A Novel Validated Stability-indicating HPTLC Method to Quantitate Forskolin as a Bulk Drug and in a Nanosuspension

NAZIA KHAN*, AMEEDUZZAFAR[1], A. ALI AND F. J. AHMAD

Department of Pharmaceutics, Faculty of Pharmacy, Jamia Hamdard, New Delhi-110 062, India, [1]College of Pharmacy, Aljouf University, Sakaka, Aljouf, KSA

Khan *et al*.: Quantification of Forskolin by a Novel Validated HPTLC Method

A novel, reproducible, precise, accurate and dependable high-performance thin-layer chromatographic technique was developed and authenticated as per International Conference on Harmonization guiding principle for quantitative evaluation of forskolin as a bulk drug and in a nanosuspension. Chromatographic plates 60 F-254 pre-coated with silica gel and a solvent system of ethyl acetate:hexane:formic acid (7:2.9:0.1 v/v) was used for the development of the plates. Densitometric scanning of the developed plates in the reflectance mode at 555 nm using a Camag TLC scanner after exposing the plates to anisaldehyde-sulphuric acid reagent, gave a compact spot for forskolin (R_f=0.46±0.012). In accordance to the ICH guidelines, the method was validated for linearity, precision (intra- and inter-day), limit of quantification and limit of detection, robustness and accuracy. Good linear relationship was observed for the calibration plots with linear range from 100-1000 ng/spot and a coefficient of correlation r^2=0.994. Limit of detection and limit of quantification were found to be 10.83 and 36.12 ng/spot, respectively. The method appeared to be well suited for determining forskolin content in the developed nanoformulation. Forskolin was found to be susceptible to acid and alkali hydrolysis, oxidation, photochemical degradation with UV and sunlight. Statistical analysis confirmed that the process developed is precise, repeatable, accurate, and suitable for the analysis of forskolin in a formulation and could be used also for analysis of forskolin in plasma and other biological fluids. The ability of the developed method to separate the drug from its degradation products effectively confirmed that it could be a stability indicating method.

Key words: Forskolin, HPTLC, ICH, nanosuspension, validation

Herbal medicines are the oldest form of healthcare. According to World Health Organization (WHO), plants are the major sources for the drugs to be prescribed globally and constitute around 25 % and currently 121 compounds with therapeutic potential are actively used. WHO listed a total 252 drugs, which are considered essential and basic, of which 11 % are actually of plant origin[1]. Chemically, forskolin is 7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxy-labd-14-en-11-one'[2] (fig. 1). It is a diterpenoid isolated from *Coleus forskolii* belonging to the family Lamiaceae and been previously reported to have antiglaucoma potential[3]. Forskolin is a lipophilic compound that when applied topically to the eye for treating glaucoma it acts by stimulating adenylate cyclase of ciliary epithelium to increase levels of cyclic adenosine monophosphate leading reduced aqueous humor inflow and a reduction in the intraocular pressure (IOP)[4-6]. Increased IOP resulting

in optic nerve damage is primarily responsible for impairment in visualisation leading to glaucoma. It is basically an irreversible cause of blindness, however if detected, can be treated well with medications[7]. Forskolin is one of the herbal drug, which was found to have antiglaucoma potential and been used for treating glaucoma[3]. Of all the chromatographic techniques, high-performance thin-layer chromatography (HPTLC) is one of the significant planar chromatographic technique, which is widely used for the quantification of various traditional and other forms of therapeutic

*Address for correspondence
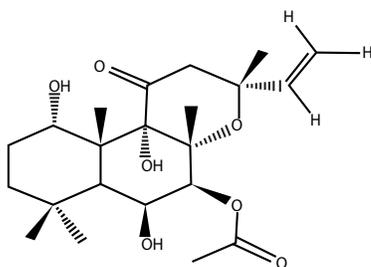E-mail: khannaz88@rediffmail.com

Fig. 1: Structure of forskolin

agents. Rapid cost-effective analysis of simple mixtures, ability to analyse various samples on a single plate run with efficient quantification[8] are some of the outstanding features of HPTLC that make it different from other chromatographic techniques. Requirement for very low quantity of mobile phase along with ease in preparation of samples for calibration plot are other advantages of HPTLC analysis of drugs. HPTLC as well as other chromatographic techniques such as HPLC, gas chromatography mass spectrometry (GC/MS) have previously being reported for forskolin determination in different formulations and natural fluids[9,10]. However, the present method developed and validated is different from the other described methods as it has been used for the analysis of drug content in the developed nanosuspension, which has not been reported till now.

The efficacy and safety of a pharmaceutical product depended a lot on the chemical behaviour of the drug molecule. Forced or stress degradation studies are basically conducted at different stress or accelearted conditions, probably thought to be more severe for a particular drug molecule to assess its degradation pattern and hence, shelf-life. Stress testing is intended for degradation product identification with simultaneous determination of inherent stability of the active compounds as mentioned in the International Conference on Harmonization (ICH) guidelines. Various tests and parameters need to be tested for stress degradation studies with minimal factors like susceptibility of the compound (solid or in liquid and suspension form) to oxidation, acid and base hydrolyis, thermal and photolytic degradation have been suggested[11-14].

In the present investigation, a novel HPTLC method was developed and validated as per the guidelines of ICH for determination of forskolin as a bulk drug and in a pharmaceutical dosage form. Stress degardation studies were performed to assess the stability of forskolin.

MATERIALS AND METHODS

Sami Labs, Bangalore, India, provided a gift sample of forskolin. The plates for TLC analysis used were pre-coated silica gel aluminium plates (E. Merck, Darmstadt, Germany) procured from Anchrom Technologies, Mumbai, India. All the reagents and chemicals used were of analytical grade and were procured from S.D. Fine-Chem Ltd., Mumbai, India.

TLC instrumentation and conditions:

The samples for chromatographic analysis comprising of the standards and forskolin extracted samples were spotted on a 60 F 254 (10×10 cm with 250 µm thickness) aluminium plate precoated with silica gel with a sample applicator Camag Linomat IV (Camag, Muttenz, Switzerland) and a Hamilton syringe of 100 µl capacity. Prior to application of spots on TLC plates, plate's pre-treatment and activation was done at 60±5° in a hot air oven to make them absolute moisture free for about 5 to 10 min so as to obtain better compact spots.

Application of the samples was done using nitrogen aspirator at a constant 150 nl/s application rate and spotted with first application position X=10 mm and position Y=10 mm in the form of distinct bands of length 5 mm with each spot being 11.4 mm apart. Linear ascending twin trough glass chamber (Camag Muttenz, Switzerland) of the dimensions 10× 10 cm saturated with mobile phase was used for linear ascending development of the plates. The optimized chamber saturation time for mobile phase was 20 min at room temperature and the length of chromatogram run was 85 mm.

The plate was dried by air dryer. The plates were sprayed with anisaldehyde-sulphuric acid reagent comprising of 0.5 ml anisaldehyde+5 ml sulphuric acid+85 ml methanol+10 ml glacial acetic acid with subsequent drying at 110° in a hot air oven (10-15 min) till the appearance of dark pink spots. Slit dimensions of length 4.00 mm and thickness 0.45 mm with a 20 mm/s scanning speed were employed and scanning of the dried chromatogram was done by Camag TLC scanner III in the reflectance mode 555 nm[15]. The radiation source employed was halogen tungsten lamp and Win CATS planar chromatography version 1.1.3.0 was used for its operation. The concentrations of chromatographed compounds were determined from the potency of dispersed reflected light. The peak areas with linear regression were determined[16-19].

Calibration curve of forskolin:

Methanol was used for preparing forskolin stock solution (1000 µg/ml), which was prior to spotting filtered through a membrane filter of thickness 0.25 µm. Spotting was performed in triplicate with different volumes ranging from 0.1 to 1.0 µl on the HPTLC plate corresponding to concentrations of 100-1000 ng of forskolin and by linear least square regression analysis the peak area vs. drug concentration data was treated.

Method validation:

Linearity, precision, limit of detection (LOD) and limit of quantification (LOQ), robustness and accuracy were the parameters validated as per ICH guidelines[20]. A standard curve of forskolin was constructed with the concentration versus peak area plotted between 100-1000 ng/spot.

Evaluation of precision was done with respect to intra- and inter-day precision. Two hundred and 600 ng/spot were the two concentration levels of sample solution at which the inter- and intra-day variation were determined. The sample solutions (200 and 600 ng/spot) were analysed three times on the same day for intra-day variation study whereas for inter-day variation the sample solutions (200 and 600 ng/spot) were analysed thrice each day for three consecutive days. The mean, standard deviation (SD) and % relative standard deviation (% RSD) values were calculated with the peak areas obtained.

Small changes in mobile phase composition and amount of mobile phase were the parameters primarily studied at concentration levels of 600 and 1000 ng/spot to assess the robustness of the developed method. Changes in mobile phase composition involved small variations in the ratios of mobile phase. Ethyl acetate:hexane:formic acid of ratios (6.5:3.4:0.1 v/v and 7.5:2.4:0.1 v/v) were the mobile phases composition studied. Amount of mobile phase was another parameter for which the robustness study was carried out with two volumes of mobile phase 15 and 20 ml. The TLC plate was spotted with blank methanol six times for estimating the LOD and LOQ with subsequent determination of noise level. Signal-to-noise ratio of 3:1 is considered as LOD while 10:1 as LOQ. SD was determined for the analytical response and LOD and LOQ were thus experimentally verified. Samples previously analysed were added with an additional 50, 100 and 150 % of the standard forskolin to determine the accuracy of the method. The testing was carried out in triplicate and the recovery

of forskolin at different levels in the formulations was determined.

Analysis of forskolin from the formulation:

Quantification of drug from developed novel nanosuspension was performed using the proposed validated HPTLC method as previously described earlier. Single emulsion technique was employed for formulating forskolin nanosuspension. Polylactic glycolic acid (PLGA) and chitosan were the polymers primarily employed for the formulation process. Dichloromethane (DCM), was used as an organic solvent to solubilise the required amount of polymer (PLGA 50:50) and hence, constituted the organic phase while chitosan (mucoadhesive polymer) and polyvinyl alcohol, used as a stabilizer were dissolved in 1 % acetic acid with pH adjusted (pH 5.0) constituted the aqueous phase. The organic phase was emulsified in aqueous phase by means of probe sonicator for specified period of time. The resultant O/W emulsion was kept on a magnetic stirrer for evaporating DCM at room temperature for 5-6 h till the solvent completely evaporated. The nanosuspension was centrifuged at 15 000 rpm for 30 min and was thrice washed to remove excess of stabilizer. The pellet obtained was redispersed in 2-3 ml water and was lyophilized to obtain freeze dried nanoparticles. Extraction of the nanoparticles obtained was done with 10 ml methanol and for complete extraction of the drug, sonication for 30 min followed by centrifugation at 15 000 rpm for 15 min were performed. The supernatant obtained from the formulation was filtered and applied in triplicate on the TLC plate followed by development and scanning as described in the previous section. Other than analysis of drug content from the nanosuspension, the chances of excipients interference with the drug peak was also studied.

Stability studies:

The stability-indicating property of the HPTLC method developed was assessed by conducting forced degradation studies of forskolin according to ICH guidelines. Forskolin was subjected to acid and base hydrolysis, oxidation, photochemical degradation by UV and sunlight. Forskolin solution containing 50 mg/50 ml of methanol was prepared as the stock solution, which was further used for stress degradation studies.

Preparation of acid and base-induced degradation:

To 5 ml of 1M HCl and 1M NaOH, 5 ml of stock solution

was added separately and the mixtures were refluxed in different containers at 90° for 6 h with the whole experiment performed in dark to avoid the possible side effects of light. 2 µl of the resultant solution (800 ng/spot) was applied on TLC plate and as per the method described in previous section the chromatograms were run.

Preparation of hydrogen peroxide-induced degradation:

To 5 ml of hydrogen peroxide (H_2O_2; 30 %, v/v), 5 ml of stock solution was added in dark to eliminate the possible effects of light with the solution being refluxed for 6 h at 90°. 2 µl of the resultant solution was applied smoothly on TLC plate as a single spot. The experimentation for chromatogram development and analysis was executed as per method described earlier.

Sunlight and UV light-induced degradation:

The stock solution of forskolin was exposed to direct sunlight and UV irradiation at 254 nm for 48 h to carry out photochemical-induced degradation, respectively. Diluted samples of the resultant solutions were properly prepared with the spots applied to the TLC plate and were analysed with the chromatogram run as per the method described previously.

RESULTS AND DISCUSSION

A HPTLC method for analysis of forskolin was developed with proper selection and optimization of the mobile phase. Compact and dense spots with considerably different R_f values of the solvent system were particularly desired for forskolin. Various combinations of mobile phases were investigated and evaluated for HPTLC analysis of forskolin and finally plates were developed using a mobile phase of composition ethyl acetate:hexane:formicacid (7:2.9:0.1 v/v). Formic acid was added after thorough optimization since, before the addition of formic acid, tailing problem was observed in the chromatogram developed (fig. 2); however, after addition of 0.1 ml of formic acid the problem was overcome and more compact spots, with normal symmetrical peak and R_f value of 0.46 with good resolution for forskolin were obtained (fig. 2).

Calibration curves of forskolin (n=3) studied over a range of 100-1000 ng/spot showed a good linear relationship with respect to peak area as shown in fig. 3. The slopes of the standard curves showed no significant difference (ANOVA; p<0.05). The graph was found
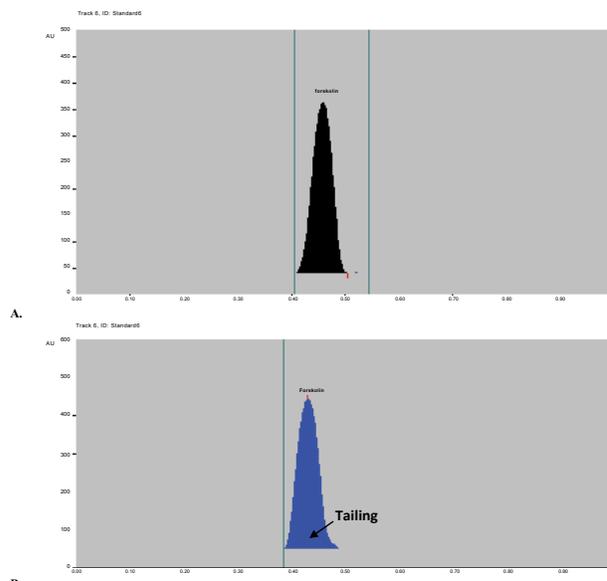

Fig. 2: HPTLC chromatogram
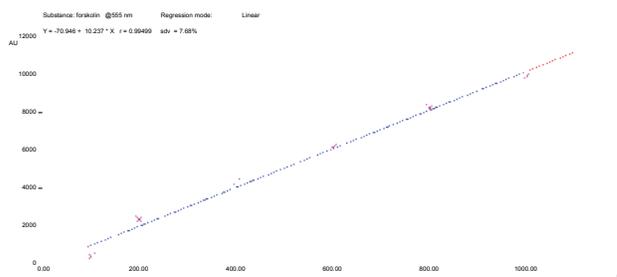**(A) Forskolin and (B) forskolin before addition of formic acid**

Fig. 3: Calibration curve
Area vs. concentration

to be linear. A coefficient of correlation r^2=0.994 was obtained for the calibration plots (100-1000 ng/spot), which indicated good linear relationship with slope and intercept values obtained as; slope=10.237±1.27 and intercept=70.946±2.65 (n=3) over the concentration range studied. The slopes of the standard curves in this range depicted no significant differences (ANOVA; p<0.05). The linear regression data is as shown Table 1.

Peak area measurement and sample application repeatability were expressed in terms of % RSD values. The % RSD for two concentration levels 400 and 600 ng/spot was found to be 0.64 and 0.51, respectively. Results for inter-day and intra-day variation of forskolin peak area for both concentrations are as shown in Table 2. The % RSD values were found to be <2 % in all instances, which indicated that the developed method is of high precision.

Amount of the mobile phase and small changes in mobile phase were the parameters studied for determining robustness of the method. Percent RSD values were calculated for each parameter from the

TABLE 1: LINEAR REGRESSION DATA FOR CALIBRATION PLOT FOR FORSKOLIN (n=3)

Parameters	Values
Linearity range (ng/spot)	100-1000 ng/spot
Regression equation	Y= -70.946+10.237×X
Correlation coefficient	0.994
Slope±SD	10.237±1.27
Intercept±SD	70.946±2.65
R_f value	0.46±0.012

TABLE 2: INTRA- AND INTER-DAY PRECISION OF FORSKOLIN (n=3)

Amount (ng/spot)	Mean (area)	SD (area)	% RSD (area)	SEM (area)
Intra-day				
200	2369.4	32.3529	1.36545	18.69
600	6161.5	31.5013	0.51136	18.2
Inter-day				
200	2373.6	27.6892	1.16654	16
600	6170.4	39.6752	0.64298	22.9

TABLE 3: ROBUSTNESS OF FORSKOLIN (n=3)

Forskolin	Mobile phase composition	
Amount	Ethyl acetate:hexane:formic acid	Ethyl acetate:hexane:formic acid
(ng/spot)	(6.5:3.4:0.1, v/v) % RSD (area)	(7.5:2.4:0.1, v/v) % RSD (area)
600	0.89	0.55
1000	0.36	0.23
Forskolin	**Amount of mobile phase (±5 %)**	
Amount (ng/spot)	15 ml, % RSD (area)	20 ml, % RSD (area)
600	0.85	0.99
1000	0.54	0.53

peak areas obtained and since it was less than 2 %, the developed method was found to be robust. Table 3 showed the respective results.

LOD is measured as 3:1 signal-to-noise ratio while LOQ as 10:1. The values for LOD and LOQ were found to be 10.83 and 36.12 ng/spot, respectively. Fifty, 100 and 150 % of the standard forskolin were added to previously analysed forskolin samples for carrying out a recovery study. The proposed TLC method afforded good recovery ranging from 99.04 to 100.24 % as shown in Table 4, and indicated that the method was adequate. In Table 5, a summary of the validation parameters have been shown.

The chromatogram of the samples extracted from developed nanoparticulate formulation showed a single spot of R_f value of 0.47. The drug content i.e. amount of forskolin encapsulated in the formulation was found to

be 94.1 % with a % RSD of 0.0357 for three replicates. The suitability of the method is indicated by low value of % RSD and thus, can be used for routine analysis of forskolin in various dosage forms. No degradation of the drug occurred in formulation as the developed chromatogram showed a single peak of drug with little or no interference of the excipients during the analysis.

Additional peaks at different R_f values were obtained for the chromatograms of the acid-degraded samples and base-degraded samples. Three peaks for acid-induced degraded sample were observed at R_f values 0.12, 0.37, 0.39 as shown in Table 6 and fig. 4a while for base-induced degraded sample two peaks were observed at R_f values 0.02 and 0.57 as shown in Table 6 and fig. 4b. The forskolin spot was well resolved from the degraded products from acid and base-induced degradation. The three additional peaks apart from forskolin peak were obtained as degraded product peaks from hydrogen peroxide 30 % v/v treatment

TABLE 4: RECOVERY STUDIES (n=3)

Excess drug added to analyte (%)	Theoretical content (ng)	Recovery (%, area)	RSD (%, area)	SEM (area)
0	400	99.04	1.69	42.06
50	600	99.83	0.16	5.25
100	800	100.24	0.24	11.56
150	1000	100.13	0.41	23.97

TABLE 5: SUMMARY OF VALIDATION PARAMETERS

Parameter data	Data
Linearity range (ng/spot)	100-1000 ng/spot
Correlation coefficient	0.994
Limit of detection	10.83 ng per spot
Limit of quantification	36.12 ng per spot
Recovery studies (accuracy, n=3)	99.04 to 100.24 %
Robustness	Robust
Precision (% RSD)	
Inter-day (n=3)	0.64
Intra-day (n=3)	0.51

TABLE 6: DEGRADATION PRODUCTS OF FORSKOLIN WITH R_f VALUES

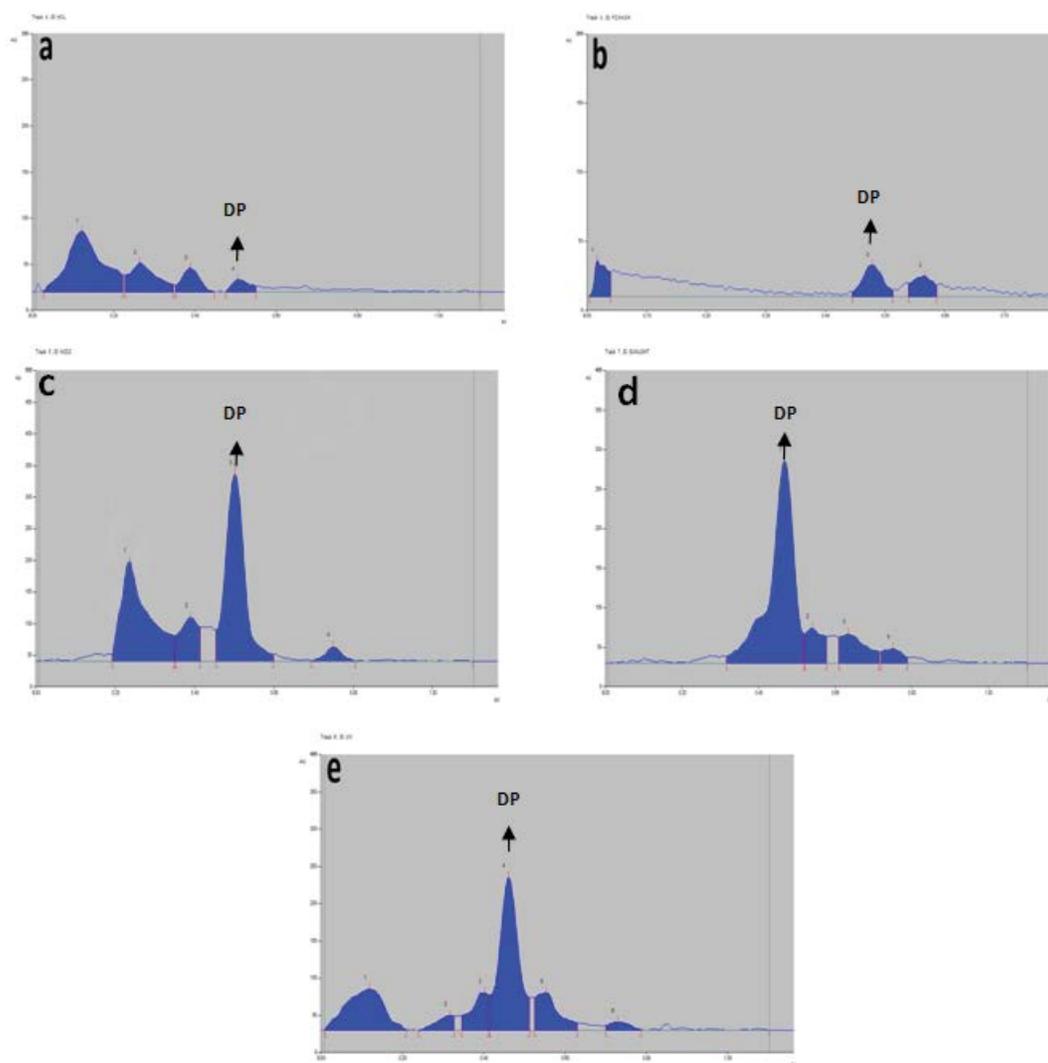| Stress condition	No. of degradation products R_f value	Forskolin R_f value	% Recovery
Acid (1 M HCl)	3 (0.12, 0.27, 0.39)	0.51	2.85
Base (1 M NaOH)	2 (0.02, 0.57)	0.48	5.65
Peroxide (30% H_2O_2)	3 (0.23, 0.39, 0.75)	0.5	69.4
Sunlight	3 (0.54, 0.64, 0.75)	0.47	77.2
UV light	5 (0.12, 0.32, 0.40, 0.56, 0.73)	0.46	47.3

Fig. 4: Forced degradation of forskolin
(a) Acid degradation (b) base degradation (c) H_2O_2 degradation (d) sunlight degradation (e) UV light degradation. *DP indicates drug peak

at different R_f values, 0.23, 0.39, 0.75 as depicted in Table 6 and fig. 4c. Forskolin spot was well resolved from among the degraded products.

Chromatograms for sunlight and UV-induced degraded products were obtained with variable peaks at different R_f values. Three peaks for sunlight-induced degradation products at R_f values 0.54, 0.64, 0.75 as depicted in Table 6 and fig. 4d whereas five peaks for UV-induced degradation products at R_f values 0.12, 0.32, 0.40, 0.56, 0.73 were observed as shown in Table 6 and fig. 4e with the drug spots for respective parameters well resolved from the other peaks (degraded products).

In conclusion, after thorough optimization and validation, a validated HPTLC method for the analysis of forskolin has been developed. The developed method was found to be simple, precise, accurate, less time consuming and cost-effective, reproducible and selective as evidenced by the statistical analysis of data and could be used for routine analysis of forskolin. As compared to other densitometric methods, the developed HPTLC method has been found to be more accurate since it has been properly optimized, developed and validated. Purity of the drug can be assessed by this method as the drug is available from a variety of sources and detection of associated impurities can also be done with developed approach. Statistical analysis has proved that the method is repeatable, accurate and precise and therefore, can be employed for analysis of forskolin in various formulations. No degradation of drug occurred in formulation and least interaction of excipients occurred as single chromatogram of forskolin was observed with R_f value of 0.47. Quantitative estimation of the said drug

i.e., forskolin can also be done in plasma and other biological fluids. Prediction of shelf life of forskolin containing pharmaceuticals by accelerated stability testing can also be done by employing the developed method. Forskolin can be well effectively used in the formulation of nano-dosage forms and can be used for glaucoma treatment in future.

Acknowledgements:

Conflicts of interest:

There are no conflicts of interest.

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