sulfate (Erythromycin A 2''-propionate dodecyl sulfate).

Having a molecular formula of $C_{40}H_{71}NO_{14}$, $C_{12}H_{26}O_4S$ and molecular weight is 1056 g/Mol and melting point is 135 - 140 ºC. Practically insoluble in water and dilute hydrochloric acid, freely soluble in ethanol (96 percent), soluble in acetone. The PKA value is 6.9 and stable at normal temperature and pressure [2, 3]. Erythromycin estolate is specified especially for those patients who are allergic or sensitive to sulfa drugs or Penicillin.

However, Erythromycin is degraded to inactive anhydrous form in acidic fluids. Several analogs of EM (Azithromycin and Clarithromycin) have been developed that have a chemical substitution at the location where internal dehydration is first initiated to prevent the degradation reaction from starting, resulting in slightly increased acidic stability and therapeutic efficacy.

Hence, ester type prodrugs are preferred such as stearate salts, which are poorly soluble in water and suitable for oral administration. Thus, the Erythromycin estolate **Fig. 1** has been formulated both in liquids *e.g*. suspension and solid dosage forms like tablets and capsules. LC represents a tremendous growth that makes it the most popular method used in the pharmaceutical analysis. Literature review reveals numerous analytical methods for the determination of erythromycin and its analogs either alone, or in combination with other antibacterial agent and its related substances in bulk, pharmaceutical formulations [4 - 16] and biological fluids [17 - 21], these spectrophotometric, HPLC, LCMS / MS and FTIR techniques. The stability indicating method is a process that identifies the degradation products of analytes. A very few stability indicating LC methods were reported for ester forms of erythromycin in dosage forms and along with some alkaloids [22 - 27].



Erythromycin (estolate)	Mol. Formula	M_r	R1	R2
A	$C_{52}H_{97}NO_{18}S$	1056	OH	CH_3
B	$C_{52}H_{97}NO_{17}S$	1040	H	CH_3
C	$C_{51}H_{95}NO_{18}S$	1042	OH	H

FIG. 1: CHEMICAL STRUCTURE OF ERYTHROMYCINE ESTOLATE

So, the need was felt to develop an analytical technique for the estimation of Erythromycin estolate in a capsule formulation. In the present research, a very simple and rapid isocratic RP-UPLC method for the routine analysis of Erythromycin estolate in capsules was developed. The anticipated method was validated with respect to specificity, linearity, precision, accuracy, and robustness. In addition, strain testing of the drug was also conducted, as required by the International Conference on Harmonization (ICH, 2003) to support the suitability of the method [28, 29, 30].

MATERIALS AND METHODS: All the reagents were of LC grade except stated or else Milli-Q-water was used throughout the research. Operational standard of Erythromycin estolate was procured from M/S ADCOCK INGRAM, RD & I, Sabax Road, Aero ton, Johannesburg, 2013, South Africa. Dipotassium hydrogen phosphate and acetonitrile were procured from Merck, Mumbai.

Apparatus: The UPLC system consisted of high pressure pump, photodiode array detector and 10 µl capacity injector loops. The column used was BEH C18; 50 × 2.1 mm; 1.7 µm column. The output signal was monitored and processed using Empower software.

Chromatographic Conditions: BEH C18; 50 × 2.1 mm, 1.7 µm column was used for separation. Chromatographic separation was attained using a mobile phase comprising of 0.002M di-potassium hydrogen phosphate buffer and acetonitrile 53:47 v/v. The flow rate of the mobile phase was 0.6 ml/min with detection at 210 nm. The column temperature was kept at 40 ºC and the injection volume was 2 µl solution preparations.

Preparation of 0.002M of Di-Potassium Hydrogen Phosphate Buffer: Buffer solution was prepared by dissolving 0.348 gm of di-potassium hydrogen phosphate in DID water.

Preparation of Mobile Phase: 530 ml of 0.002M of di-potassium hydrogen phosphate was mixed with 470 ml of acetonitrile. The solution was degassed in an ultrasonic water bath for 5 min and filtered through 0.45 µm filter under vacuum.

Preparation of Stock Solution: Exactly weighed 400 mg of Erythromycin estolate running standard into a 50 ml volumetric flask; 35 ml of diluent was added and sonicate for 5 min to dissolve completely. Cool to room temperature; make up the volume with diluent and mix

Preparation of Standard Solution: Precisely weighed 80 mg of Erythromycin estolate operational standard into a 20 ml volumetric flask. approximately 15 ml of diluent was added and

sonicate for 5 min to dissolve completely; cool to room temperature, make up to volume with diluent and mix.

Preparation of Sample Solution: Weighed and finely powdered not fewer than 20 capsules. An accurately weighed quantity of powder corresponding to about 250 mg of Erythromycin base from Erythromycin 250 mg capsule powder (about 440 mg) was transferred into 100 ml volumetric flask; approximately 75 ml of diluent was added and sonicate for 5 min to dissolve completely; cool to room temperature and makeup the volume with diluent and mix.

Diluent: 0.002M of di-potassium hydrogen phosphate buffer and acetonitrile in the ratio of 53:47 v/v was selected as diluents. Since the Erythromycin estolate is also soluble in acetonitrile.

Method Development: Any analytical method was not reported in the stability studies of Erythromycin estolate in a capsule formulation. Hence, it was noteworthy to commence the method development using Reverse Phase Liquid Chromatography as it is commonly used and C-18 columns are also available. Different columns were used with different mobile phases during the development of UPLC method suitable for the analysis of Erythromycin estolate in a capsule formulation. 0.002M of dipotassium hydrogen phosphate in water and the organic modifier acetonitrile was preferably chosen as appropriate mobile phase for ideal separation as no interference was found with the solvent. Several isocratic and gradient elution were tried to separate Erythromycin B and Erythromycin.

Finally, the mobile phase composition of 53:47% v/v of 0.002M of dipotassium hydrogen phosphate and acetonitrile was found to be most suitable for separation of Erythromycin B and Erythromycin with a resolution of greater than 2.0. The sample was injected with an injection volume of 2 µl and the injector port temperature was maintained at 40°C ± 2°C and the flow rate of 0.6 ml/min. The column BEH C18; 50 × 2.1 mm; 1.7 µm column was selected. The column was equilibrated by pumping the mobile phase through the column for at least 30 min prior to the injection of the drug solution. 2 µl of the standard, sample solutions were injected into the chromatography system and measure the area of the erythromycin estolate peak. The detection of the drug peak was monitored at 210 nm. The runtime was set at 12 min. Under these optimized chromatographic conditions, the retention time obtained from the drug was 2.69 min. A typical chromatogram showing the separation of the drug is given in **Fig. 2**.

Stress Degradation Study: To determine the developed analytical method was stability indicating, Erythromycin estolate standard solution was stressed under various conditions includes.

Oxidative Degradation: Erythromycin estolate solution was prepared in 3% hydrogen peroxide and kept in a mechanical shaker at at 50 °C for 1h to facilitate the oxidation of the drug.

Acid Hydrolysis: Erythromycin estolate solution was prepared in 0.01N hydrochloric acid and kept in a mechanical shaker at 50 °C for 15 min

Alkaline Hydrolysis: Erythromycin estolate solution was prepared in 0.01N sodium hydroxide and kept at room temperature for 1h.

Temperature Stress Studies: Erythromycin 250mg capsules were exposed to dry heat (105 °C) in a hot air oven for 2 h, 42 min. The drug solution was prepared and subjected to analysis

Photostability Studies: Erythromycin 250 mg capsules were exposed to light to reach greater than 1.2 million Lux hours. The drug solution was prepared and subjected to analysis

Thermal / Humidity Studies: Placebo, Erythromycin 250 mg capsules is subjected directly at 50 °C/ 75% Rh for 7 days. The samples were analyzed.

Method Validation: The optimized method was validated as per International Conference on Harmonization (ICH) guidelines. The validated parameters were system suitability, specificity, and linearity, accuracy, precision, and robustness.

RESULTS AND DISCUSSION:

Method Optimization: A variety of chromatographic conditions were practiced to develop the stability-indicating LC method. The UPLC method was optimized through the

assessment of different buffers and organic solvents. The use of 0.002M of dipotassium hydrogen phosphate and acetonitrile in a ratio of 53:47% v/v on BEH C18; 50 × 2.1 mm; 1.7 µm column was found to furnish sharp, well-defined peak with good resolution and low retention time of about 2.69 min at a flow rate 0.6 ml/min. The peak was monitored at 210 nm.

System Suitability: After equilibration of the column with mobile phase, five replicate injections of 2.0 µl of standard solution through an auto-sampler injector were injected and the chromatograms were recorded. The system suitability parameters were measured and results are revealed in **Table 1**. The results have been confirmed with respect to % RSD, resolution and USP tailing factor. The % RSD was less than 1. The results will make sure transferability of the method and raise the consistency of the results obtained.

TABLE 1: SYSTEM SUITABILITY RESULTS FROM STANDARD SOLUTION

Injection no.	RT (min)	Area	Tailing	Plate count
1	2.694	595311	1.40	1196
2	2.687	595536	1.39	1184
3	2.686	596179	1.40	1181
4	2.676	593458	1.39	1172
5	2.672	593643	1.39	1169
Mean	2.683	594826		
Std dev	0.0088	1208.46		
% RSD	0.33	0.20		

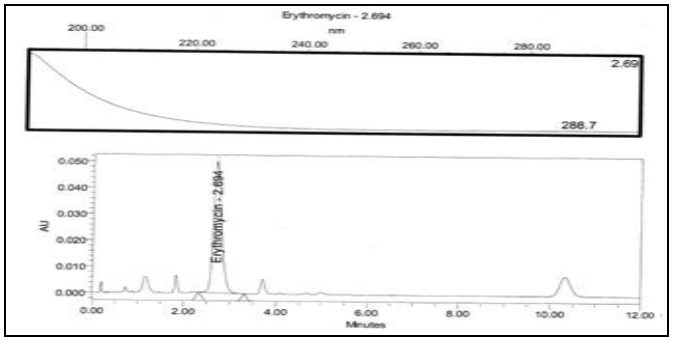


FIG. 2: TYPICAL CHROMATOGRAM OF ERYTHROMYCIN ESTOLATE FROM STANDARD SOLUTION

Degradation Studies: The results of the degradation studies state the specificity of the method as there was no interference from the sample, placebo and degrading peaks and also reveal that the method was selective and stability-indicating. The % assay of the drug was calculated after exposure of Erythromycin estolate solutions to various stress conditions. The drug undergoes degradation of oxidative and thermal / humidity stress conditions. The degrading peaks were well resolved from the drug peak. The drug was stable in thermal and photolytic conditions. The results are shown in **Table 2**.

TABLE 2: STRESS STUDY RESULTS OF ERYTHROMYCIN ESTOLATE

Stress condition	%Assay
Normal	103.81
0.01N HCl; at 50 °C for 15 min	98.32
0.01N NaOH; at Room temperature for 1 h	101.37
3% H_2O_2, at 50 °C for 1h	90.27
105 °C for 2 h 42 min	99.47
Exposure to fluorescent NLT 1.2 million Lux hours	113.70
50 °C / 75% Rh for 7 days	92.76

Method Validation: The designed technique was validated according to ICH guidelines for specificity, linearity, accuracy, and precision, and robustness. Specificity was carried out in which no interference of the excipients was observed at retention time of the analytical peak. A calibration curve was constructed by plotting concentration *vs* plot area. It showed that there was a good linear relationship in the concentration range of 40% to 160% with > 0.999 as the value of correlation coefficient. The accuracy of the method was studied by analyzing the drug solutions at 80%, 100% and 120% concentration level. The mean percentage recovery was found to be 101.7% for precision the sample solution at working concentration was analyzed in replicate as per the method. The percentage relative standard deviation was found to be less than 1%. Robustness of the method shows no significant change in system suitability parameters and mean % assay for modifying chromatographic conditions from the original method.

Specificity: The Specificity of the method was established by injecting a blank, (diluent) Placebo and sample preparations into the chromatograph. No interference was observed.

This indicates that the solvent does not interfere with drug peak and shows a good resolution.

Linearity: The peak area responses of all solutions over concentration levels ranging from 40% to 160% of target concentration were measured in

triplicate. A linear correlation was obtained between peak area and concentration of Erythromycin estolate for LC method. The linearity was validated by the value of correlation coefficients of the regression (r = 0.9999). The curves are shown in **Fig. 3**.

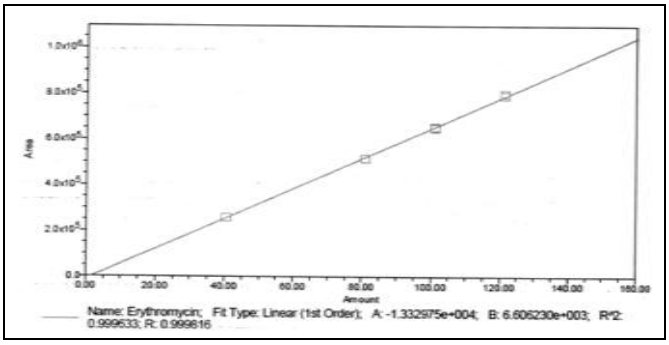


FIG. 3: LINEARITY GRAPH FOR ERYTHROMYCIN ESTOLATE FROM LINEAR CURVE STANDARD SOLUTIONS

Accuracy: The accuracy of the method was determined by recovery experiments and was performed in triplicate by standard addition method at 80%, 100% and 120% of test concentration and analysis precision was expressed as % RSD. A known amount of measuring analyte was added to placebo preparations and was subjected to the proposed UPLC method. Results of recovery studies are shown in **Table 3**. The mean percentage recovery was 101.7% for Erythromycin estolate and % RSD was found to be less than 1.0%. The slope of Erythromycin estolate was 1.02 from 10% to 150% of accuracy levels and the confidence level 95% of accuracy of the method was 0.32. The results are shown in **Table 3**.

TABLE 3: ACCURACY RESULTS OF ERYTHROMYCIN ESTOLATE

Accuracy level (%)	% Assay	% Standard amounts added	% Recovery
80	81.32	81.7	99.53
	80.42	80.73	99.62
	81.27	81.52	99.69
100	102.82	101.05	101.75
	103.85	102.18	101.63
	102.60	100.52	102.07
120	124.00	121.15	102.35
	123.93	122.22	101.40
	125. 0	120.49	103.91

Precision: Method repeatability (intra - day precision) was evaluated by assaying six injections of sample preparation of same the batch. The mean % assay was 104.78% and was within the acceptance criteria. The % RSD was found to be 0.41%. The results are shown in **Table 4**. The difference in the assay of Erythromycin estolate in Erythromycin 250 mg capsules between the preparations are less than 2.0% of % RSD. The results are given away in **Table 3**.

TABLE 4: PRECISION RESULTS OF ERYTHROMYCIN ESTOLATE

Preparation no.	% Assay results
1	104.44
2	104.45
3	105.57
4	104.62
5	104.67
6	104.97
Mean	104.78
Standard deviation	0.429
%RSD	0.41

Robustness: Robustness of the method was established by determining the assay of a sample under small but deliberately modified chromatographic conditions specified under the method like flow rate, column temperature, pH of buffer or buffer strength in % v/v, mobile phase composition and wavelength on lower and higher side of the actual values.

TABLE 5: ROBUSTNESS STUDY RESULTS FOR SYSTEM SUITABILITY STUDIES

Robust Conditions	System suitability results			
	RT (min)	Peak Tailing	USP Plate count	% RSD
Normal	2.694	1.40	1196	0.33
FR- 0.59 ml/min	2.701	1.34	1119	0.19
FR-0.61 ml/min	2.676	1.33	1079	0.26
CT-38 ºC	2.654	1.40	890	0.38
CT-42 °C	2.671	1.35	1245	0.25
MPC-54:46 v/v	3.037	1.32	1051	0.16
MPC-52:48 v/v	2.419	1.40	980	0.51
BS-0.0018M K_2HPO_4	2.680	1.34	1159	0.06
BS-0.0022M K_2HPO_4	2.677	1.33	1141	0.19
WL-208.0 nm	2.681	1.39	1168	0.23
WL-212.0 nm	2.681	1.39	1168	0.23

TABLE 6: ROBUSTNESS STUDY RESULTS FOR % ASSAY

Robust Conditions	%Mean Assay
Flow rate – 0.59 ml/min	105.20
Flow rate – 0.61 ml/min	105.32
Column temperature – 38 °C	105.11
Column temperature – 42 °C	104.96
Mobile phase composition-54:46 v/v	105.97
Mobile phase composition-52:48 v/v	105.88
Buffer strength – 0.0018M K_2HPO_4	105.49
Buffer strength – 0.0022M K_2HPO_4	105.23
Wave length-208.0nm	104.72
Wave length-212.0nm	104.68

The drug concentration was analyzed under these changed experimental conditions. There was no significant change in the retention time and assay of the drug when the flow rate and composition of the mobile phase were changed. The results are illustrated in **Table 5**.

CONCLUSION: In view of the fact that the Erythromycin peak was not found in the existing HPLC method, will pose less of a problem using the proposed UPLC method because it is extracted in the solvent. The proposed UPLC method for the assay of Erythromycin estolate in Erythromycin 250 mg capsules was accurate, precise, robust, specific, and stability-indicating thus demonstrating a quality by design approach to method development.

Due to its shorter run time, use of an economical and readily available mobile phase, optimized UV detection the method has been proven to be a significant importance compared with the reported methods and is compliant with current regulatory requirements. Owing to shorter run time this method enable rapid quantification of many samples in routine and quality-control analysis of various formulations containing Erythromycin estolate.

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CONFLICT OF INTEREST: Nil

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