ISSN : 2249-5746

International Journal of Ayurvedicand Herbal Medicine 7:3 (2017) 2605–2614

Journal homepage: http://www.interscience.org.uk
UGC Approved Journal
Impact factor: 4.415

Stability Indicating Hplc Method For Forskolin And Glycyrrhetinic Acid

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Abstract

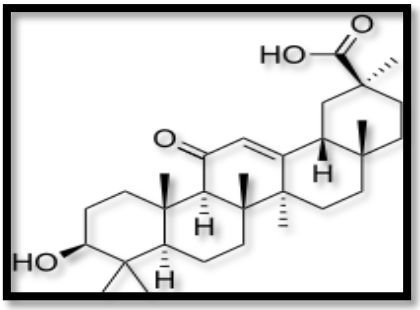
A stability indicating high performance liquid chromatographic (HPLC) method for the estimation of Glycyrrhetinic acid and Forskolin has been developed. The operating condition were C-8 reversed phase column (250×4.6 mm, 5 mm) at room temperature, acetonitrile: water (pH 3 with formic acid) 50:50 as mobile phase, at flow rate of 1.0ml/min and the detection was achieved by PDA detector at a wavelength of 210 nm. The linear regression analysis data for the calibration plots showed good linear relationship with R^2 = 0.9973 and 0.9982 for Glycyrrhetinic acid and Forskolin in the concentration range 10–50 µg/ml of Glycyrrhetinic acid as well as Forskolin. Stress degradation studies were done as per ICH Q1A(R2) guidelines. The method can be employed for routine analysis and as a stability-indicating method.

Key words: High performance liquid chromatography, Forskolin, Glycyrrhetinic acid, stress degradation.

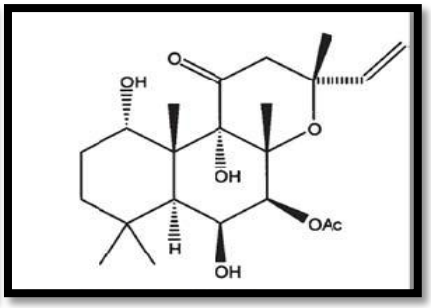
Introduction

Coleus forskohli is a plant official in Indian Pharmacopoeia 2014 and *Glcyrrhiza glabra* is a plant official in Indian Herbal Pharmacopoeia. Plants *Coleus forskohli* and *Glycyrrhiza glabra* has utility in various ways. Each and every part of the plant has 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 is recorded in *Ayurvedic Materia Medica* under the Sanskrit name *Makandi* and *Mayani*[4]. *Forskolin* is a 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.[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 herb contains the pentacyclic triterpenoid *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 in various preparations and has shown antimicrobial and anti-tumor, anti-inflammatory activities.[8,9]



Chemical Structure of Glycyrrhetinic acid



Chemical Structure of Forskolin

Sophisticated modern technique of standardization such as HPLC provide quantitative and semi-quantitative information about the main active constituents or marker compounds. HPLC offers better resolution and estimations of active constituents can be done with reasonable accuracy in a short time, extensive study is required for estimation of their stability-indicating properties. The parent drug stability test guidelines Q1A(R2) is used by International Conference on Harmonization (ICH) requires the stress testing to elucidate the inherent stability characteristics of the active substance. This guideline emphasizes the testing of those features which are susceptible to change during the storage under the influence of various environmental factors. (temperature, light, humidity, oxidation etc.)

Extensive literature survey reveals that few HPTLC and HPLC methods have been reported for estimation of FSK and GA individually and in combination with other marker compounds. To the best of our knowledge no reports were found for simultaneous estimation of FSK and GA by stability indicating HPLC method. Hence, main objective was development of stability indicating method.

Materials and Methods

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.

Instrumentation:

An HPLC (JASCO0 with MD 2010 PDA detector , Borwin –PDA software was used. The analysis was carried out on HiQ Sil C8 (250x4.6 mm) column with 5µ m particle size as a stationary phase. Rheodyne injector with a 20 µl loop was used for the injection of sample solution and mobile phase.

Preparation of Standard Stock Solution:

Standard stock solution of Glycyrrhetinic acid and Forskolin was prepared by dissolving 10 mg of drug in 10 ml of ACN to get concentration of 1000 µg/ml (A). From the standard stock solution, working standard solution was prepared in mobile phase containing 100 µg/ml of Glycyrrhetinic acid and Forskolin (B).

Preparation of sample solution of Formulation:

Weighed accurately 1 gm of gel (containing 10 mg of each of Glycyrrhetinic acid and Forskolin) and dispersed in 10 ml of methanol to get concentration of 1000µg/ml. The content was sonicated for 15 min. filtered through whatmann filter paper no. 41 (125mm) and centrifuged the solution. After appropriate dilution, the final concentration was made 30 µg/ml for Glycyrrhetinic acid and Forskolin. These sample

solution were injected six times. After development, peak areas were measured at 210 nm and the amount of drugs present in sample were estimated from the respective calibration curve.

Chromatographic condition:

Selection of analytical wavelength:

From the standard stock solution further dilutions were done using mobile phase and scanned over the range of 200 – 400 nm. The spectrum was obtained. It was observed that the both the drugs showed considerable absorbance at 210 nm so it was selected as detection wavelength.

Selection of mobile phase:

The standard solution of Glycyrrhetinic acid and Forskolin (10 µg/ml) was injected into the HPLC system and run in different solvent systems. Initially, trials were carried out using various solvents in various proportions of mobile phase components were tried in order to obtain the desired system suitability parameters for the Glycyrrhetinic acid and Forskolin. After several trials, acetonitrile and water (adjusted pH 3 with formic acid) in the ratio of 50: 50 v/v was selected as the mobile phase for analysis, which gave good resolution and acceptable peak parameters.

Stress Degradation Studies:

The degradation was carried out under hydrolytic, oxidative, photolytic and thermal stress conditions as per ICH guidelines. As per Q1A(R2) guidelines, the stress conditions were optimized with respect to strength of reagent and duration of exposure, so as to achieve 10-30% degradation.

Alkali hydrolysis:

0.5mL standard stock solution of Glycyrrhetinic acid (1000 µg ml^{-1}) was mixed with 0.5mL of 0.1 N NaOH and volume was made upto 10 ml with mobile phase. The solution was kept at room temperature for 1 hr.

For alkali hydrolysis, same like acid hydrolysis 0.5mL standard stock solution of Forskolin (1000 µg ml^{-1}) was mixed with 0.5mL of 0.01 N NaOH, volume was made upto 10 ml with mobile phase. The solution was kept at room temperature for 10 min. 50 µg/ml of the resultant solution of Glycyrrhetinic acid and Forskolin was then injected into HPLC system.

Acid hydrolysis:

0.5mL standard stock solution of Glycyrrhetinic acid (1000 µg ml^{-1}) was mixed with 0.5 mL of 0.5 N HCl, volume was made upto 10 ml with mobile phase. The solution was kept at room temperature for overnight. 50µg/ml of the resultant solution was then injected into HPLC system.0.5mL standard solution of Forskolin (1000 µg ml^{-1}) was mixed with 0.5mL of 0.01 N HCl, volume was made upto 10 ml with mobile phase. The solution was kept at room temperature for 10 min.

Oxidative Hydrolysis:

0.5mL standard stock solution of Glycyrrhetinic acid (1000 µg ml^{-1}) was mixed with 0.5ml of 30 % solution of H_2O_2v/v and volume was made upto 10 ml with mobile phase. The solution was kept at room temperature for 1 hrs. and it was injected into HPLC system.

0.5mL standard stock solution of Forskolin (1000 µg ml^{-1}) was mixed with 0.5mL of 3% solution of H_2O_2 v/v and volume was made upto 10 ml with mobile phase The solution was kept at room temperature for 10 min.

Degradation under dry heat:

Dry heat degradation was performed by keeping drugs in solid state individually in oven, initially Glycyrrhetinic acid exposed at 60^oc for 4 hr. and Forskolin at 60^oc for 1 hr. A sample was withdrawn at

appropriate times, weighed and mixed with mobile phase to get solution 50 µg/ml of the resultant solution of Glycyrrhetinic acid and Forskolin was then injected into HPLC system.

Photo degradation Studies:

(a) First method of Photolytic studies were carried out by exposure of drugs individually to UV light up to 200 watt h square meter^{-1} Samples were weighed, dissolved in mobile phase to get concentration of 50 µg/ml for Glycyrrhetinic acid and Forskolin.

(b) Second method were carried out by exposure of drugs individually to Fluroscence light up to 1.2 million Lux/hr. Samples were weighed, dissolved in mobile phase to get concentration of 50 µg/ml for Glycyrrhetinic acid and Forskolin.

Neutral hydrolysis:

0.5mL standard stock solution of Glycyrrhetinic acid (1000 µg ml^{-1}) was mixed with 0.5mL of purified water and volume was made upto 10 ml with mobile phase. The solution was kept at room temperature for overnight. 50 µg/ml of the resultant solution was then injected into HPLC system.

0.5mL standard stock solution of Forskolin (1000 µg ml^{-1}) was mixed with 0.5mL of purified water and volume was made upto 10 ml with mobile phase. The solution was kept at room temperature for overnight.

Results

It was observed that both drugs showed considerable absorbance at 210nm. Hence these wavelengths were chosen for detection of chromatogram. Figure 1 is showing UV spectra of Glycyrrhetinic acid at 254 nm and Forskolin at 210 nm.

Optimization of Chromatographic Conditions:

Mobile phase was prepared by mixing acetonitrile and HPLC grade water (adjusted pH 3 with formic acid) in the ratio of 50:50 v/v. It was then filtered through 0.45 µm membrane filter paper using vacuum filtration assembly and then sonicated on ultrasonic water bath for 15 min. Figure 2 is showing chromatogram of standard solution of Glycyrrhetinic acid and Forskolin

Chromatogram and system suitability parameter of drug:

The column was equilibrated with the mobile phase (indicated by constant back pressure at desired flow rate). Working standard solution of drug (10µg/ml) was injected into the system. The retention time for the drug was found to be: Glycyrrhetinic acid = 15.4 ± 0.2 min, Forskolin = 7.0 ± 0.2 min.

System suitability parameters of Glycyrrhetinic acid and Forskolin are summarized in Table 1.

Result of Forced Degradation Studies:

After optimization of the different stress conditions, Glycyrrhetinic acid and Forkolin were found to degrade not more than 21% and 23%.There was no separate peak for product of degradation observed for either of the two. The optimized conditions of stress degradation studies are summarized in Table 2.

Validation of Analytical Method:

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

Specificity:

The specificity of the method was checked for the interference of impurities and excipients in the analysis of drug solution under optimized chromatographic condition. The peak purity values were found to be more than 990 as shown in table 3, indicating the non interference of any other peak of degradation product or impurity. Hence, the method was found to be specific.

Linearity:
Linearity was tested for the range of concentrations 10-50µg/ml. The response factors were plotted against the corresponding concentrations of Glycyrrhetinic acid and Forskolin to obtain the calibration curve. The equation of the calibration curve found for Glycyrrhetinic acid was y = 4035.5x + 77814 and y=2859.3x + 68342 for Forskolin. The coefficient of correlation (r^2) was found to be 0.9973 and 0.9982 for Glycyrrhetinic acid and Forskolin at respectively shown in Figure 3.

Range:
Glycyrrhetinic acid = 10-50µg/ml
Forskolin = 10-50µg/ml

Assay:
Assay studies were carried out by addition of standard drug to blank gel base Assay was determined by extrapolation of peak area from linearity equation which was found to be 101.56% for Forskolin and 99.96% for Glycyrrhetinic acid respectively.

Accuracy:
Accuracy of method was evaluated by the percent recovery study at 80, 100, 120 % levels. The recoveries were verified by estimation of drugs in triplet at each specified level. Result of recovery study were given in table 4.

Precision:
Precision of the method was tested by performing intra-day and inter-day studies. For intra-day studies, triplet of sampleswere analyzed within same day. For inter-day validation, analysis was carried out on three separate days. % RSD of 0.63 % and 0.74 % for Glycyrrhetinic acid and Forskolin for intraday whereas % RSD of 0.82 % and 1.06% for Glycyrrhetinic acid and Forskolin respectively for interday study was obtained. The smaller values of % R.S.D. (< 2) indicated that method was found to be precise.

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 = 0.75 µg/ml and LOQ = 2.30 µg/ml
LOD of Forskolin = 1.04 µg/ml and LOQ = 3.16 µg/ml

Robustness:
Robustness of the method was determined by carrying out the analysis under conditions during which flow rate, mobile phase pH variation and mobile phase variation were altered and the effects on the peak area were noted Table (5).

Summary of validation study:
The result of all validation parameters are summarized in table 6.

Discussion
Ahmad S. *et al*[14] have reported A Validated Stability- Indicating TLC Method for Determination of Forskolin in Crude Drug and Pharmaceutical Dosage Form. In this article, , they have not followed ICH guidelines for UV (200 watt hrs/square meter) and florescence (1.2 million Lux. hrs) degradation, Drug was

directly exposed to sunlight instead of photostability and for visualization they have used derivatization technique, where as our method is simpler.

Syed G.M *et al*[17] have reported Stress degradation studies and stability –indicating TLC –densitometry method of glycyrrhetic acid. In this reported article of Glycyrrhetinic acid for stress degradation has been down by use of reflux conditions for every stress degradation.

We have developed method that follows ICH guidelines and Stress conditions did not involved refluxing and visualization did not involved derivatization technique.

Conclusion

The proposed method is simple, sensitive and rapid hence, can be used for routine analysis of Glycyrrhetinic acid and Forskolin. The proposed work is stability indicating and can be used in determination of Glycyrrhetinic acid and Forskolin for stability testing. Glycyrrhetinic acid showed extensive degradation in hydrolytic stress conditions, while stable or less degradation to acidic, neutral, hydrolytic, oxidative, photochemical stress conditions. Forskolin is sensitive to all stress condition but highly sensitive to hydrolytic condition. The validation of the analytical method indicated its suitabilility for the routine use.

Acknowledgement

Authors are thankful to the Principal and the management of AISSMS College of Pharmacy, Pune for providing the necessary facilities for research work and to Yucca Enterprises, Wadala (E), Mumbai-400 037 for providing Glycyrrhetinic acid and Forskolin.

Table 1: System suitability parameter

Name	RT (min.)	Conc. (µg/ml)	Area	Plates	Asymmetry
Glycyrrhetinic acid	15.4	10	119793	1929.16	1.33
Forskolin	7.0	10	99706.77	1942.93	1.56

Table 2: Summary of stress degradation of Glycyrrhetinic acid and Forskolin

Stress degradation conditions at 210nm	Glycyrrhetinic acid %degradation	Stress degradation conditions at 210nm	Forskolin %degradation
Initial	-	Initial	-
Base (0.1N NaOH kept for 1 hour)	21.53	Base (0.01N NaOH kept for 10 min.)	18.87
Acid (0.5 N HCl overnight)	20.80	Acid (0.01N HCl kept for 10 min.)	23.58
H_2O_2 30%v/v (kept for 1 hour)	15.63	H_2O_2 3% v/v (kept for 10 min.)	23.42
Dry Heat (60^{o}C, 4 hour)	20.95	Heat dry (60^{o}C 1 hour)	17.13
Photo stability UV, 200 watt hour/square meter	7.69	Photo stability UV, 200 watt hour/square meter	17.55
Florescence 1.2million Lux. Hour	10.21	Florescence , 1.2 million Lux. Hour	20.41
Neutral (for overnight)	1.28	Neutral (for overnight)	0.09

Table 3: Peak purity values for specificity

Peak purity				
Stress condition	**Glycyrrhetinic acid (at 210nm)**		**Forskolin (at 210nm)**	
	Peak front	**Peak tail**	**Peak front**	**Peak tail**
Initial	998.8	999.7	998.8	997.6
Base	997.1	996.0	996.2	997.1
Acid	991.6	993.2	994.2	995.1
Oxidative	996.4	998.4	995.8	994.2
Heat dry	995.8	997.4	994.9	997.3
Photo stability UV, 200 watt hrs/square meter	997.5	996.1	993.1	996.0
Florescence , 1.2 million Lux. Hrs	996.4	996.7	995.7	997.2
Neutral	998.3	998.8	997.9	996.4

Table 4: Recovery studies

Drug	**Amount added per 1 gm of gel base (mg)**	**Total amount found (µg/ml)**	**% Recovery (µg/ml)**
Forskolin	8	23.96	99.86
	10	30.18	100.61
	12	35.58	98.85
Glycyrrhetinic acid	8	23.93	99.74
	10	29.59	100.31
	12	35.71	99.21

Table 5: Results of Robustness Study Glycyrrhetinic acid and Forskolin

Sr. no.	**Parameters**	**Variation**	**Glycyrrhetinic acid (%RSD)**	**Forskolin (%RSD)**
1.	Flow rate (1ml/min)	0.8ml/min	1.24	0.24
		1.2ml/min	0.94	0.40
2.	Mobile phase (50:50::ACN:WATER) pH 3	pH 2.8	1.99	0.52
		pH 3.2	1.54	1.02
3.	Mobile phase (50:50::ACN:WATER)	48:52	0.86	0.62
		53:47	1.64	0.72

Table 6: The summary of validation parameters

Sr. no.	Validation parameters	Glycyrrhetinic acid	Forskolin
1.	Linearity Equation (R^2) Range	y=4035.5x+77814 R^2=0.9973	y=2859.3x+68342 R^2=0.9982
2.	Precision (% RSD) Interday Intraday	 0.82 0.63	 1.06 0.74
3.	Assay	% assay- 99.96 % RSD- 0.89	% assay- 101.56 % RSD- 1.17
4.	Accuracy	% recovery	
	80% Level	99.74%	99.86%
	100%Level	100.31%	100.61%
	120%Level	99.21%	98.85%
5.	LOD	0.75 µg/ml	1.04 µg/ml
6.	LOQ	2.30 µg/ml	3.16 µg/ml
7.	Specificity	Specific	Specific
8.	Robustness	robust	robust

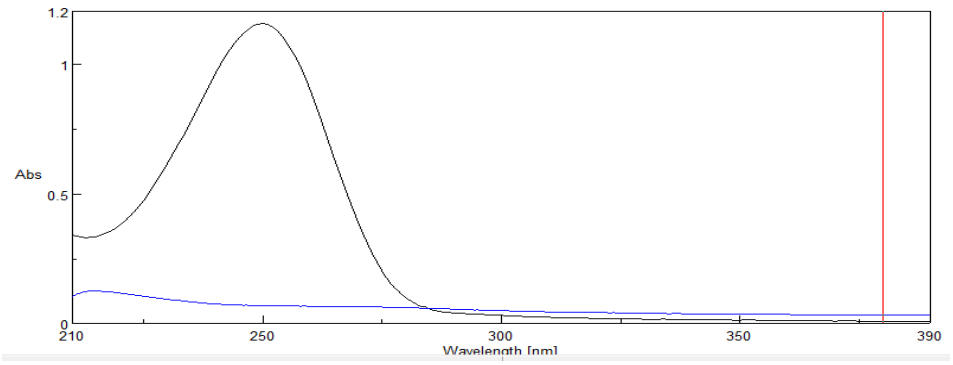


Fig.1: UV Spectra of Glycyrrhetinic acid at 254nm and Forskolin at 210nm

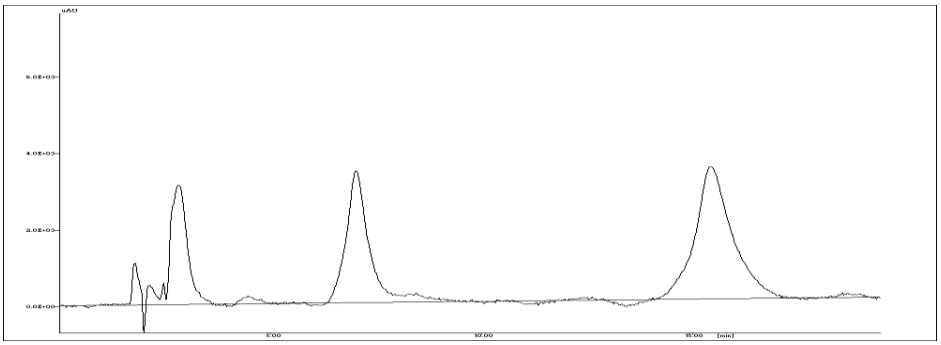
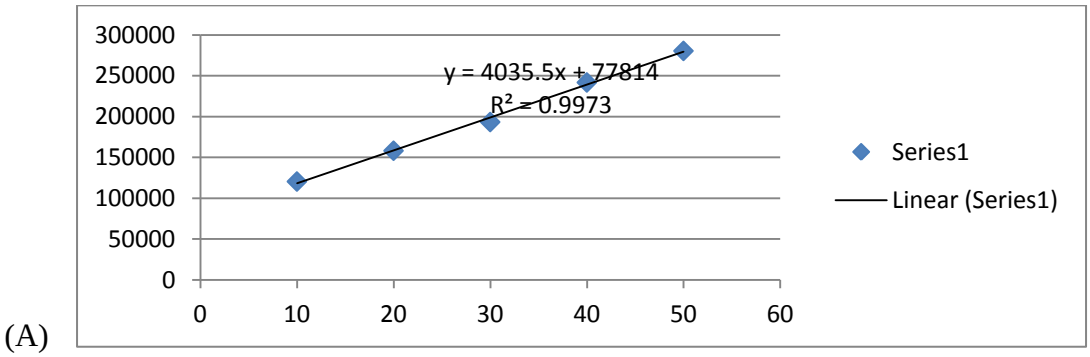
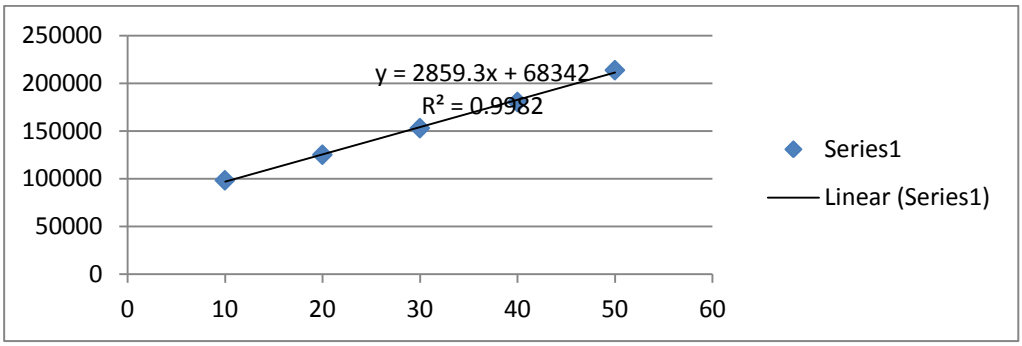


Fig. 2: Representative Chromatogram of standard solution of Glycyrrhetinic acid at 15.4 ± 0.2 min and Forskolin 7.0 ± 0.2 min.



(A)



(B)

Fig. 3: calibration curve for linearity of Glycyrrhetinic acid(A) and Forskolin(B) 10-50 (µg/ml)

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