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Stability Indicating HPTLC method for Forskolin and Glycyrrhetinic acid

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ABSTRACT

A simple, specific and rapid high performance thin-layer chromatography (HPTLC) method was developed for quantification of Forskolin (FSK) and Glycyrrhetinic acid (GA) and also for the determination of stress degradation product as per the ICH guidelines. The compounds were chromatographed on precoated silica gel G 60254 plates using chloroform: methanol (9.5:0.5 v/v), as the mobile phase. The linear regression analysis of data for the calibration plots showed good linear relationship with R^2 = 0.996 and 0.991 for Glycyrrhetinic acid and Forskolin with respect to peak height and peak area, respectively, in the concentration range of 4–20 µg per spot of Glycyrrhetinic acid, and 40-200 µg per spot Forskolin. Mean recovery for Forskolin and Glycyrrhetinic acid was found to be 100.46% *w/w*, 100.87% *w/w*. Statistical analysis proves that the method is repeatable, selective and accurate for the estimation of Forskolin and Glycyrrhetinic acid in pharmaceutical dosage forms as well as their degradation products hence, it can be employed for routine analysis and as a stability-indicating method.

KEYWORDS

Thin layer chromatography, Validation, Forskolin, Glycyrrhetinic acid, Stress degradation

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INTRODUCTION

Plants have utility in various ways. Each and every part of the plant has applicability, and use for different purposes. On the basis of literature survey number of medicinal uses has been reported for Forskolin and Glycyrrhetinic acid. The genus coleus forskohli (Lamiaceae) commonly known as Mainmula, includes 150 species of small shrubs occurring in tropical Asian countries. It grows wild in the sub-tropical warm temperate climates of India, Nepal, Burma, Sri Lanka and Thailand. It is recorded in Ayurvedic Materia Medica under the Sanskrit name Makandi and Mayani. C. forskohlii is a perennial herbaceous plant that grows to about 45 -60 cm tall. The entire plant is aromatic[4]. Forskolin is a labdane diterpene isolated from Coleus forskohlii. Forskolin increases the amount of cyclic AMP (cAMP) (adenosine monophosphate) in cells by activating adenylate cyclase enzyme[3,1]. cAMP is one of the most important secondary messengers in the cell and considered as one of the most important cell regulating compounds. Forskolin has been found to increase lipolysis by increasing cAMP and also inhibit fat storage by stimulating thyroid hormone production and release[2]. It has been used for the treatment of heart and lung diseases, psoriasis, intestinal spasms, insomnia, and convulsions and widely used in several biochemical studies related to cAMP and adenyl cyclase pathways[5,6].

The genus Glycyrrhiza glabra (Leguminosae) commonly known as liquorice includes 150 species. The liquorice plant is a herbaceous perennial legume native to southern Europe and part of Asia, such as India. The roots are stoloniferous. Countries producing liquorice include India, Iran, Italy, Afghanistan, Iraq, Azerbaijan, Uzbekistan, Turkmenistan, turkeyand England. The herb contains the pentacyclic triterpenoid 18-β glycyrrhetinic acid and also contains flavonoids, isoflavonoids[8,10]. Liquorice used worldwide as a natural sweetener and in certain cases, used as a flavour additive in the preparative of candies and foods[7]. Moreover, powdered Liquorice root is widely used in herbal drugs in the formulation of Ayurvedic and Chinese medicines. This herb has been reported with various biological activities including antitumor, expectorant, antiulcer, immunomodulatory, antimalarial, and anti-hypercholesterolemia. Glycyrrhetinic acid used as an in various preparations has shown antimicrobial and anti-tumor, anti-inflammatory activities[8,9].

Coleus forskohli is the plant official in Indian Pharmacopoeia 2014 and Glycyrrhiza glabra is the plant official in Indian Herbal Pharmacopoeia. The WHO has emphasized to ensure the quality of medicinal plant products using modern controlled technique like HPTLC.

Extensive literature survey reveals that few HPTLC and HPLC methods have been reported for estimation of Forskolin and Glycyrrhetinic acid individually and in combination with other marker compounds. To the best of our knowledge no reports were found for simultaneous estimation of Forskolin and Glycyrrhetinic acid by stability indicating HPTLC method.

MATERIALS AND METHODS

Sample Collection

Working standards of Glycyrrhetinic acid and Forskolin were purchased from Yucca Enterprises, Wadala (E), Mumbai-400 037. Methanol (AR grade), Chloroform (AR grade), Hydrochloric acid (HCl), Hydrogen Peroxide (H_2O_2 30% v/v and 3% v/v), Sodium Hydroxide (NaOH) were purchased from LOBA CHEMIE PVT. LTD. Mumbai.

Preparation of Standard Stock Solution

Standard stock solution of Glycyrrhetinic acid and Forskolin were prepared separately. Accurately weighed 100 mg of Glycyrrhetinic acid and Forskolin were separately dissolved in 10 ml of methanol to get concentration of 10,000 µg/ml and 20,000 µg/ml, respectively. Working standard solution of both the drugs were prepared separately containing 1000 µg/ml of Glycyrrhetinic acid and 10,000 µg/ml of Forskolin using Methanol.

Preparation of sample solution of Formulation:

Weighed accurately 1 gm of gel and dispersed in 10 ml of methanol. The content was sonicated for 15 min. Filtered through Whatmann filter paper (125mm) and the filtrate centrifuged. Forskolin (40 µL) and Glycyrrhetinic acid (8 µL) solution was applied on TLC plate. After development, peak areas of the bands were measured at 210 nm and the amount of drug present in samples were estimated from the respective calibration curve. Procedure was repeated six times for the analysis of homogenous sample.

Selection of mobile phase and chromatographic conditions

Chromatographic separation studies were carried out on the working standard solution of Glycyrrhetinic acid (1000 µg/ml) and Forskolin (10,000 µg/ml). Initially, trials were carried out using various solvents in various proportions on normal TLC plates,

to obtain satisfactory resolution, desired R_f and shape for drug peak. After several trials Chloroform: Methanol (9.5:0.5 v/v) was chosen as the mobile phase, which gave acceptable peak parameters.

Solution Application

Solution of Glycyrrhetinic acid (1000 µg/ml) and Forskolin (10,000 µg/ml) was prepared. A

4 µl (4 and 40 µg/band) of solution was applied on precoated silica gel 60F254 Aluminum sheets with the help of Hamilton syringe (100 µl), using Linomat 5 applicator attached to CAMAG HPTLC system, which was programmed through WIN CATS software.

Development of Chromatogram

After the application of sample, the chromatogram was developed in Twin trough glass chamber 10 x 10 cm saturated with mobile phase for 20 min. The spotted plate was placed in the saturated chamber and developed up to 90 mm distance.

Detection of Spots

The plate was dried and scanned by densitometer at 210 nm. The R_f values data were recorded by WINCATS software (Version 1.4.3,) slit dimensions were 5.00 x 0.45 mm and Deuterium lamp was used as a radiation source.

Stress Degradation Studies

Stress testing studies were carried out separately on each drug to provide evidence on how the quality of drug varies under the influence of variety of stress conditions like hydrolytic, oxidative, photolytic and thermal stress conditions as per ICH guidelines. As per Q1AR2 guidelines, the stress conditions were optimized with respect to stress of condition and duration of exposure, so as to achieve 10-30% degradation.

Alkali Catalyzed Hydrolysis

1 ml standard stock solution of Glycyrrhetinic acid (10,000 µg/ml respectively) was mixed with 1 ml of 0.1 N NaOH and volume was made up to 10ml with methanol. Solution was kept for 1hour and applied on TLC plate.

1 ml standard stock solution of Forskolin (20,000 µg/ml respectively) was mixed with 1 ml of 0.01 N NaOH. Solution was kept for 10min and applied on TLC plate.

Acid Catalyzed Hydrolysis

1 ml standard stock solution of Glycyrrhetinic acid (10,000 µg/ml, respectively) was mixed with 1 ml of 0.5 N HCl and volume was made up to 10ml with methanol. Solution was kept for overnight and applied on TLC plate.

1 ml standard stock solution of Forskolin (20,000 µg/ml respectively) was mixed with

1 ml of 0.01 N HCl. Solution was kept for 10min and applied on TLC plate.

Oxidation Degradation

1 ml standard stock solution of Glycyrrhetinic acid (10,000 µg/ml respectively) was mixed with 1 ml of 30% v/v H_2O_2 and volume was made up to 10ml with methanol. Solution was kept for 1 hour and applied on TLC plate.

1 ml standard stock solution of Forskolin (20,000 µg/ml respectively) was mixed with 1 ml of 3% v/v H_2O_2. Solution was kept for 10min and applied on TLC plate.

Degradation under Dry Heat

Dry heat study was performed by keeping both drugs in oven at 60^0 C. A sample of Glycyrrhetinic acid was withdrawn after 4 hrs, weighed and dissolved in methanol to get solution of 1000µg/ml and sample of Forskolin was withdrawn after 1 hrs, weighed and dissolved in methanol to get solution of 10,000µg/ml and then applied on TLC plate.

Photo-Degradation

Photolytic studies were carried out by exposure of drug to UV light up to 200 watt hrs/square meter and subsequently to cool fluorescent light to achieve an illumination of 1.2 million Lux hrs. Sample was weighed, dissolved and diluted get 1000µg/ml of Glycyrrhetinic acid and 10,000µg/ml of Forskolin as final concentration and was applied on TLC plate.

Neutral degradation

1 mL standard stock solution of Glycyrrhetinic acid (10,000 µg ml^{-1}) was mixed with 1 mL of purified water and 8 mL of methanol. The solution was kept at room temperature for overnight and applied on TLC plate

1 mL standard stock solution of Forskolin (20,000 µg ml^{-1}) was mixed with 1 mL of purified water. The solution was kept at room temperature for overnight and applied on TLC plate to get concentration 80 µg $band^{-1}$ and analyzed under optimized chromatographic conditions.

RESULTS AND DISCUSSION

It was observed that both drugs showed considerable absorbance at 254nm of Glycyrrhetinic acid and 210nm of Forskolin. Hence these wavelengths were chosen for scanning the TLC plate. (Figure 1)

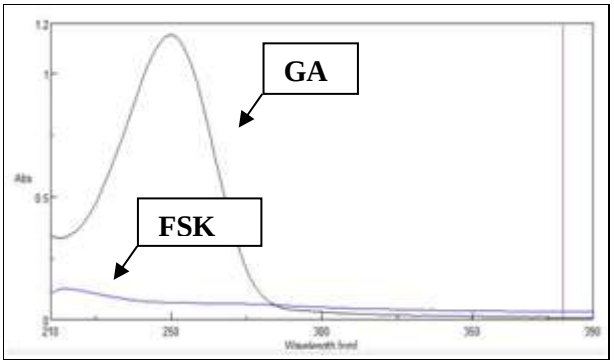


Figure 1 UV Spectra of GA at 254nm and FSK at 210nm

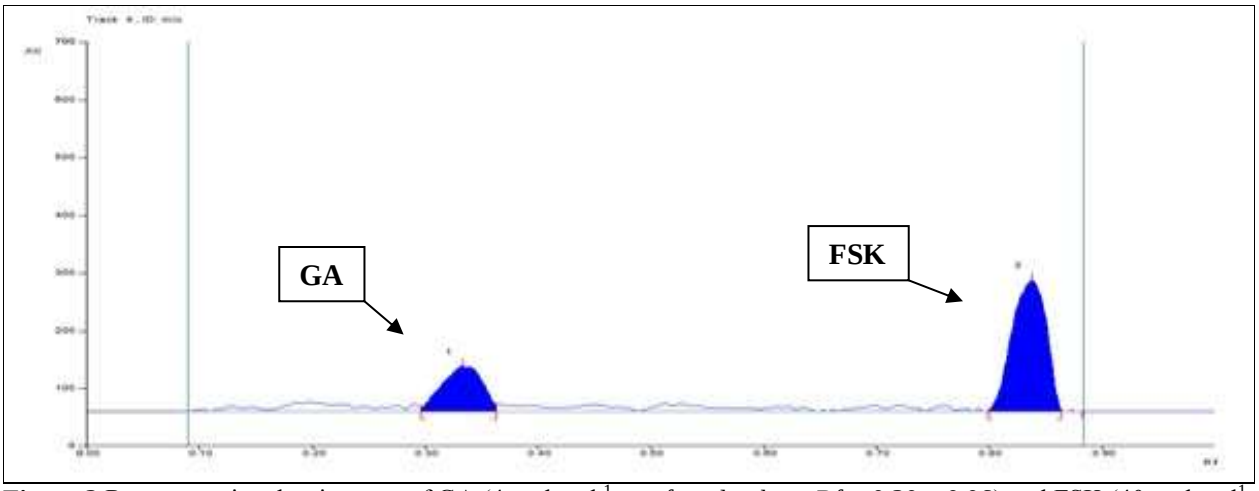


Figure 2 Representative densitogram of GA (4 µg band^{-1} was found to be at Rf = 0.30 ± 0.06) and FSK (40 µg band^{1} was found to be Rf = 0.80 ± 0.03)

Optimization of Chromatographic Conditions

The chromatographic separation was achieved by linear ascending development in 10 cm × 10 cm twin trough glass chamber using Chloroform: Methanol (9.5:0.5 v/v). As mobile phase and detection was carried out at 254 nm for Glycyrrhetinic acid and at 210 nm for Forskolin. The retention factor for Glycyrrhetinic acid and Forskolin was found to be 0.30 ± 0.02and 0.80 ± 0.02 respectively. Representative densitogram of standard solution of Glycyrrhetinic acid and Forskolin is shown in figure 2.

Result of Forced Degradation Studies

After optimization of the different stress conditions, Glycyrrhetinic acid and Forkolin were found to degrade not more than 22% and 25% (Table 1).

Table 1 Summary of stress degradation of GA and FSK

Stress degradation conditions at 254nm	Glycyrrhetinic acid % Assay	% degradation	Stress Degradation conditions at 210nm	Forskolin % Assay	% degradation
Initial	100	-	Initial	100	-
Base (0.1N NaOH kept for 1 hour)	77.81	22.18	Base (0.01N NaOH kept for 10 min.)	79.88	20.11
Acid (0.5 N HCl overnight)	78.96	21.03	Acid (0.01N HCl kept for 10 min.)	74.95	25.04
H_2O_2 30%v/v (kept for 1 hour)	84.30	15.69	H_2O_2 3% v/v (kept for 10 min.)	75.94	24.05
Dry Heat (60ºC, 4 hour)	77.75	22.24	Heat dry (60ºC 1 hour)	84.80	15.19
Photo stability UV, 200 watt hour/square meter	92.86	7.13	Photo stability UV, 200 watt hour/square meter	83.86	16.13
Florescence	90.27	9.72	Florescence	80.27	19.72

1.2million Lux. Hour			1.2 million Lux. Hour		
Newtral (for overnight)	98.56	1.43	Newtral (for overnight)	99.93	0.06

There was no separate peak for product of degradation observed for either of the two. It was confirmed by applying 10 times higher concentration (4000ng /band for Glycyrrhetinic acid and 40,000ng/band for Forskolin) and further confirmed by multiwavelenght scanning to observe if any degradation products were present (Figure 3).

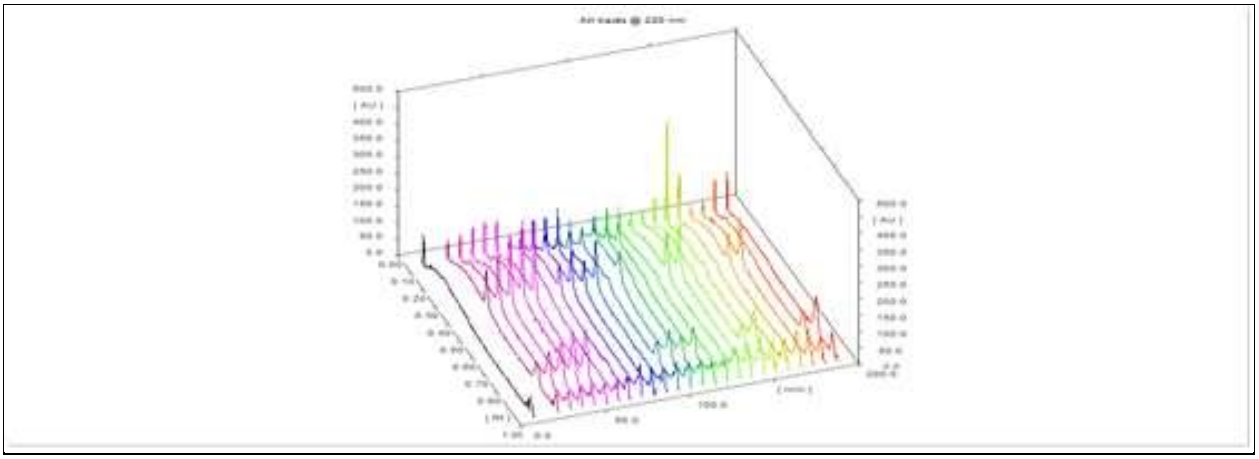


Figure 3: Densitogram of 40µg/band and 400µg/band for GA and FSK respectively, Track 1-methanol, track 2-6 linearity of GA (at Rf 0.30±0.02) and FSK (at Rf 0.80±0.02), track 7-acid blank, track 8-9 GA acid degradation (4 and 40µg/band), track 10-11 GA thermal degradation (4 and 40µg/band), track 12-13 FSK acid degradation (40 and 400µg/band), track 13-14 FSK thermal degradation (40 and 400µg/band), track 15 –H_2O_2 blank, track 16-17 GA oxidative degradation (4 and 40µg/band), track 18-19 FSK oxidative degradation (40 and 400µg/band)

Peak purity was comparison of absorbance spectra from the start to middle (s,m) and from middle to end (m,e) of the peak to determine if they are homogenous peaks.

VALIDATION OF ANALYTICAL METHOD

The method was validated as per ICH Q2 (R1) guidelines,

Linearity

The calibration curve was obtained in the range of 4-20µg/band for Glycyrrhetinic acid and 40-200µg/band for Forskolin by applying different volumes on TLC of stock solution 1000µg/ml and 10,000µg/ml respectively and peak areas were recorded (Figure 4).

The standard calibration graph was plotted between peak area versus concentration applied. The equation of the calibration curve found for Glycyrrhetinic acid was y = 1577.5x + 10152 and y=142.4x + 1447.1 for

Forskolin. The coefficient of correlation (r^2) was found to be 0.996 and 0.991 for Glycyrrhetinic acid and Forskolin at respectively shown in Figure 5.

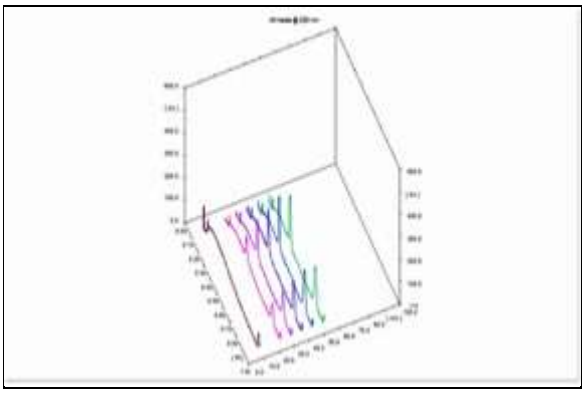
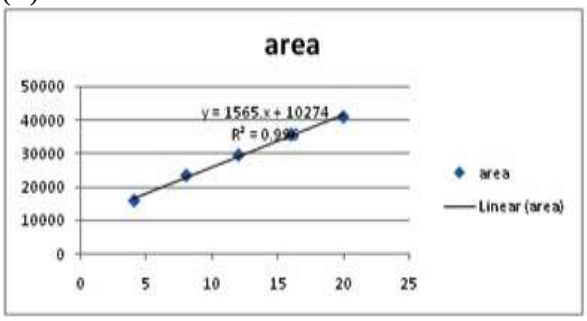


Figure 4 Densitogram of linearity of GA (Rf 0.30 ± 0.02) 4-20µg/band and FSK (Rf 0.80 ± 0.02) 40-200µg/band

(A)



(B)

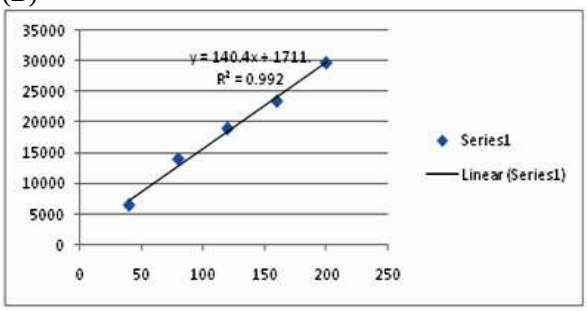


Figure 5 Calibration curve for linearity of GA(A) And FSK(B) (4-20 µg band^{-1}, 40-200 µg band^{-1})

Precision

The precision of the system was demonstrated by intra-day and inter-day studies. In the intraday studies three sets of one standard concentration (8 µg band^{-1} for Glycyrrhetinic acid and 80 µg band^{-1} for Forskolin) were analyzed in a day and percentage RSD was calculated. For the inter day study, same concentrations of the standard solutions in linearity range were analyzed and percentage RSD was calculated. For intraday precision %RSD was found to be 1.54 % and 1.58 % for Glycyrrhetinic acid and Forskolin respectively. For interday %RSD was found to be 1.40 % and 1.32% for Glycyrrhetinic acid and Forskolin, respectively.

Assay

Assay was carried out by addition of standard drug to blank gel base. It was determined by extrapolation of peak area from linearity equation which was found to be 98.59% for Forskolin and 100.03% for Glycyrrhetinic acid, respectively.

Accuracy

To check accuracy of the method, recovery studies were carried out by addition of standard drug to assayed at three different levels 80, 100 and 120 %. The drug concentrations were calculated from respective linearity equation. The results of the recovery studies indicated that the method is accurate for estimation of drug in the gel (Table 2).

Table 2 Recovery studies

Drug	Amount added per 1 gm of gel base (µg band^{-1})	Total amount found (µg band^{-1})	% Recovery
FSK	8	31.99	99.98
	10	40.03	100.09
	12	48.62	101.31
GA	8	6.32	98.86
	10	8.06	100.83
	12	9.8	102.94

Specificity

The specificity of the method was ascertained by peak purity profiling studies. The peak purity values were found to be more than 0.9980, indicating the non-interference of any other peak of degradation product or impurity (Table 3).

Table 3 Peak purity values for specificity

	Peak purity			
Degradation	**GA (at 254nm)**		**FSK (at 210nm)**	
Initial	0.9998	0.9996	0.9997	0.9995
Base	0.9996	0.9994	0.9992	0.9983
Acid	0.9988	0.9990	0.9996	0.9997
Oxidative	0.9995	0.9993	0.9987	0.9990
Heat dry	0.9989	0.9995	0.9994	0.9997
Photo stability UV, 200 watt hours/square meter	0.9989	0.9992	0.9993	0.9991
Florescence , 1.2 million Lux. Hours	0.9997	0.9989	0.9981	0.9990
Neutral	0.9997	0.9995	0.9996	0.9999

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ were calculated as 3.3 σ/S and 10 σ/S, respectively; where σ is the standard deviation of the concentration response and S is the slope of the calibration plot. The LOD and LOQ were found to be.

LOD of Glycyrrhetinic acid = 0.54 µg/band and LOQ of GA = 1.65 µg/band

LOD of Forskolin = 2.56 µg/band and LOQ of FSK = 7.78 µg/band

Robustness

Robustness of the method was determined by carrying out the analysis under conditions during which chamber saturation time were altered. Time was also changed from spotting to development and development to scanning and the effects on the peak area was noted (Table 4).

Summary of validation study

Summary of validation parameters given below (Table 5).

CONCLUSION

The developed and validated TLC-densitometric method is precise, accurate, and stability-indicating for the quantification of Glycyrrhetinic

acid and Forskolin in the presences of its degradation products. Glycyrrhetic acid showed extensive degradation in hydrolytic and thermal stress conditions, while stable or less degradation to acidic, neutral, oxidative, photochemical stress conditions. Forskolin is sensitive to all stress condition. This method was developed as per ICH guidelines, cost effective and can be used for routine analysis of these two markers in other formulations containing the same.

Table 4 Results of Robustness Study GA and FSK

Sr. no.	Parameter	(% RSD)	
		GA	FSK
1	Time from application to development (after 10min, 20min, 30min)	0.22	0.81
2	Detection wavelength (±2nm)	1.62	1.44
3.	Chamber saturation time (20min)± 2 min.	1.00	1.55
4.	Time from development to scanning ▪ ▪i.e., after 30 min, 1 hour	0.72	0.61

Table 5 Summary of validation study

Sr. No	Validation Parameters	GA (Rf=0.30±0.02)	FSK (Rf=0.80±0.02)
1.	Linearity Equation (r^2) Range	y = 1577.5x + 10152 R^2 = 0.996 4-20µg/band	y =142.4x + 1447.1 R^2 = 0.991 40-200µg/band
2.	Precision (% RSD) Interday Intraday	 1.42, 1.18, 1.62 1.50, 1.43, 1.69	 1.50,1.30, 1.16 1.88, 1.27, 1.60
3.	Assay	% assay- 100.03 % assay- 98.59	% RSD- 1.29 % RSD- 1.98
4.	Accuracy 80% 100% 120%	 98.86 100.83 102.94	 99.98 100.09 101.31
5.	Limit of Detection (µg/band)	0.54	2.56
6.	Limit ofQuantitation (µg/band)	1.65	7.78
7.	Specificity	Specific	Specific
8.	Robustness	Robust	Robust