WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 6.805
Volume 5, Issue 5, 1107-1117.
Research Article
ISSN 2277– 7105

DEVELOPMENT AND VALIDATION OF HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS ESTIMATION OF ROFLUMILAST AND SALMETEROL IN SYNTHETIC MIXTURE

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Article Received on
01 March 2016,
Revised on 22 March 2016,
Accepted on 13 April 2016
DOI: 10.20959/wjpr20165-6131

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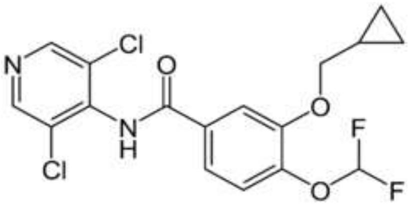
ABSTRACT

A simple, precise, sensitive, rapid high performance thin layer chromatographic method has been developed and validated for the simultaneous estimation of the Roflumilast and Salmeterol in synthetic mixture. The stationary phase used was precoated silica gel 60F$_{254}$ plate. The mobile phase used was a mixture of toluene: acetonitrile: triethayamine (6:3:1 v/v/v). The detection of spots was carried out densitometrically using a UV detector at 254 nm in absorbance mode. This system was found to give compact spots for Roflumilast (R$_f$ value of 0.13) and Salmeterol (R$_f$ value of 0.63). The method was validated according to ICH guidelines in terms of linearity, accuracy, precision and specificity. The calibration curve was found to be linear between ^{20}0 to 1400 ng/spot for Roflumilast and 100-700 ng/spot for Salmeterol with significantly high value of correlation coefficient ($r^2 > 0.99$). The limits of detection and limits of quantitation were found to be 161.74 and 35.59 ng/spot respectively for Roflumilast and 8.96 and 27.14 ng/spot respectively for Salmeterol. All the validation parameters were found to be within acceptable limits. The method was found to be rapid, cost effective, accurate and precise therefore be utilized for the routine analysis of both the drugs in synthetic mixture. The proposed method can be used in the quality control of both the drugs in synthetic mixture.

KEY-WORDS: Roflumilast, Salmeterol, HPTLC, Synthetic mixture, Validation.

INTRODUCTION

Roflumilast (ROF) is chemically 3-(cyclopropylmethoxy)-N-(3, 5-dichloropyridin-4yl)-4 (difluoromethoxy) benzamide (Figure 1), is a Phosphodiesterase-4 (PDE-4) inhibitor orally acting Anti-inflammatory.[1] It is official in Indian Pharmacopeia.[2] Literature survey reveals UV Spectrophotometry[3] and HPLC[4] methods for determination of ROF in pharmaceutical dosage forms. Salmeterol (SAL) is chemically (RS)-2-(hydroxymethyl)-4-{1-hydroxy-2-[6-(4-phenylbutoxy) hexylamino] ethyl} phenol (Figure 2), Long acting beta$_2$ adrenorecepter agonist.[5] It is official in Indian Pharmacopeia[6], United State Pharmacopeia[7], European Pharmacopeia[8], and British Pharmacopeia.[9] Literature survey reveals UV Spectrophotometry[10], HPLC[11] and UPLC[12] methods for determination of SAL in pharmaceutical dosage forms. Literature survey also reveals Spectrophotometric[13], HPLC[14] and HPTLC[15] methods for determination of SAL with other drugs in combination. The combination of these two drugs is not official in any pharmacopoeia; hence no official method is available for the simultaneous estimation of ROF and SAL in their combined mixture. Literature survey does not reveal any simple spectrophotometric or other method for simultaneous estimation of ROF and SAL in combined dosage forms. The combination of Roflumilast and Salmeterol are study under clinical trial phase Identifier No: NCT00313209 by Takeda Pharmaceuticals.[16] This combination is used in the treatment of chronic obstructive pulmonary disease (COPD). Literature survey uncover only few reported spectrophotometric method for simultaneous estimation of ROF and SAL in synthetic mixture.[17-18] The present work describes simple, rapid, accurate, precise and cost effective HPTLC method for simultaneous estimation of ROF and SAL in synthetic mixture.



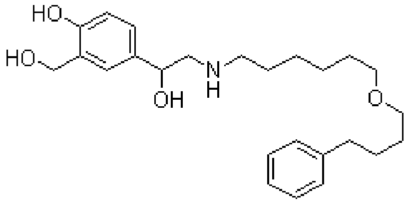


Figure 1: Chemical structure of Roflumilast. Figure 2: Chemical structure of Salmeterol

MATERIALS AND METHODS

Apparatus

Pre-coated silica gel aluminum plate 60F$_{254}$, (10 x 20 cm, layer thickness 0.2 mm, E. Merck, Darmstadt, Germany) was used as stationary phase, Camag (Muttenz, Switzerland) flat bottom and twin-trough flat-bottom TLC developing chamber (10 x 20 cm), Volumetric flasks and pipettes.

Materials and Reagents

ROF and SAL standard drug powder given as a gift sample from Cadila Zydus Ltd., Ahmedabad. Chemical used during Experimental works were Methanol, Toluene, Acetonitrile and Triethayamine. All chemicals and reagents used were of analytical grade.

Instruments

A Camag HPTLC system (Switzerland) with Linomat V automatic sample applicator and Camag TLC Scanner IV, Camag TLC Visualizer, Ultrasonic bath (Frontline ultrasonic bath, Mumbai, India), Analytical balance (CP224S, Sartorius, and Gottingen, Germany).

Software

Win-CATS 1.4.6 planar chromatography manager software.

Preparation of standard stock solution

ROF (20 mg) and SAL (10 mg) were individually weighed accurately, dissolved and diluted with methanol to obtain the final concentration of 200 µg/ml for ROF and 100 µg/ml SAL in separate 100 ml volumetric flask. For SAL preparation amber colored volumetric flask was used.

Preparation of Synthetic mixture

Synthetic mixture (130 mg) was prepared by using ROF (50 mg), SAL (10 mg) and excipients (70 mg) like starch, magnesium stearate, lactose and talc.

Chromatographic conditions

The experiment was performed on stationary phase which was silica gel $60F_{254}$ aluminum sheets (10 x 20 cm) layer thickness 0.2 mm. Mobile phase was consist of mixture of Toluene: Acetonitrile : Triethayamine (6:3:1 v/v/v). TLC plates were prewashed with methanol and activated in an oven at 50^{o} for 5 min prior to experimental work. The sample solutions were applied on TLC plate in the form of bands of 6 mm width under a stream of nitrogen gas using a Camag Linomat V automatic sample applicator. A constant application rate of 0.1 µl/s was employed and space between two bands was fixed at 16 mm. Ascending development to 70 mm was performed in 10 cm x 20 cm Camag twin trough glass chamber

(Muttenz, Switzerland) saturated with the mobile phase for 20 min at room temperature. The developed TLC plate was dried by dryer and then scanned between 200 to 400 nm using Camag TLC scanner IV using WinCATS 1.4.6 planar chromatography manager software. Both the drugs show reasonably good response at 254 nm keeping the slit dimension of 4 × 0.30 mm and scanning speed of 20 mm/s. The monochromatic band width was set at 20 nm, each track was scanned thrice and baseline correction was used. Two and five microliters of standard and sample solutions of ROF and SAL were spotted and developed.

Calibration curves of ROF and SAL

Calibration curve were plotted over a concentration range of 200-1400 ng/spot for Roflumilast and 100-700 ng/spot for Salmeterol. Aliquots of 1, 2, 3, 4, 6, and 7 µl from 200µg/ml and 100µg/ml standard stock solution of ROF and SAL respectively were over spotted on the TLC plate. TLC plate was dried by dryer, developed in TLC chamber and analyzed photometrically as described earlier. The calibration curves were obtained by plotting peak area Vs concentration (ng/spot) corresponding to each spot.

VALIDATION OF THE DEVELOPED METHOD[19]

Linearity

Calibration curve were plotted over a concentration range of 200-1400 ng/spot for Roflumilast and 100-700 ng/spot for Salmeterol. For this, 1, 2, 3, 4, 5, 6 and 7µl of 200µg/ml and 100µg/ml standard stock solution of ROF and SAL respectively was spotted in band width 6 mm using Hamilton syringe (100 microliter) for both the drugs individually on precoated silica gel aluminum plate 60 F_{254} using automatic application device.

Precision

The repeatability of the method was checked by repeated scanning and measuring the peak area of solutions (n = 6) of ROF (800 ng/spot) and SAL (400 ng/spot) without changing the parameters of the developed method It showed low % RSD of peak area of ROF and SAL. The inter-day and intra-day variation was determined at three different concentration levels on three different days over a period of one week (interday precision) and three different times on same day (intraday precision). Concentration level taken were 800, 1000, 1200 ng/spot for ROF and 400, 500, 600 ng/spot for SAL.

Limit of detection and limit of quantification

LOD and LOQ of the drug were calculated by using the following equations designated by ICH guideline: LOD = 3.3 X σ/S and LOQ = 10 X σ/S

Where, σ = the standard deviation of the response.

S = slope of the calibration curve.

Specificity

The specificity of the method was ascertained by analyzing standard drugs and the sample. The spots for ROF and SAL in the samples were confirmed by comparing the R_f and spectra of the spots with that of the standards.

Recovery studies

The accuracy of the method was determined by calculating recoveries of ROF and SAL by the standard addition method. Known amounts of standard solution of ROF and SAL were added at 80 %, 100 % and 120 % levels to pre quantified sample solutions of ROF and SAL. Accuracy was determined in terms of percentage recovery. The experiment was conducted in triplicate.

Analysis of ROF and SAL in synthetic mixture

The amount equivalent to 50 mg ROF and 10 mg SAL from synthetic mixture was transferred to 100 ml volumetric flask and 50 ml methanol was added to it and sonicated for 15 minutes. The solution was filtered through Whatman filter paper No.41 and volume was diluted up to mark with methanol. The resulting solution had final concentration of 500 µg/ml of ROF and 100 µg/ml of SAL. The peak area of final sample solution was measured densitometrically at 254 nm for quantitation of ROF and SAL. The amount ROF and SAL present in the sample solutions were determined by fitting the response into the respective regression line equation for ROF and SAL.

RESULTS AND DISCUSSION

Development of the optimum mobile phase: The TLC procedure was optimized with a view to develop an assay method. Both the pure drugs were spotted on the TLC plates and run in different solvent systems as mention in below Table 1. . Finally, the mobile phase consisting of Toluene: Acetonitrile: Triethylamine (6:3:1, v/v/v) gave sharp and symmetrical peak with Rf values of 0.12 for ROF and 0.63 for SAL. A 3D chromatogram showing peak of ROF and

SAL in different concentration at 254 nm as shown in figure 3 and chromatogram of standards are shown in Figure 4 and Figure 5 respectively.

TABLE 1: Optimization of Mobile Phase

Trial No.	Mobile phase	Observation
1	Toluene: Ethyl acetate (8:2, v/v)	Very Poor separation.
2	Toluene: Methanol (6:4, v/v/v)	Both drugs were separated with but SAL shows tailing.
3	Toluene: Methanol: Ethyl acetate (7:2.5:0.5, v/v/v)	Both drugs were separated with poor Rf (Rf <1) and SAL shows tailing.
4	Toluene: Methanol: Triethylamine (7:2.5:0.5, v/v/v)	Both drugs well separated but SAL shows tailing.
5	Toluene: Ethyl acetate; Triethylamine (7:2.5:0.5, v/v/v)	Both the drugs were separated but with higher Rf (Rf >9)
6	Toluene: Acetonitrile: (7:3:0.2, v/v/v)	Both the drugs were separated but poor Rf (Rf <1)
7	Toluene: Acetonitrile: Triethylamine (6:3:1, v/v/v)	Both drugs were separated with good Resolution and good intensity.

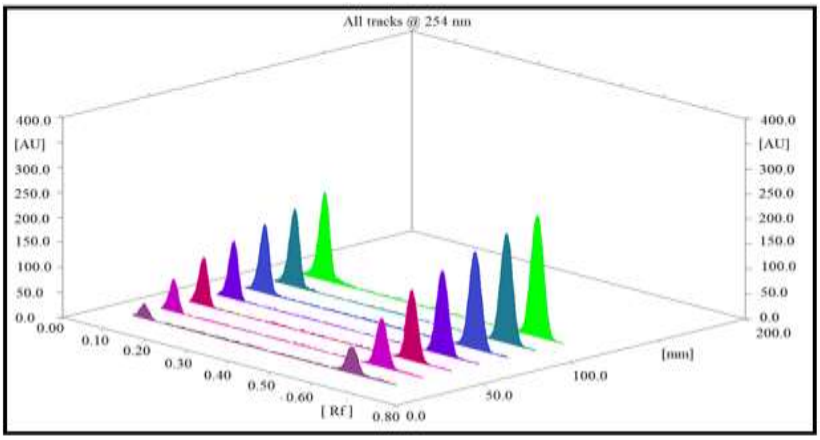


Figure 3: 3D Densitogram of standard ROF and SAL at 254 nm

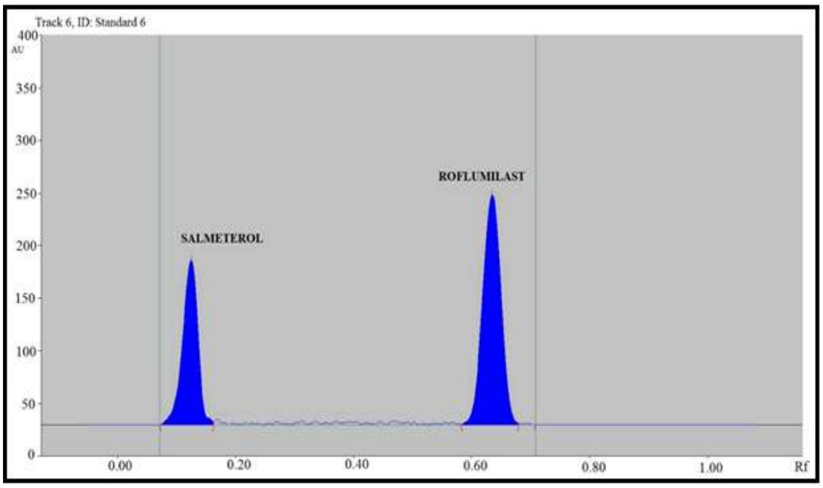


Figure 4: Chromatogram of ROF (1200 ng/spot) and SAL (600 ng/spot)

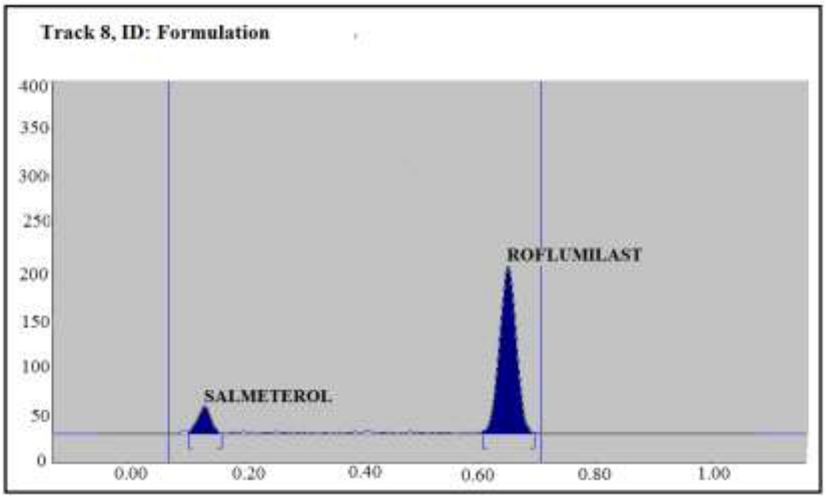


Figure 5: Chromatogram of ROF (500 ng/spot) and SAL (100 ng/spot) in Synthetic Mixture

Validation data of the method

Calibration graph was found to be linear over the concentration range of 200-1400 ng/spot for Roflumilast and 100-700 ng/spot for salmeterol both the drugs as per beer lambert law[20]. Peak area and concentration was subjected to least square linear regression analysis to calculate the calibration equations. Equations were ($y = 3.6994x + 488.04$) (r^2) 0.9974 for ROF and ($y = 4.8986x + 78.351$) (r^2) 0.9944 for SAL. In precision the repeatability of developed were expressed in terms of % RSD. The % RSD was found to be < 2 %.The results of inter and intraday variation were found to be in acceptable limit**.** LOD and LOQ were found to be 11.74 and 35.58 ng/spot respectively for ROF and 8.96 and 27.14 ng/spot respectively for SAL. The summary data of validation parameter are as mentioned in Table 2. For specificity it was observed that the excipients present in the formulation did not interfere with the peaks of ROF and SAL. The peak purity of ROF and SAL were assessed by comparing the spectra of standard and sample at peak start, peak apex and peak end positions of spot. For ROF standard spot and sample spot r (S, M) = 0.99924 & 09.99941 and r (M, E) = 0.99991 & 0.99912 respectively. For SAL standard spot and sample spot r (S, M) = 0.99935& 0.99985 and r (M, E) = 0.99929 & 0.99931 respectively. Good correlation was also found between standards and sample chromatogram of ROF and SAL as shown in figure 6 and figure 7 respectively. In recovery study average recovery was found to be 99.69 ± 1.21 for ROF and 99.80 ± 1.97 for SAL is listed in Table 3. Analysis of synthetic mixture was performed two spots at R_f 0.13 and R_f 0.63 were observed in chromatogram of the drugs samples extracted from synthetic mixture. There was no interference from the excipients commonly present in the synthetic mixture. The average drug content as per label claim was found to be 100.01 % with S.D of 1.51 for ROF and 100.01 % with a S.D of 1.8 for SAL as mentioned in Table 4. The low values of standard deviation indicates the suitability of this method for routine analysis of ROF and SAL in synthetic mixture.

Table 2: Regression Analysis Data and Summary of Validation Parameters For The Developed Method

PARAMETERS	**ROF**	**SAL**
Wavelength (nm)	254	254
Beer's law limit (ng/spot)	200-1400	100-700
Regression equation (y = a+bc) Slope (b) Intercept (a)	3.6994x + 488.04 3.6994 488.04	4.8986 + 78.351 4.8986 78.351
Correlation Coefficient (r^2)	0.9974	0.9944
Method precision (Repeatability) (% RSD), (n = 6)	0.83	1.20
Intraday (n = 3) (%RSD)	0.52 – 1.12	0.57 – 1.21
Interday (n = 3) (%RSD)	0.93 – 1.73	0.79 – 1.84
LOD (ng/spot)	11.74	8.96
LOQ (ng/spot)	39.58	27.14
Accuracy (Mean % Recovery± S.D.) (n =3)	99.69 ± 1.21	99.80 ± 1.97
% Assay ± S.D. (n = 5)	100.1 ± 1.52	100.1 ± 1.81

TABLE – 3 Results of Recovery Study of ROF and SAL by Developed Method.

Drug	**Level**	**Amount taken (ng/spot)**	**Amount added (%)**	**% Mean recovery ± S.D. (n = 3)**
ROF	I	500	80	97.21 ± 1.08
	II	500	100	101.2 ± 1.27
	III	500	120	100.5 ± 1.26
SAL	I	100	80	99.53 ± 1.87
	II	100	100	99.55 ± 2.02
	III	100	120	100.3 ± 1.98

TABLE – 4 Analysis of ROF and SAL in Synthetic Mixture by Developed Method

Synthetic Mixture	**Label claim (µg)**		**Amount found (µg)**		**% Label claim ± S. D. (n =5)**	
	ROF	SAL	ROF	SAL	ROF	SAL
I	500	100	500.9	100.0	100.1 ± 1.21	100.0 ± 1.81

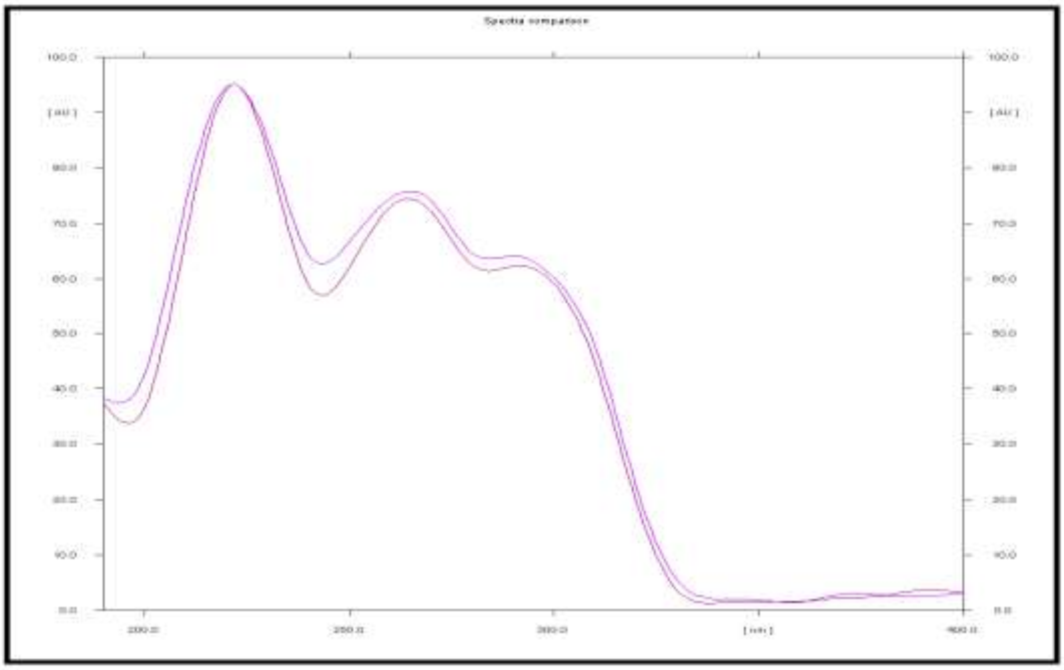


Figure 6: Spectrum comparison of standard and sample spot of ROF at 254 nm

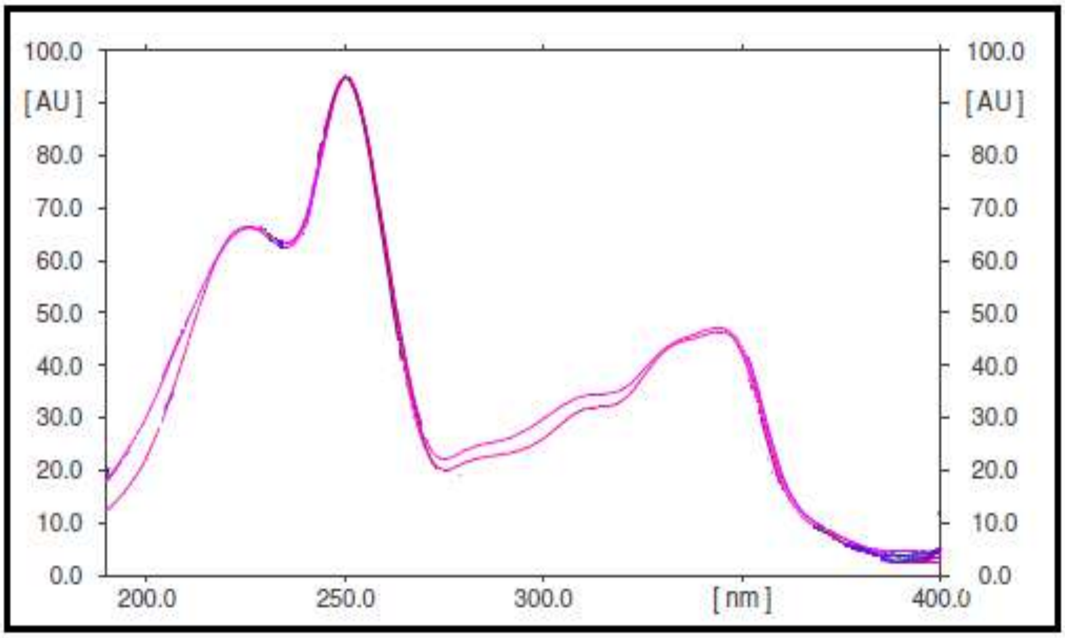


Figure 7: Spectrum comparison of standard and sample spot of SAL at 254 nm

CONCLUSION

The High performance thin layer chromatography method was developed for simultaneous determination of ROF and SAL in synthetic mixture. Method was found to be precise and accurate as can be reflected from validation parameter data. Developed method was efficiently applied for determination of ROF and SAL in synthetic mixture and there for method can be extended for the analysis of formulation.

ACKNOWLEDGMENT

The authors are thankful to Cadila Zydus Ltd., Ahmedabad, India for gifting pure API drug sample of ROF and SAL. Author also wish heartfelt thanks to S. K. Patel College of Pharmaceutical Education and Research for providing all the facilities to carry out the work.

REFERENCES

1. O'Neil MJ. The Merck Index: An encyclopedia of chemicals, drugs and biologicals, 14th ed. New Jersey: Published by Merck Research Laboratories, Division of Merck and Co., Inc. Whitehouse station: Roflumilast, 2006; 8249.
2. Indian Pharmacopoeia. Government of India, Ministry of Health and Family Welfare Ghaziabad, Indian Pharmacopoeia Commission, 7th ed., 2014; 3: 2682-2683.
3. Patel Krishna R, Desai D G, Zanwar Aarti, Sen Ashim Kumar and Seth A K. Development and validation of Uv Spectroscopy method for estimation of Roflumilast in bulk and tablet dosage form. Int. J. Pharm. Sci., 2013; 4(3): 274-280.
4. Ladani JJ, Bhimani RD, Vyas KB, Nimavat KS. Analytical method development and validation of RP-HPLC for estimation of Roflumilast in bulk drug and tablet dosage form. Int. J. Pharm. Res. Scholars, 2012; 1(3): 28-32.
5. O'Neil MJ. The Merck Index: An encyclopedia of chemicals, drugs and biologicals, 14th ed., New Jersey: Published by Merck Research Laboratories, Division of Merck and Co., Inc. Whitehouse station: Salmeterol, 2006; 8337.
6. Indian Pharmacopoeia. Government of India, Ministry of Health and Family Welfare Ghaziabad, Indian Pharmacopoeia Commission, 7th ed., 2014; 3: 2708-2709
7. USP 36, NF 31 United State Pharmacopoeia. U.S. Pharmacopeia convention, Twin brook Parkway, Rockville, MD., 2013; 3: 4063.
8. European Pharmacopoeia. European Directorate for the Quality of Medicines & Healthcare, Council of Europe, Brussels, 6th ed., 2008; 2: 2860-2862.
9. British Pharmacopoeia. The Department of Health, Social Services and Public Safety, London Her Majesty's. Stationary office, 6th ed., 2010; 2: 1872, 1873.
10. Chowdary K.P.R., Devala Rao G., New spectroscopic methods for the determination of Salmeterol xinafoate. Indian J. Pharm. Sci., 1998; 60(5): 294-296.
11. Jain Pritam, Gorle Ashish, Patil Smita, Chavan Rashmi, Bari Pankaj and Surana Sanjay. RP-HPLC method for estimation of Salmeterol xinafoate in bulk and in pharmaceutical formulation. Int. J. Pharm. Chem. Res., 2015; 4(1): 6-12.

12. Patel Pratibha, Sahu Kapendra, Karthikeyan Chandrabose, Hari Narayana, Moorthy N.S., Trivedi Piyush. Determination of Salmeterol xinafoate and its degradation products according to ICH Guidelines with use of UPLC Technique. Lat. Am. J. Pharm., 2011; 30 (5): 985-990.
13. Kondawa M.S, Shah R.R, Waghmare J.J, Malusare M.K, Shah N.D. UV spectrophotometric method for simultaneous estimation of Salmeterol xinafoate and Fluticasone propionate in bulk and dosage form. Int. J. Pharm. Tech. Res., 2011; 3(3): 1801-1806.
14. Katari Srinivasaro, Vinayk Gorule, Venkata Krishna Akula. Development and validation for simultaneous estimation of Budesonide and Salmeterol xinafoate in metered dose inhalation form by RP-HPLC, Int. J. Pharm. Phytopharmacol. Res., 2012; 1(5): 271-275.
15. Lantider Kasaye, Ariaya Hymete, Abdel-Maaboud I. Mohamed. HPTLC Densitometric method for simultaneous determination of Salmeterol xinafoate and Fluticasone Propionate in dry powder inhalers. Saudi Pharmaceutical Journal, 2010; 18: 153–159.
16. A service of a US National Institute of Health Effect of Roflumilast on Lung Function in Chronic Obstructive Pulmonary Disease (COPD) Patients Treated With Salmeterol. (https://clinicaltrials.gov/ct2/show/NCT00313209) [Access: September 2015].
17. Patel Ushma B., Patel Paresh U. Development and Validation of Spectrophotometric Method for Simultaneous Determination of Roflumilast and Salmeterol in Synthetic Mixture. Ijppr. Human, 2016; 5(2): 67-77.
18. Patel Ushma B., Patel Paresh U. Development and Validation of Q-Absorbance Method for Simultaneous Determination of Roflumilast and Salmeterol in Synthetic Mixture. Int. Res. J. Pharm, 2016; 7(1): 58-61.
19. ICH Harmonized Tripartile Guideline, 2006. Validation of Analytical Procedures: Text and Methodology Q2B (R1), International Conference on Harmonization, Geneva, Switzerland.
20. Beckett AH, Stenlake JB. Practice Pharmaceutical Chemistry, 4th ed., New Delhi: CBS Publisher, 1997; 285.