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Research Article

Quantitative estimation of piperine, 18- beta glycyrrhetinic acid and 6- gingerol from Suryacid tablet formulation by HPTLC method

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ABSTRACT

A high-performance thin-layer chromatographic (HPTLC) method has been established for simultaneous analysis of piperine, 18- beta glycyrrhetinic acid and 6- gingerol in ayurvedic proprietary formulation Suryacid tablet. TLC was performed on aluminum foil-backed silica gel 60F $_{254}$ plates with toluene: ethyl acetate: glacial acetic acid (7:3:0.1) as mobile phase. The plates were scanned and the compounds were quantified at their wavelengths of maximum absorption at 340 nm for piperine at Rf 0.48, 254nm for 18 beta- glycyrrhetinic acid at Rf 0.26 and 284nm for 6- gingerol at Rf 0.43 respectively. Calibration plots for all three standards were linear in the range of 0.2 µg/spot – 1.0 µg/spot. The polynomial regression data for the calibration plots were indicative of good relationships with r^2 = 0.997, r^2 = 0.991, r^2 = 0.991 for piperine, 18 beta- glycyrrhetinic acid and 6- gingerol respectively. Average recovery was 95.88% for piperine, 96.72% for 18 beta- glycyrrhetinic acid and 96.91% for 6- gingerol. The method enabled simple, sensitive, precise, accurate, and specific analysis of all the three marker compounds in the formulation.

Keywords: Piperine; 18- beta glycyrrhetinic acid; 6- gingerol; ayurvedic proprietary formulation; Simultaneous estimation

INTRODUCTION

Standardization of herbal formulations is essential in order to assess the quality of drug, based on the concentration of their active principles. The growing use of botanicals by the public is forcing scientists to evaluate the health claims of these agents and to develop standards of quality and manufacture. Chromatographic fingerprint have been suggested to check for authenticity or provide quality control of herbal medicine (Biresh S., 2011). Quantitative estimation of chemical markers of each ingredient in the polyherbal preparation required ideal separation technique by which these markers are separated with highest purity and with least inferences from each other. For botanicals and herbal preparations, there is a requirement for scientific proof and clinical validation with chemical standardization, biological assays, animal models and clinical trials (Vishvnath G *et al.,* 2011).

Suryacid tablet is an ayurvedic proprietary medicine mainly used for the treatment of hyperacidity and nonulcer dyspepsia. The Suryacid tablet has 13 ingredients; Amla, Mulethi, Nishoth, Harad, Lavang, Nagarmotha, Pippali, Kali mirch, Sonth, Baheda, Elachi small, Tejpatra, Vidanga. For the present study Suryacid tablet markers selection, were based on the active constituents and their pharmacological activity. Piperine active constituent of pippali and marica,18- beta glycyrrhetinic acid of liquorice and 6- gingerol active constituent of ginger were majorly responsible for anti ulcer activity of tablet.

Literature reveals various methods for the identification and quantification of piperine, 18- beta glycyrrhetinic acid and 6- gingerol alone in herbal raw material. Methods so far reported for, combined estimation are, simultaneous estimation of glycyrrhetinic acid and apigenin using ethyl acetate: Ethanol: Water: Ammonia as a solvent system (Permender R., 2011). Some of other analytical techniques like HPLC and second order UV spectroscopy have been documented for 18- beta glycyrrhetinic acid (S.B. Song *et al.*, 1990). A number of literature also suggest High-performance liquid chromatography (HPLC) analysis of piperine (S.C. Jain *et al.*, 1988) and estimation using toluene and methanol solvent system (Ganesh T. *et al.*, 2009). HPTLC method was developed to determine the quantity of 6-gingerol using *n*-hexane and diethyl ether as a solvent system (R. Sujay, 2006). However, the literature does not reveal any method for quantification of piperine, 18- beta glycyrrhetinic acid and 6- gingerol. The current work is an attempt to develop a validated, simple, rapid and precise chromatographic method for simultaneous extraction and quantitative determination of piperine, 18- beta glycyrrhetinic acid and 6- gingerol simul-

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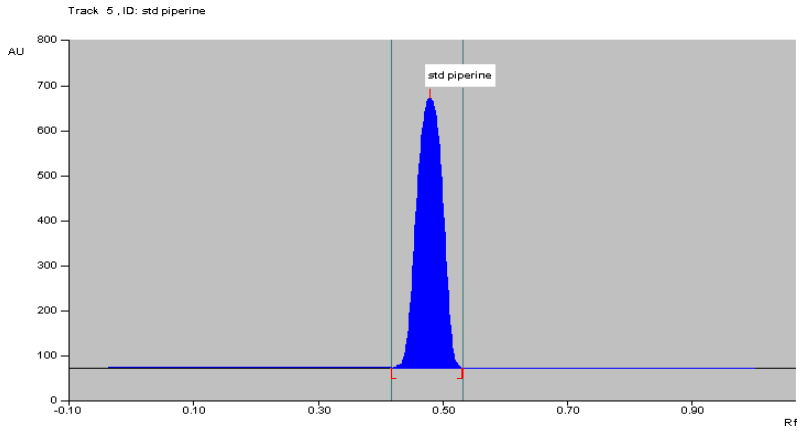

Figure 1: HPTLC Chromatogram of standard piperine at 340nm

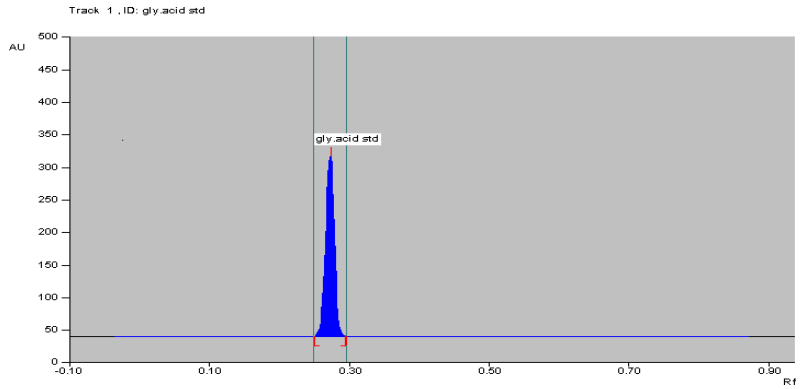

Figure 2: HPTLC Chromatogram of standard 18 beta- glycyrrhetinic acid at 254nm

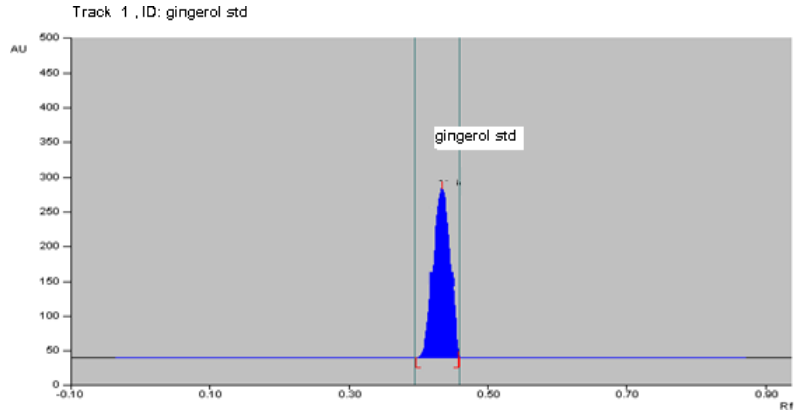

Figure 3: HPTLC Chromatogram of standard 6- gingerol at 284nm

taneously from their crude powder and polyherbal formulation Suryacid tablet.

MATERIALS AND METHODES

Reagents, chemicals and plant marterials

Standards 18 beta- Glycyrrhetinic acid (97%, CAS 471-53-4) and 6- Gingerol (98%, G 1046) were purchased from Sigma-Aldrich, of Spain. Piperine was extracted and isolated from the fruits of *Piper nigrum* using dichloromethane. It was purified by preparative TLC and its identity was confirmed by comparison of its melting point, U.V., NMR and IR spectra with the standard data given in literature (Epstein W *et al.,* 1993). This piperine was used as a reference standard in HPTLC analysis. Analytical grade reagents: toluene, ethyl acetate, glacial acetic acid and methanol (Merck Chemicals, India) were used. Liquorice, Marica, Pippali and Ginger were purchased from the local crude drug market. The authenticity of these crude drugs was estimated by comparison of its morphological characteristics to those given in the literature (Kokate CK *et al* 2005). Polyherbal formulation Suryacid was purchased from local market.

Chromatographic Conditions

HPTLC analysis was performed on aluminium-backed silica gel 60, GF254 plates (10 cm x 10 cm, with 0.2 mm thickness, E. Merck, Germany) with a Camag Linomat V. Samples are applied as 6 mm bands by use of a camag (Switzerland) linomat V sample applicator fitted with a microliter syringe. Liner ascending development of the plates to a distance of 80 mm was performed in

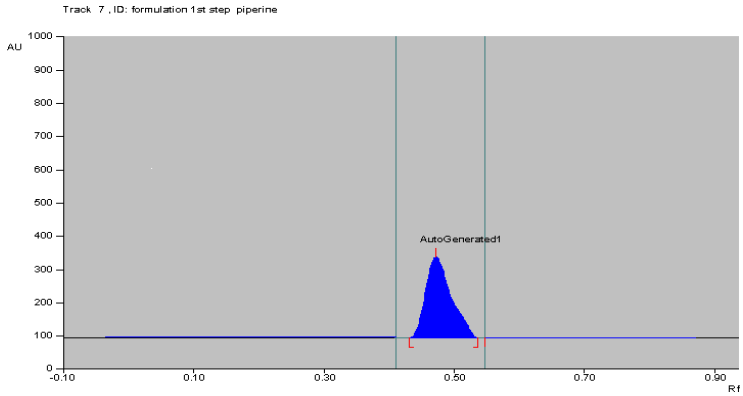

Figure 4: HPTLC chromatogram of M1 formulation for piperine at 340nm

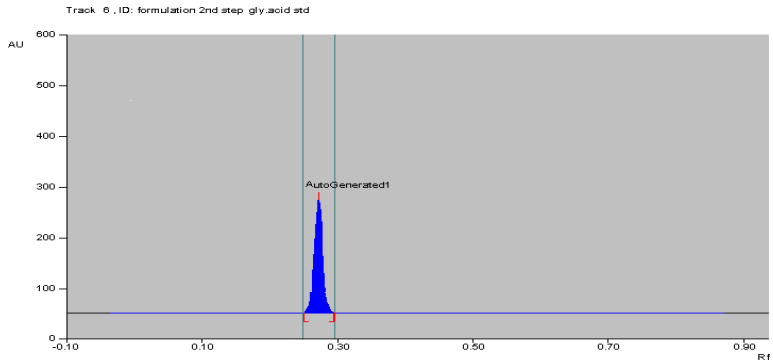

Figure 5: HPTLC chromatogram of M1 formulation for 18 beta- glycyrrhetinic acid at 254 nm

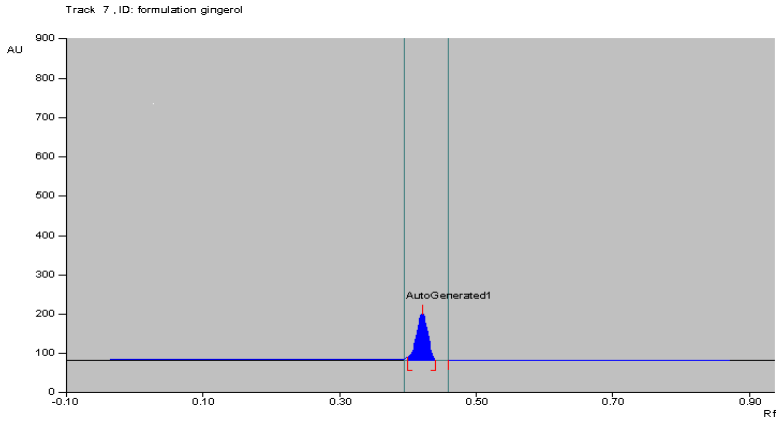

Figure 6: HPTLC chromatogram of M1 formulation for 6- gingerol at 284 nm

the solvent system Toluene: ethyl acetate: glacial acetic acid (7:3:0.1 v/v.) in a twin-trough glass chamber previously saturated with mobile phase vapor for 10 min. A constant application rate of 150 nL s^{-1} was employed. After development plate was scanned using Camag TLC scanner 3 and winCATS software 1.4.4.6337 with a slit dimension of 5x0.45 nm and scan speed of 200 mm/s. A deuterium lamp was used as source of radiation and wave length used was 340 nm for piperine, 254 nm for 18 beta- glycyrrhetinic acid and 284 nm for 6- gingerol. .

Preparation of standard stock solutions

A stock solution of piperine, 18 beta- glycyrrhetinic acid and 6- gingerol (1 mg/ml) was prepared by dissolving 10 mg of accurately weighed respective standards in 10 ml methanol in volumetric flask. 5 ml of this stock solution was further diluted to 50 ml with methanol to give a test solution of concentration 100µg/ml respectively. For calibration different concentrations of piperine (0.2-1.0 µg mL^{-1}), 18 beta- glycyrrhetinic acid (0.2-1.0 µg mL^{-1}), 6- gingerol (0.2-1.0 µg mL^{-1}) were prepared individually from respective stock solution (100µg/ml). 10 µl of each sample was applied on plate and developed as per the chromatographic conditions. After development of plates, peak area and concentration data were treated by linear regression analysis.

Preparation of sample solutions

Test samples under study were: Pippali fruit powder (S1), Marica fruit powder (S2), Liquorice root powder (S3), Ginger root powder (S4), Marketed polyherbal formulation (M1).

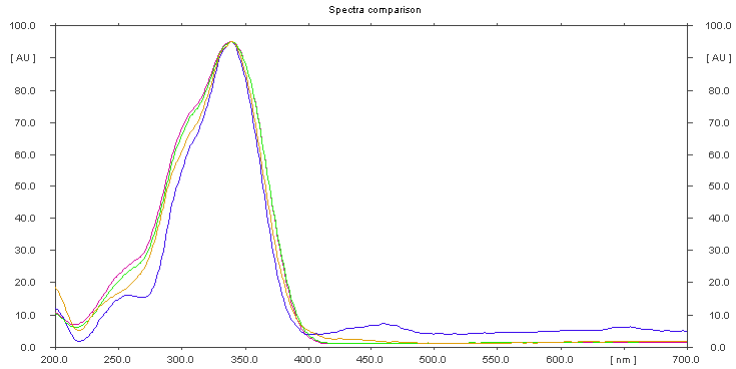

Figure 7: Spectral overlay of Standard piperine and extract of S1, S2 and M1 (λ max 340nm)

Brown: Std piperine, Green: Pippali fruit extract, Yellow: Marica fruit extract, Blue: Marketed formulation (M1)

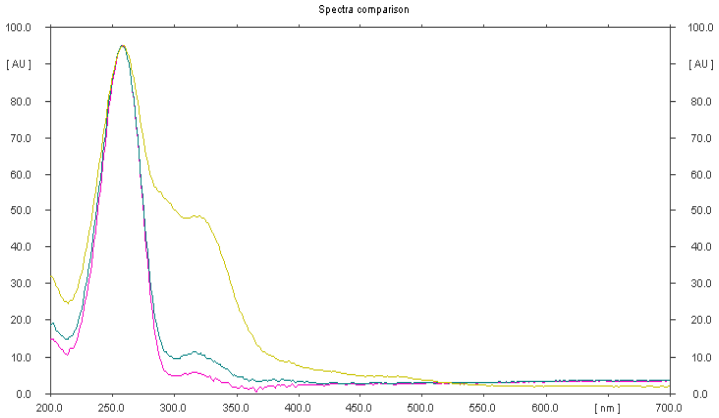

Figure 8: Spectral overlay of Standard 18 beta- glycyrrhetinic acid and extract of S3 and M1 (λ max 254nm)

Green: std 18 beta- glycyrrhetinic acid, Pink: liquorice root extract, Yellow: Marketed formulation (M1).

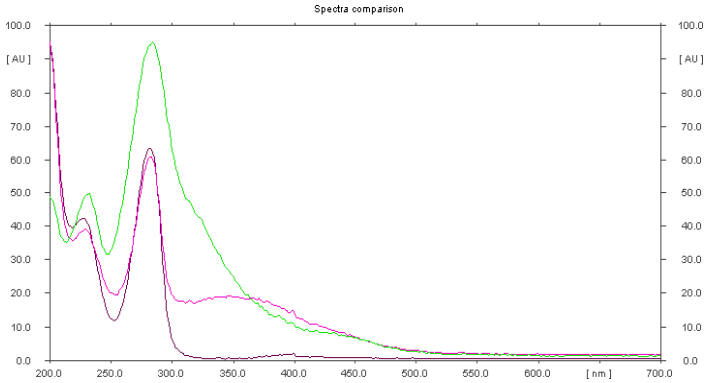

Figure 9: Spectral overlay of Standard 6- gingerol and extract of S4 and M1 (λ max 284nm)

Brown: 6- gingerol std, Pink: ginger extract, Green: M1 formulation

Extraction of biomarkers from crude drugs

For extraction of 6- gingerol from Ginger root and piperine from pippali fruit and marica fruit, about 1 gm of each powdered drug (S1,S2,S4) was separately refluxed with 60 ml methanol for one hour and filtered through Whatman filter paper. The residue was refluxed again with 40 ml fresh methanol for half hour. The extract was filtered. Both the filtrates were combined and evaporated to make final volume of 10ml. 1ml of this solution was diluted to 10ml with methanol. This was used as a test solution for determination of 6- gingerol content in Ginger root and piperine content in pippali fruit and marica fruit.

For the extraction of 18 beta - glycyrrhetinic acid from liquorice powder, 1 gm of the powder (S3) was macerated with chloroform for 30 minutes and filtered. The residue was refluxed with 0.5 M sulfuric acid for 2 hrs. This acidified solution was fractionated in chloroform (20 x 6) times. The combined chloroform extract was completely evaporated and the residue was dissolved in the solvents, methanol: chloroform (1: 1) and the volume was made up to 50 ml with the same solvent

Table 1: Precision studies for piperine, 18 beta- glycyrrhetinic acid, 6- gingerol

Sample	Amount (X) (µg)	Intraday (N=3)		Interday (N=3)	
		MEAN ± %RSD	SD	MEAN ± %RSD	SD
Piperine Standard	0.4	0.3996 ± 0.25	0.0010	0.4125± 0.60	0.0025
	0.6	0.5907± 0.23	0.0014	0.5844± 0.37	0.0022
	0.8	0.7925± 0.18	0.0015	0.8018± 0.12	0.0010
Pippali Fruit	2.6019	2.5836± 0.09	0.0024	2.6530± 0.07	0.0019
Marica Fruit	3.0926	3.0845± 0.07	0.0023	3.1048± 0.09	0.0029
M1 (for piperine)	0.2573	0.2459± 0.65	0.0016	0.2529± 0.83	0.0021
18 beta- glycyrrhetinc acid Standard	0.4	0.3982± 0.25	0.0010	0.3922± 0.48	0.0019
	0.6	0.5980± 0.28	0.0017	0.5866± 0.32	0.0019
	0.8	0.7972± 0.27	0.0022	0.7845± 0.29	0.0023
Liquorice root	0.2153	0.2167± 0.96	0.0021	0.2060± 1.21	0.0025
M1(for 18 beta glycyrrhetinc acid)	0.1700	0.1721± 0.98	0.0017	0.1715± 0.75	0.0013
6- gingerol Standard	0.4	0.4132± 0.62	0.0026	0.4047± 0.74	0.0030
	0.6	0.6223± 0.32	0.0020	0.6122± 0.44	0.0027
	0.8	0.8042± 0.36	0.0029	0.7931± 0.26	0.0021
Ginger root	2.2874	2.2950± 0.16	0.0038	2.2868± 0.08	0.0019
M1 (for 6- gingerol)	0.1089	0.1091± 1.28	0.0014	0.1077 ± 0.92	0.001

combination. This solution was used for estimation of 18 beta - glycyrrhetinic acid.

Extraction of biomarkers from formulation

Six tablets of marketed polyherbal formulation (M1) was triturated in clean motor and refluxed with 50 ml of methanol for 30 minutes and filtered using Whatman filter paper. The marc was refluxed once again with 50 ml methanol for complete extraction of piperine and 6- gingerol. The refluxed solution was filtered and the combined filtrate were evaporated to make final volume of 10ml. 1ml of this solution was diluted to 10ml with methanol. This was used as a test solution for determination of piperine and 6- gingerol in marketed formulation. The residue remained after the second reflux was macerated with chloroform for 30 minutes. The chloroform was filtered and the marc refluxed with 0.5 M sulfuric acid for 2 hrs. for complete hydrolysis of glycoside. The acidified solution was extracted with chloroform (20x6) times, the chloroform extract was completely evaporated and the residue was dissolved in solvents methanol: chloroform (1: 1) and the volume made upto 50 ml with the same solvents. This was further used for estimation of 18 beta-glycyrrhetinic acid in formulation.

The test samples (S1, S2, S3, S4, M1) prepared as mentioned above were applied as bands and developed as per chromatographic conditions. The plate was scanned at 340 nm for piperine, 254 nm for 18 beta-glycyrrhetinic acid and 284 nm for 6- gingerol. The area of peak that corresponds with the Rf of standards was recorded and amount present in the test solution was calculated from regression equation obtained from the calibration plot.

Method validation

The linearity of the method for piperine, 18- beta glycyrrhetinic acid and 6- gingerol was checked between 0.2 µg - 1µg per spot and concentration was plotted again peak area. Instrumental precision was checked by repeated scanning of the same spot of piperine (0.6µg/spot), 18 beta- glycyrrhetinic acid (0.8µg/spot) and 6- gingerol (1µg/spot) seven times each. Intraday and interday variation were determined by analysing three different concentrations of the standards and test solution on the same day and also by analysing it over a period of 3 days. The analysis was carried out seven times, and all the results were expressed as mean ± % RSD (relative standard deviation). Accuracy as recovery studies were carried out using standard addition method at three different level, preanalyzed samples of the formulations were each spiked with extra piperine standard (0, 12.86, 25.73, 51.46 µg) and the mixtures were reanalyzed. A second set of formulations was spiked with extra 18- beta glycyrrhetinic acid standard (0, 8.5, 17,34) similarly, the last set of formulation was spiked with extra 6- gingerol (0, 5.44, 10.89, 21.78) and were reanalyzed. Limits of detection (LOD) and quantiication (LOQ) were determined by the standard deviation (SD) method from the slope (S) of the calibration plot and the SD of a blank sample, by use of the equations LOD = 3.3 × SD/S and LOQ =10 × SD/S.

RESULTS AND DISCUSSION

Method Development

The mobile phase composition was optimized to established a suitable and accurate densitometric HPTLC method for analysis of piperine, 18- beta glycyrrhetinic acid and 6- gingerol. The solvent system of Toluene: ethyl acetate: gla.acetic acid (7:3:0.1 v/v) resulted in a sharp, symmetrical and well resolved peak at Rf 0.48 for piperine under 340 nm (Fig.no.1), Rf 0.26 for 18-beta glycyrrhetinic acid at 254 nm (Fig.no.2), Rf 0.43 for 6- gingerol at 284 nm (Fig.no.3).

Table 2: Recovery study of piperine, 18 beta glycyrrhetinic acid and 6- gingerol in Suryacid(M1)

Sample	Initial amount (µg/ml)	Externally added piperine (µg)	Recovered piperine (µg)	% recovery	SD (n=3)	Average % recovery
Piperine in M1	25.73	--	--	--	0.0014	95.88%
	25.73	12.86	11.93	92.76%	0.0028	
	25.73	25.73	24.61	95.64%	0.0021	
	25.73	51.46	51.07	99.24%	0.0029	
18 beta glycyrrhetinic acid in M1	17.00	--	--	--	0.0011	96.72%
	17.00	8.50	7.94	93.41%	0.0026	
	17.00	17.00	16.82	98.94%	0.0024	
	17.00	34	33.26	97.82%	0.0019	
6- gingerol in M1	10.89	--	--	--	0.0012	96.91%
	10.89	5.44	5.27	96.87%	0.0027	
	10.89	10.89	10.64	97.70%	0.0018	
	10.89	21.78	20.95	96.18%	0.0023	

Calibration

The calibration plots were linear in the range 0.2 µg - 1µg per spot. The correlation coefficient and linear regression equation for piperine was r2=0.997 and Y=11808x+776.6, for 18 beta- glycyrrhetinic acid r2=0.991 and Y=3591x+918.8 for 6- gingerol r2=0.994 and Y=559.78x+881.63 respectively.

Validation

Instrumental precision The method was validated in terms of instrument precision gave a mean ± % RSD of 0.5997% for piperine, 0.8123% for 18 beta glycyrrhetinic acid and 1.0249% for 6- gingerol. The repeatability of the method was confirmed by analysis of seven spot of standards piperine (0.6µg/spot), 18 beta- glycyrrhetinic acid (0.8µg/spot) and 6- gingerol (1µg/spot) which gave the % RSD of 0.41% for piperine, 0.29% for 18 beta glycyrrhetinic acid and 0.27 %for 6- gingerol.

Intra-day and inter-day precision

Results from determination of interday and intraday precision for all three standards were expressed % RSD, as shown in (Table no.1). % RSD lower than 2 indicated that the method was précised.

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

The LOD and LOQ was found to be 0.01243 µg and 0.03767 µg respectively for piperine and 0.01132 µg and 0.03430 µg respectively for 18 beta glycyrrhetinic acid , and 0.001274 µg and 0.00386 µg for 6- gingerol respectively for which indicate adequate sensitivity of the method.

Accuracy (Recovery studies)

The average % recovery of piperine, 18 beta glycyrrhetinic acid and 6- gingerol in Suryacid (M1) marketed formulation were found to be 95.88%, 96.72%, 96.91% respectively which are satisfactory. (Table no.2).

Quantitative estimation

The solvent system of Toluene: ethyl acetate: gla. acetic acid (7:3:0.1 v/v) was found to be ideal mobile phase for separation of piperine, 18- beta glycyrrhetinic acid and 6- gingerol. Standard piperine shows Rf 0.48 at 340nm, 18 beta- glycyrrhetinic acid shows Rf 0.26 at 254nm and 6- gingerol shows Rf 0.43 at 284nm (Figure 1,2,3) same Rf was observed in the test samples S1, S2, S3, S4 and M1. (Figure 4,5,6,). There was no interference from other components present in the samples. The content of markers obtained from the calibration curve was for piperine in pippali fruit 2.60 % w/w, marica fruit 7.7 %w/w, 18 beta - glycyrrhetinic acid in liquorice was 0.10%w/w and 6- gingerol in ginger root 2.28%w/w. Based on theoretical calculation, as per the formula of Suryacid tablet the amount of markers in formulation should be, piperine 0.05%w/w, 18 beta-glycyrrhetinic acid 0.014%w/w and 6- gingerol in formulation should be 0.013%w/w. The variation in content of markers may be due to factors like drug variety, geographic variation, and age of the plant at the time of harvest, genetic and environmental factors.

CONCLUSION

The proposed HPTLC method for simultaneous estimation of piperine, 18 beta glycyrrhetinic acid and 6- gingerol in Suryacid polyherbal formulation is quite simple, accurate, precise, reproducible, sensitive and less time consuming. The validation procedure confirms that this is an appropriate method for their quantification in the plant material and formulation. So, it can definitely be used in routine quality control of the raw materials as well as formulations containing any or all of these compounds.

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