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Validated HPTLC Method for Simultaneous Estimation of Isotretinoin and Erythromycin in Bulk Drug and Topical Gel Form

Atul S. Rathore, Lohidasan Sathiyanarayanan, Kakasaheb R. Mahadik*
Department of Pharmaceutical Chemistry, Poona College of Pharmacy, Bharati Vidyapeeth University, Pune, India
E-mail: *krmahadik@rediffmail.com*
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Abstract

A simple, precise and accurate high performance thin layer chromatographic method has been developed for the simultaneous estimation of Isotretinoin and Erythromycin in pharmaceutical gel. The separation was carried out on Merck TLC aluminum sheets of silica gel 60 F_{254}, (20 × 10 cm) with 250 µm thickness using toluene: DMSO: methanol (6.5:0.2:2.5, v/v/v) as a mobile phase. HPTLC separation of the two drugs followed by densitometric measurement of their spots at 340 nm for Isotretinoin before derivatization and 410 nm for Erythromycin after derivatization with 10% H_2SO_4 and heating at 100°C for 15 min. The drugs were satisfactorily resolved with R_F values of 0.38 ± 0.02 and 0.55 ± 0.02 for Isotretinoin and Erythromycin, respectively. The accuracy and reliability of the method was assessed by evaluation of linearity (30-150 ng spot^{-1} for Isotretinoin and 1200-6000 ng spot^{-1} for Erythromycin), precision (intra-day RSD 0.62-0.79% and inter-day RSD 0.43-0.71% for Isotretinoin and intra-day RSD 0.47-1.71% and inter-day RSD 0.42-1.49% for Erythromycin), accuracy (98.91 ± 0.92% for Isotretinoin and 99.27 ± 0.72% for Erythromycin), and specificity, in accordance with ICH guidelines.

Keywords: Isotretinoin, Erythromycin, HPTLC, Validation

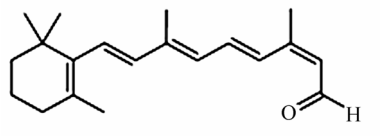
1. Introduction

Isotretinoin (**Figure 1(a)**) is chemically (13cis)-retinoic acid. Isotretinoin is a member of the large group of Vitamin A related compounds. It alters DNA transcription and decreases the size and sebum output of the sebaceous glands. It also stabilizes keratinization, Due to its effect on regulating cell differentiation it has been used for the treatment of cystic and nodular acne and also as an inhibitor of neoplasic cells proliferation [1-7]. Erythromycin (**Figure 1(b)**) is chemically (3R, 4S, 5S, 6R, 7R, 9R, 11R, 12R, 13S, 14R)-6-{[(2S, 3R, 4S, 6R)-4-(di- methylamino)-3-hydroxy-6-methyloxan-2-yl]oxy}-14-ethyl-7, 12, 13-trihydroxy-4-{[(2R, 4R, 5S, 6S)-5-hydroxy-4-methoxy-4,6-dimethyloxan-2-yl]oxy}-3, 5, 7, 9, 11, 13-hexamethyl-1-oxacyclotetradecane-2,10-dione. It is an antibiotic that possess bactericidal activity, particularly at higher concentrations. It acts by binding to the 50s subunit of the bacterial 70s rRNA complex, protein synthesis and subsequently structure/function processes critical for life or replication are inhibited [8,9]. Literature review reveals that several analytical methods have been reported for Isotretinoin [10-12] and Erythromycin [13-18] as individual determination or in biological fluids or in combination with other drugs in pharmaceutical dosage forms. A comprehensive literature survey revealed that no method has been reported for simultaneous estimation of Isotretinoin and Erythromycin by HPTLC in pharmaceutical dosage forms. So, the present study is designed for the development and validation of simple HPTLC method for the simultaneous estimation of Isotretinoin and Erythromycin in their combined topical gel formulation. The proposed method is validated as per ICH guideline [19].

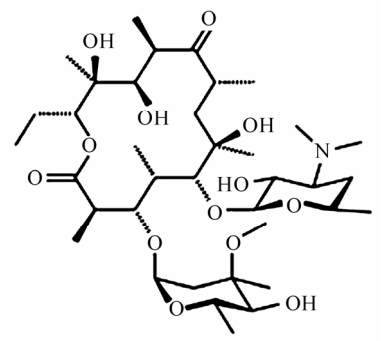
2. Experimental

2.1. Materials

Working standards of pharmaceutical grade Isotretinoin



(a)



(b)

Figure 1. (a) Structure of Isotretinoin; (b) Structure of Erythromycin.

and Erythromycin were obtained as a gift sample from Ranbaxy laboratory ltd. Dewas, India. Formulation in the form of gel (Isotrexin) was procured as a gift sample from Stiefel Laboratories (Ireland) Ltd., Silgo, Ireland containing 0.05% Isotretinoin and 2% Erythromycin (w/w). All chemicals and reagents of analytical grade were purchased from Merck Chemicals, Mumbai, India. High purity deionized water was obtained from Millipore, Milli-Q (Bedford, MA, USA) water purification system.

2.2. Preparation of Standard Stock and Working Solutions

Stock standard solution containing Isotretinoin (150 µg mL^{-1}) and Erythromycin (6000 µg mL^{-1}) was prepared by dissolving 7.5 mg Isotretinoin and 300 mg Erythromycin in methanol in a 50 mL volumetric flask. Working standard solution of Isotretinoin and Erythromycin was prepared at concentration of 15 ng μL^{-1} and 600 ng μL^{-1} respectively, by diluting the stock standard solution in methanol. The stock solution was stored at 2-8°C protected from light.

2.3. Instrumentation

The samples were spotted in the form of bands 6 mm width with a Camag 100 microlitre sample syringe (Hamilton, Bonaduz, Switzerland) on silica gel precoated aluminum plate 60 F_{254}, [(20 × 10 cm) with 250 µm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai] using a Camag Linomat IV applicator (Switzerland). The plates were pre-washed with methanol and activated at 110°C for 5 min prior to chromatography. A constant application rate of 0.1 μLs^{-1} was used and the space between two bands was 6 mm. The slit dimension was kept at 5 mm × 0.45 mm and the scanning speed was 10 mm s^{-1}. The mobile phase was consisted of toluene: DMSO: methanol (6.5:0.2:2.5, v/v/v) and 18.4 mL were used per chromatography run. Linear ascending development was carried out in 20 cm × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 30 min at room temperature (25 ± 2°C). The length of chromatogram run was 8 cm. Densitometric scanning was performed using a Camag TLC scanner III in the reflectance-absorbance mode and operated by CATS software (V 3.15, Camag). The sources of radiation used were deuterium and tungsten lamp with a spectral range from 190 to 800 nm. Concentrations of the compound chromatographed were determined from the intensity of the diffused light. Evaluation was by peak areas with linear regression.

2.4. Selection of Analytical Wavelength

A UV spectrum for the solution (10 µg mL^{-1}) of Isotretinoin was recorded in a 10 mm cell over the range 200-400 nm using methanol in the reference cell. Isotretinoin showed maximum absorbance at 340 nm (**Figure 2**) while Erythromycin was detected at 410 nm after derivatization with 10% H_2SO_4 and heating at 100 °C for 15 min [13].

2.5. Optimization of HPTLC Method

The HPTLC procedure was optimized with a view to develop a simultaneous assay method for Isotretinoin and Erythromycin. The stock standard solution containing

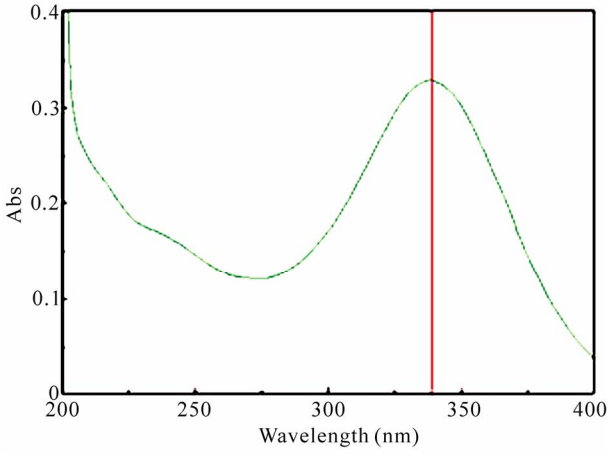


Figure 2. UV spectrum of Isotretinoin.

150 µg mL^{-1} of Isotretinoin and 6000 µg mL^{-1} of Erythromycin were spotted onto Merck HPTLC silica gel precoated aluminum plate 60 F_{254}, (20 × 10 cm) with 250 µm thickness and run in different solvent systems. Initially, toluene, ethyl acetate and methanol were tried in different ratio. (Toluene, acetone and methanol) and (hexane, acetone and methanol) were tried in various ratio. Finally, toluene, DMSO and methanol were tried. It was found that the DMSO is responsible for the elution of erythromycin. The optimum mobile phase was found to be consisted of toluene: DMSO: methanol (6.5:0.2:2.5, v/v/v). The drugs were satisfactorily resolved with RF values at 0.38 ± 0.02 and 0.55 ± 0.02 for Isotretinoin and Erythromycin, respectively. In order to reduce the neckless effect the TLC chamber was saturated for 30 min using saturation pads. The mobile phase was run up to a distance of 8 cm; which takes approximately 45 min. for complete development of the TLC plate.

2.6. Validation of HPTLC Method

The optimized HPTLC method was validated with respect to the following Parameters. The validation was performed as per the ICH guideline [19].

2.6.1. Linearity

Stock standard solution containing 150 µg mL^{-1} of Isotretinoin and 6000 µg mL^{-1} of erythromycin was further diluted with methanol to obtain a working standard solution at concentration of 15 ng μL^{-1} and 600 ng μL^{-1} respectively. From the working standard solution 2, 4, 6, 8, 10 µL volumes were spotted on HPTLC plate to obtain a final concentration range of 30-150 ng $spot^{-1}$ for Isotretinoin and 1200-6000 ng $spot^{-1}$ for Erythromycin. Each concentration was applied six times on the TLC plate. The plate was then developed using the previously described mobile phase. Curves were obtained by plotting the peak area against concentration of the drug. Linear calibration curves were generated using least-squares linear-regression analysis.

2.6.2. Precision

The precision of the method was verified by repeatability (intraday) and intermediate precision studies. Repeatability studies were performed by analysis of three different concentrations of 30, 90, 150 ng $spot^{-1}$ and 1200, 3600, 6000 ng $spot^{-1}$ for Isotretinoin and Erythromycin, respectively. Method repeatability was achieved from RSD % values obtained by repeating the assay six times on the same day for intra-day precision. The intermediate (interday) precision of the method was checked by performing same procedure on different days under the same experimental conditions.

2.6.3. Robustness

The robustness was studied by evaluating the effect of small but deliberate variations in the chromatographic conditions. Following the introduction of small changes in the mobile phase composition (± 0.1 mL for each component), the effects on the results was examined. Mobile phases having different compositions, e.g. toluene: DMSO: methanol (6.5:0.2:2.5, v/v/v), (6.5:0.2:2.4, v/v/v), (6.5:0.2:2.6, v/v/v), were tried and chromatograms were run. The amount of mobile phase was varied over the range of ±5%. The time from spotting to chromatography and from chromatography to scanning was varied from ±10 min. The robustness of the method was determined at three different concentration levels of 30, 90, 150 ng $spot^{-1}$ for Isotretinoin and 1200, 3600, 6000 ng $spot^{-1}$ for Erythromycin.

2.6.4. Specificity

The ability of an analytical method to unequivocally assess the analyte in the presence of other components can be demonstrated by evaluating specificity. The specificity of the HPTLC method was determined by analyzing standard drug and test samples. The spot for Isotretinoin and Erythromycin in the samples was confirmed by comparing the RF and spectrum of the spot to that of a standard. The peak purity of Isotretinoin and Erythromycin was determined by comparing the spectrum at three different regions of the spot i.e. peak start (S), peak apex (M) and peak end (E).

2.6.5. Accuracy

Accuracy of the HPTLC method was carried out by applying the method to drug sample [Isotretinoin 0.05% and Erythromycin 2% (w/w) combination in gel form of alcoholic base of 30 g tube] to which known amount of Isotretinoin and erythromycin standard powder corresponding to 50, 100 and 150% of label claim was added (standard addition method). The absolute recovery was calculated by comparing the peak areas obtained from standard solution of Isotretinoin and Erythromycin with the peak areas of samples of different concentration.

2.6.6. Analysis of a Marketed Formulation (Assay)

Isotrexin Gel 30 g tube (labeled to contain Isotretinoin 0.05% and Erythromycin 2% (w/w) in gel form of alcoholic base of 30 g tube, Stiefel India Ltd.) means 15 mg/30 g of Isotretinoin and 600 mg/30 g of Erythromycin. An accurate weight of the 1 gram of gel equivalent to 0.5 mg of Isotretinoin and 20 mg of Erythromycin was transferred into a 10 mL volumetric flask containing 8 mL methanol and sonicated for 30 min. The contents were restored to room temperature and diluted to volume with methanol to furnish stock test solution. The stock

solution was filtered through a 0.45 µm Nylon syringe filter. From the stock test solution (containing 50 ng μL^{-1} of Isotretinoin and 2000 ng μL^{-1} of Erythromycin), 2 µL volume was spotted for six times to achieve a final concentration of 100 ng $spot^{-1}$ and 4000 ng $spot^{-1}$ for Isotretinoin and Erythromycin, respectively.

3. Results and Discussion

In this work HPTLC method for the analysis of Isotretinoin and Erythromycin in topical gel form was developed and validated as per ICH, Q2 (R1), guideline.

3.1. Optimization of Procedures

The experimental conditions for HPTLC such as wavelength of detection and mobile phase composition were optimized to provide accurate, precise and reproducible results for the simultaneous determination of Isotretinoin and Erythromycin. A scanning wavelength of 340 nm for Isotretinoin was obtained from UV spectrum and 410 nm for Erythromycin after derivatization with 10% H_2SO_4 and heating at 100°C for 15 min [13]. A good resolution was obtained by using an optimum mobile phase consisted of toluene: DMSO: methanol (6.5:0.2:2.5, v/v/v). Isotretinoin and Erythromycin were satisfactorily resolved with RF values at 0.38 ± 0.02 and 0.55 ± 0.02, respectively (**Figure 3**).

3.2. Method Validation

3.2.1. Linearity

Linear relationships were observed by plotting drug concentrations against peak areas for each compound. Isotretinoin and Erythromycin showed linear response in the concentration range of 30-150 ng $spot^{-1}$ and 1200-6000 ng $spot^{-1}$, respectively. The corresponding linear regression equation was y = 3.774x + 468.7 and y = 2.22x – 974.1 with square of correlation coefficient (R^2) of 0.997 ± 0.066 and 0.996 ± 0.071 for Isotretinoin and Erythromycin, respectively (**Table 1**).

3.2.2. Precision

The results of the repeatability and intermediate precision experiments are shown in **Table 2**. The developed methods were found to be precise as the RSD values for repeatability and intermediate precision studies were < 2%, respectively as recommended by ICH guidelines.

3.2.3. Robustness

The standard deviation of the peak areas was calculated for each parameter and the RSD was found to be less than 2% for HPTLC. The low values of the RSD%, as shown in **Table 3**. indicated the robustness of the proposed method.

3.2.4. Specificity

The peak purity of Isotretinoin and Erythromycin was assessed by comparing their respective spectra at the peak start, apex and peak end positions of the spot i.e., r (S, M) = 0.9979 and r (M, E) = 0.9986. A good correlation (r = 0.9981) was also obtained between the standard and sample spectra of Isotretinoin and Erythromycin.

Table 1. Linear regression data for the calibration curves.

Parameters	Isotretinoin	Erythromycin
Linearity range	30-150 ng $spot^{-1}$	1200-6000 ng $spot^{-1}$
r^2 ± S.D.	0.997 ± 0.066	0.996 ± 0.071
Slope ± S.D.	3.774 ± 0.042	2.22 ± 0.099
Intercept ± S.D	468.7 ± 0.528	974.1 ± 0.984

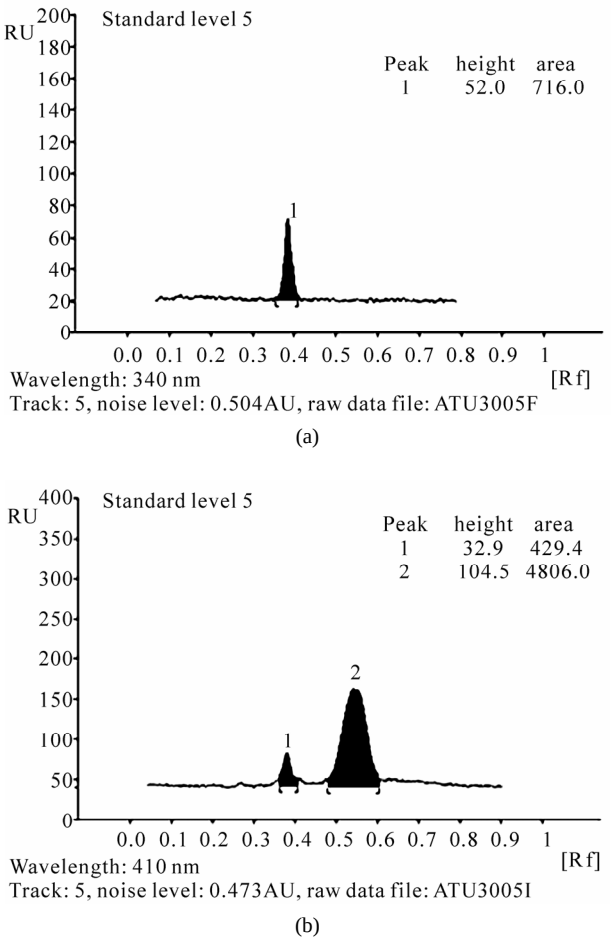


Figure 3. (a) Densitogram of Isotretinoin 60 ng $spot^{-1}$ before derivatization at 340 nm; (b) Densitogram of Erythromycin 2400 ng $spot^{-1}$ after derivatization at 410 nm.

Table 2. Precision studies of Isotretinoin and Erythromycin.

Drugs	Conc. (ng spot^{-1})	Repeatability (n = 6)		Intermediate precision (n = 6)	
		Found conc. ± SD	RSD (%)	Found conc. ± SD	RSD (%)
Isotretinoin	30	29.53 ± 0.18	0.62	29.97 ± 0.13	0.43
	90	90.08 ± 0.09	0.10	90.86 ± 0.64	0.71
	150	152.88 ± 1.21	0.79	152.14 ± 0.69	0.45
Erythromycin	1200	1067.00 ± 15.47	1.45	1038.89 ± 4.40	0.42
	3600	3690.23 ± 17.44	0.47	3637.13 ± 54.99	1.49
	6000	5993.19 ± 101.37	1.71	5934.68 ± 59.99	1.02

Table 3. Robustness testing for HPTLC method.

Parameter	SD of peak area		% RSD	
	Isotretinoin	Erythromycin	Isotretinoin	Erythromycin
Mobile phase composition (± 0.1 ml)	1.54	1.53	1.24	1.09
Amount of mobile phase (± 5 %)	1.82	0.65	1.34	1.27
Time from spotting to chromatography (± 10 min)	0.70	0.94	0.40	0.89
Time from chromatography to scanning (± 10 min)	0.72	0.59	0.48	0.40

Table 4. Accuracy of the proposed method.

Drugs	Label claim mg g^{-1} of gel	Amount added in mg (%)	Total amount (mg)	Actual conc. taken (ng spot^{-1})	For HPTLC (n = 6)		
					calculated conc. ± SD	RSD (%)	Recovery (%)
Isotretinoin	0.5	0.25 (50%)	0.75	75	73.69 ± 0.38	0.52	98.26
		0.5 (100%)	1.00	100	98.75 ± 1.29	1.31	98.75
		0.75 (150%)	1.25	125	124.65 ± 1.14	0.92	99.72
Erythromycin	20	10 (50%)	30	3000	2942.10 ± 17.36	0.59	98.07
		20 (100%)	40	4000	4062.00 ± 17.22	0.42	101.55
		30 (150%)	50	5000	4909.50 ± 56.37	1.15	98.19

3.2.5. Accuracy

As shown from the data in **Table 4**, satisfactory recoveries % with small relative standard deviations, RSD (%) were obtained at various added concentrations for both the drugs. The results indicate the methods are highly accurate for simultaneous determination of the two drugs.

3.2.6. Analysis of a Marketed Formulation (Assay)

Using the proposed chromatographic method, assay of Isotretinoin and Erythromycin in their formulation in gel form was carried out. Satisfactory results were obtained for both drugs in a good agreement with the label claims. The recovery % ± RSD % of six replicate determinations were 98.78 ± 0.99 (Isotretinoin), 101.19 ± 1.05 (Erythromycin) by HPTLC (**Table 5**).

4. Conclusions

The developed TLC technique is precise, specific and

Table 5. Analysis of marketed formulation (assay) of isotretinoin and erythromycin.

Drugs	Label claim % in 30 g gel	For HPTLC (n = 6)	
		Drug content (%) ± SD	RSD (%)
Isotretinoin	0.05 %	98.78 ± 0.98	0.99
Erythromycin	2 %	101.19 ± 1.06	1.05

accurate. Statistical analysis proves that the method is suitable for analsizing of Isotretinoin and Erythromycin as bulk drug and in pharmaceutical gel formulation. The proposed TLC method is less expensive, simpler, rapid, and more flexible than LC.

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