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SIMULTANEOUS ESTIMATION OF 11-KETO-β-BOSWELLIC ACID AND WITHAFERIN-A BY USING HPTLC METHOD FROM PAINQUIT TABLET

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ABSTRACT

Background: Herbal medicines are widely used for the management of pain and inflammation. Most of the herbal formulations present in the market are not standardized with respect to its active ingredients marker molecule. Siddhayu Ayurvedic Research Foundation Pvt. Ltd. has developed and validated Painquit Tablet to be used for pain reliving, inflammation, mobility, flexibility and strength. **Objective:** The present study was designed with an objective of simultaneous estimation of 11-Keto-β-Boswellic acid and Withaferin A by HPTLC from Tablet containing *Boswellia serrata* and *Withania somnifera*. **Methods:** High performance Thin Layer Chromatography (HPTLC) method was developed and validated for simultaneous estimation of 11-Keto-β-Boswellic acid (KBA) and Withaferin A (WA) from Painquit Tablet. Chromatographic separation was achieved on precoated silica gel HPTLC aluminium plate 60 F_{254} using Chloroform: Ethyl Acetate: Formic acid, 7.0:3.0:1.0 (v/v/v) as mobile phase followed by densito-metric measurement at two different wavelength 258 nm and 227 nm by TLC Scanner 4 (CAMAG).The method was validated according to International Conference on Harmonization guidelines. **Results:** The retention factors of KBA acid and WA were 0.58 and 0.32 respectively. The estimated amount of KBA and WA was found to be 0.15 % and 0.09 %. Results obtained in validation parameters indicate the accuracy and reliability of the developed simultaneous HPTLC method for the quantification of KBA acid and WA in Painquit Tablet. **Conclusion:** The proposed method found to be simple, sensitive, selective and accurate for routinely used in Quality Control Lab.

KEYWORDS: 11-Keto-β-Boswellic acid, Withaferin A, Painquit Tablet, HPTLC, pain reliving, inflammation.

INTRODUCTION

Joint pain was considered to be disorder affected by aged people in the past but the scenario has changed in the recent. The young adults in their 20s to 40s are increasingly falling prey to joint-related ailments and are known to suffer from acute pain in their joints, especially in shoulder, elbow, wrist, knee, ankle, feet and toe joints. Joint pain is now slowly taking the shape of an epidemic, resulting in impaired mobility and flexibility in youngsters and middle-age groups.[1]

Non-steroidal anti-inflammatory drugs (NSAIDs) are medicines that are widely used to relieve pain and reduce inflammation. But they are not suitable for everyone and can sometimes cause troublesome side effects.[2]

Complementary and alternative medicines such as herbal medicines are widely used for treating pain and inflammation which are suppose to be effective and safe alternative option for replacement of synthetic drug.[3,4]

Shodhit Salai Guggul consists of exudate of *Boswellia serrata* Roxb. (Fam. Burseraceae), a moderate sized, deciduous tree, upto 18 m in height and upto 2.4 m in girth, commonly found in the dry forests from Punjab to West Bengal and in peninsular India.[5] The resinous part of *Boswellia serrata* possesses monoterpenes, diterpenes, triterpenes, tetracyclic triterpenic acids and four major pentacyclic triterpenic acids i.e. β-boswellic acid, acetyl-β-boswellic acid, 11-keto-β-boswellic acid and acetyl-11-keto-β-boswellic acid, responsible for inhibition of pro-inflammatory enzymes.[6] A study result showed that *Boswellia serrata* Extract Containing 30% 3-Acetyl-11-Keto-Boswellic Acid Attenuates Inflammatory Mediators and Preserves Extracellular Matrix in Collagen-Induced Arthritis.[7]

Ashwagandha consists of dried mature roots of *Withania somnifera* Dunal. (Fam. Solanaceae), a perennial shrub, found in waste land, cultivated field and open grounds throughout India.[8] Various Studies has discovered that ashwagandha can potentially protect against skin

inflammation and possessed marked anti-inflammatory effect.[9,10]

Literature review revealed that various methods have been reported for analysis of 11-Keto-β-Boswellic acid (KBA) and Withaferin A (WA) in various herbal formulations by High Performance Liquid Chromatography (HPLC),[11-15] High Performance Thin Layer Chromatography (HPTLC).[16-19] The developed HPTLC method has several advantages over other available methods such as ability to analyze several samples simultaneously in parallel, as well as using small quantities of solvents as a mobile phase which reduces time and cost of analysis.

Siddhayu Ayurvedic Research Foundation Pvt. Ltd. has developed Painquit Antidiabetic Tablet and standardized with respect to its active ingredients marker molecule. The proposed analytical method is also validated as per the International Conference on Harmonization (ICH) guidelines Q2 (R1).[20]

In the present study, an attempt has been made to develop a simple, rapid, accurate, precise and cost effective HPTLC method which can be routinely used in Quality Control Lab for simultaneous estimation of 11-Keto-β-Boswellic acid (KBA) and Withaferin A (WA) in Painquit Tablet.

MATERIALS AND METHODS

Ingredients used in Painquit Tablet

Painquit Tablet was formulated using extracts of Sallaki Guggul (*Boswellia serrata*), Ashwagandha Extract (*Withania somnifera*), Rasna Extract (*Pluchea lanceolata*), Chopchini Extract (*Smilax china*), Haridra Extract (*Curcuma longa*), Pipali Extract (Piper nigrum), powder of Sonth (*Zingiber officinale*) and bhawana of Nirgundi Kwath (*Vitex negundo*), Prasarini Kwath (*Paederia foetida*). All these raw materials were screened for identity, purity and strength before formulation of tablet.

Table 1: Instrumentation details.

Instruments	Specification
HPTLC instrument	Camag, Switzerland
Sample applicator	Camag Linomat 5
Detection by	Camag TLC scanner 4
Visualizer	TLC Visualizer
Heating by	TLC Plate Heater III
Spray Cabinet	TLC Spray Cabinet II
Syringe	Hamilton (100 µl)
Software	winCATS (ver.1.4.9)
TLC Plates	Pre-coated Silica Gel 60 F_{254} TLC Plate

Reference standards

Reference standard 11-Keto-β-Boswellic acid (99.2%) and Withaferin A (98.0%) were purchased from Natural Remedies, Bangalore, India.

Chemicals and Reagents

All chemicals used throughout this work were of analytical grade or HPLC grade purchased from Merck Chemicals, India. Stationary phase was pre-coated silica gel aluminium plate 60 F_{254} was obtained from Merck, Germany.

Standard solution

The standard solution was prepared by weighing 1.2 mg of the reference standard 11-Keto-β-Boswellic acid (KBA) and then transferred to 10 ml volumetric flask and volume was adjusted with HPLC grade methanol. Also the reference standard solution of Withaferin A (WA) was prepared by dissolving 1.3 mg in 10 ml volumetric flask containing HPLC grade methanol.

Sample preparation

The contents of twenty tablets were grounded to a fine powder. An amount of 1.0 g powder was transferred to 10 ml volumetric flask containing 8 ml HPLC grade methanol and sonicated for 15 min. The solution was diluted up to the mark with HPLC grade methanol and filtered through Whatman filter paper. The resulting solution was used for the study.

Chromatographic condition

The standard and sample solutions were spotted in the form of bands, width 6 mm with a Camag 100 microliter syringe (Hamilton, Bonaduz, Switzerland) in a controlled nitrogen stream on silica gel pre-coated aluminium plate 60 F_{254} plates, using a Camag Linomat V (Switzerland) sample applicator. The plates were prewashed with methanol and activated at 110˚C for 5 min prior to chromatography. The slit dimension was kept at 5 mm × 0.45 mm and the scanning speed was 10 mm/s. The mobile phase was Chloroform: Ethyl acetate: Formic acid (7.0: 3.0: 1.0 v/v/v). Linear ascending development was carried out in a 20 cm × 10 cm twin trough glass chamber (Camag, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 20 min at room temperature (25˚C ± 2) at relative humidity 33%, saturated with $MgCl_2$. The length of each chromatogram run was 7 cm. Following the development, the TLC plates were dried in a current of air with the help of a TLC plate heater. Densitometric scanning was performed using a Camag TLC scanner 4 in the reflectance/absorbance mode at two different

wavelengths 258 nm and 227 nm. Evaluation was done using linear regression analysis through peak areas. The amount of KBA and WA were computed from peak areas. The chromatograms are shown in Figure 2-4.

Sample assay

Sample and standard solution were spotted in triplicates on a plate, developed under the same conditions. After development and drying of the plates, the analyte was found to be completely separated from other components; hence, the linear and compact zones were scanned at two different wavelengths 258 nm and 227 nm and peak areas KBA and WA were recorded.

Validation of the method

The proposed analytical method was validated for the parameters like linearity, specificity, accuracy, precision, limit of detection, limit of quantification, and robustness as per the International Conference on Harmonization (ICH) guidelines Q2 (R1).

RESULTS AND DISCUSSION

The mixtures of several mobile phases were tried on silica gel TLC plates for simultaneous estimation of KBA and WA. A mobile phase consisting Chloroform: Ethyl acetate: Formic acid (7.0: 3.0: 1.0 v/v/v) gave good separation. Standard KBA (*R*f = 0.58) and WA (*R*f = 0.32) mentioned in Table 2, showed single peak in HPTLC chromatogram in Figures 2, 5 and HPTLC chromatogram of Painquit Tablet in Figures 3,6.

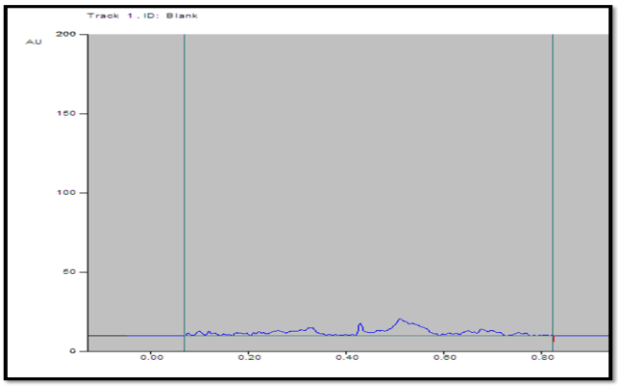

Figure 1: HPTLC chromatogram of blank solution.

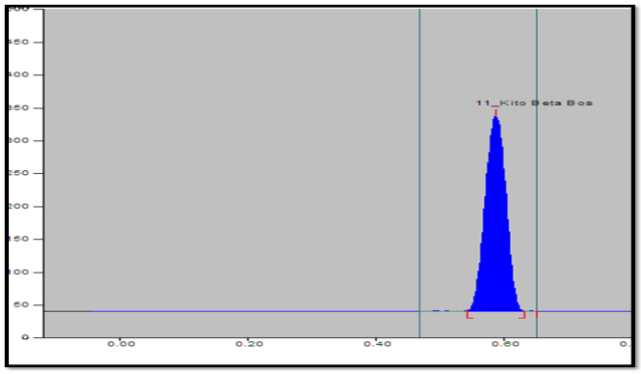

Figure 2: HPTLC chromatogram of standard 11-Keto-β-Boswellic acid (KBA) solution.

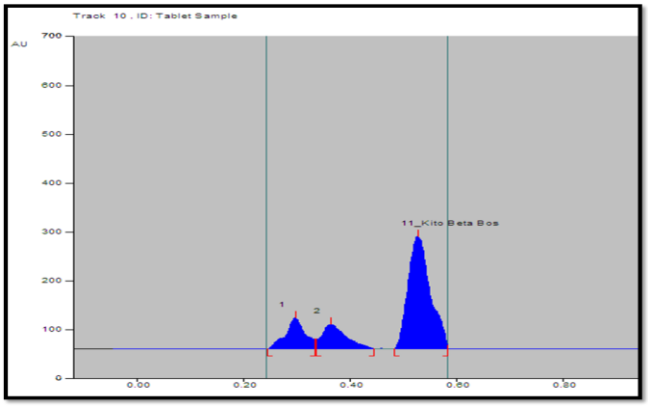

Figure 3: HPTLC chromatogram of Painquit Tablet solution.

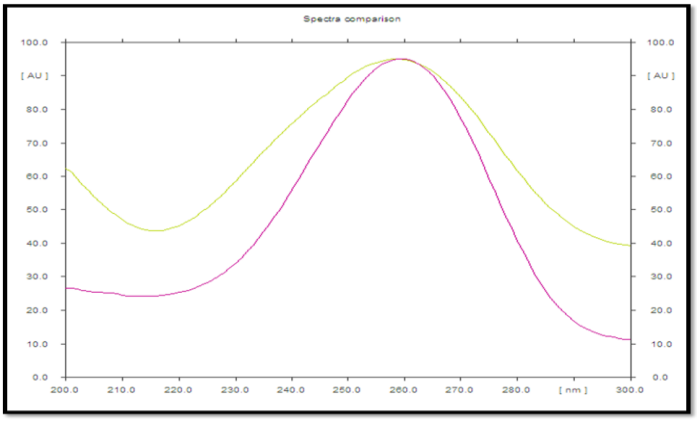

Figure 4: Overlain spectrum of standard 11-Keto-β-Boswellic acid (KBA) solution and Painquit Tablet solution.

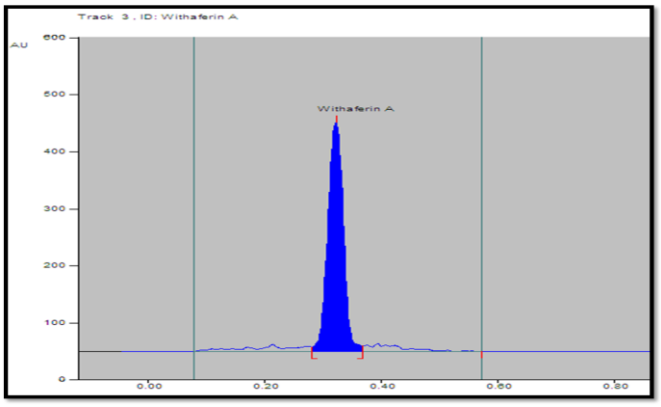

Figure 5: HPTLC chromatogram of standard Withafern A solution.

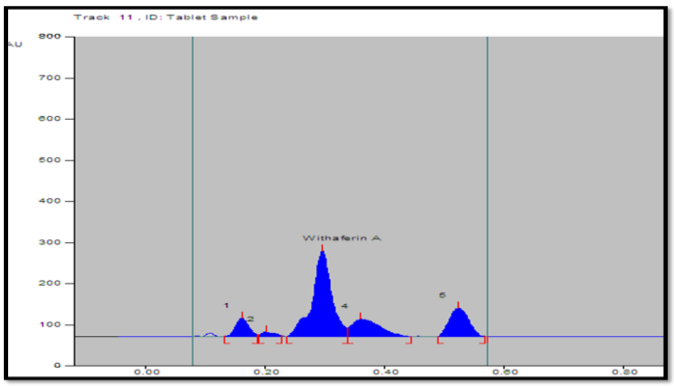

Figure 6: HPTLC chromatogram of painquit tablet solution.

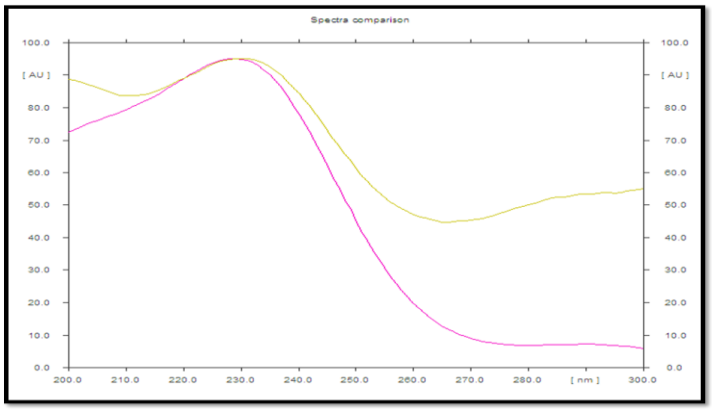

Figure 7: Overlain spectrum of standard Withaferin A (WA) solution and Painquit Tablet solution.

Linearity

Linearity was performed by applying standard solution of different concentrations of KBA and WA. Linearity curve was obtained by plotting a graph of peak area vs. applied concentration. Linear regression data for the calibration curves of standard KBA showed a good linear relationship over the concentration range of 300 - 2100 ng/band. The correlation coefficient (*R*2) was 0.998 and linear regression equation was found to be: Y = 3.413 x + 95.01, and WA showed a good linear relationship over the concentration range of 325 - 2275 ng/band. The correlation coefficient (*R*2) was 0.998 and linear regression equation was found to be: Y = 3.146 x + 84.71. All data were calculated and given in Table 2 and figure 8, 9.

Table 2: Linear regression data for calibration plots of the analyzed drugs using the proposed HPTLC methods.

Parameters	Results	
	11-Keto-β-Boswellic acid (KBA)	Withaferin A (WA)
Wavelength (nm)	258	227
Retention factor (*R*f)	0.58	0.32
Linearity range (ng/band)	300 - 2100	325 - 2275
Correlation coefficient (*r*2)	0.998	0.998
Slope	3.403	3.24
Intercept	95.01	84.71
LOD (ng/spot)	91.97	93.33
LOQ (ng/spot)	278.70	282.81

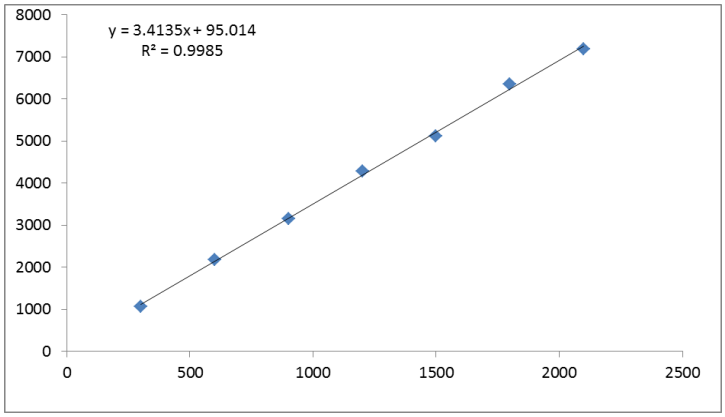

Figure 8: Calibration plot for standard 11-Keto-β-Boswellic acid (KBA) solution.

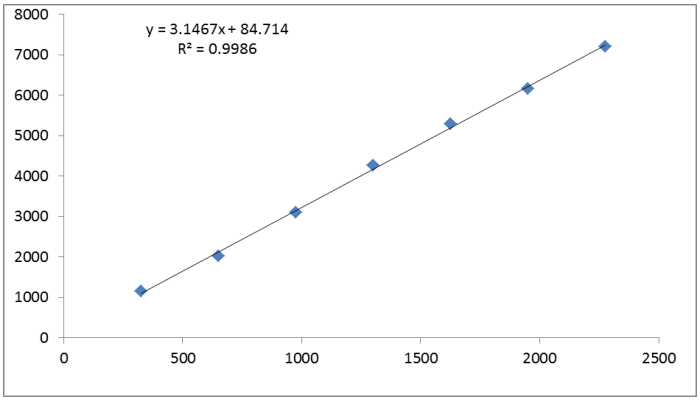

Figure 9: Calibration plot for standard Withaferin A (WA) solution.

Specificity

The specificity of method was ascertained by applying methanol as blank, standard solutions and test sample on TLC plate. The solutions were spotted on TLC plate in triplicate and run. The bands of KBA and WA in the samples were confirmed by comparing the R_f values and spectrum with that of standards. The chromatogram of the Painquit tablet obtained using the developed method showed peaks at R_f 0.58 and 0.32 for KBA and WA, respectively, and was found to be at the same R_f for both standard drugs chromatogram shown in figure 1-6.

Precision

The present method was validated for intraday and interday precision. The intra-day precision was assessed based on three replicates of three different concentration (900, 1200 and 1500 ng/band for KBA; 975, 1300 and 1625 ng/band for WA) on same day, and the measured

peak area is expressed in terms of the per cent relative standard deviation (% RSD). The inter-day precision study was performed on three different days using the aforementioned concentrations of both drugs in triplicate. The repeatability of the sample application and calculation of the peak area for the analyte were articulated in terms of the % relative standard deviation (RSD) which was found to be less than 2.0 in all cases indicate no significant variations. The results were illustrated in Table 3.

Table 3: Intra-day and inter-day precision of KBA and WA.

Analyte	Conc. (ng/band)	Avg. Peak Area	SD	% RSD
Intra-day Precision				
KBA	900	3163.87	14.65	0.46
	1200	4164.80	11.91	0.29
	1500	5042.40	42.97	0.85
WA	975	3113.87	45.66	1.47
	1300	4196.66	56.30	1.34
	1625	5145.40	80.51	1.56
Inter-day Precision				
KBA	900	3130.23	18.05	0.58
	1200	4128.53	19.90	0.48
	1500	5061.97	30.27	0.60
WA	975	3156.90	49.72	1.58
	1300	4202.87	53.46	1.27
	1625	5241.30	72.16	1.38

Accuracy

The accuracy of the methods was determined by calculating recoveries of KBA and WA by the standard addition method. Known amounts of standard solution of KBA (900, 1200, 1500 ng) and WA (975, 1300, and 1625 ng) were added to pre-quantified sample solutions. Analysis was performed in triplicates and the average percentage recovery at each concentration level was evaluated. The results are illustrated in Table 4. The amount of drug recovered in accuracy study indicates that the method is accurate.

Table 4: Recovery Study of KBA and WA.

Compound	Amount Found (ng)	Amount of Std. Added (ng)	Theoretical amount of standard found (ng)	Amount of standard Recovered (ng)	Recovery (%)	Average Recovery (%)
KBA	1504.36	900	2404.36	2397.38	99.71	99.34
	1504.36	900	2404.36	2387.69	99.31	
	1504.36	900	2404.36	2380.19	98.99	
	1504.36	1200	2704.36	2691.65	99.53	99.40
	1504.36	1200	2704.36	2681.13	99.14	
	1504.36	1200	2704.36	2691.41	99.52	
	1504.36	1500	3004.36	2979.72	99.18	99.22
	1504.36	1500	3004.36	2968.70	98.81	
	1504.36	1500	3004.36	2994.28	99.66	
WA	870.35	975.00	1845.35	1829.11	99.12	98.83
	870.35	975.00	1845.35	1816.18	98.42	
	870.35	975.00	1845.35	1825.78	98.94	
	870.35	1300.00	2170.35	2154.94	99.29	99.17
	870.35	1300.00	2170.35	2150.06	99.07	
	870.35	1300.00	2170.35	2152.25	99.17	
	870.35	1625.00	2495.35	2474.88	99.18	99.28
	870.35	1625.00	2495.35	2476.28	99.24	
	870.35	1625.00	2495.35	2481.24	99.43	

Limit of detection (LOD) and Limit of quantification (LOQ)

The LOD and LOQ were calculated by using the values of slopes and intercepts of the calibration curve. The limit of detection (LOD) values for KBA and WA were 91.97 and 93.33 ng, respectively, and the limit of quantification (LOQ) values were 278.70 and 282.81 ng, respectively, which shows the adequate sensitivity of the method all results given in Table 2.

Robustness

The estimations were performed by introducing variations in the mobile phase distance development, saturation time in development chamber and change in mobile phase composition; the effects on the results were examined. Mobile phase development distance was changed by ±1 cm. The saturation time of mobile phase in the chamber was varied by ±1 min. The composition of Methanol in mobile phase was changed by ± 0.2 ml. The % RSD was found to be less than 1.0 in all cases indicates no significant variations in the analysis of KBA at the concentration 300 ng and for WA at the concentration of 300 ng. The summary of the robustness study is given in Table 5 and 6.

Table 5: Robustness evaluation of the method for KBA.

Std. Conc. (ng/band)	Robustness Parameter		Average Peak Area	SD	RSD (%)	Average RSD (%)
300	Development Distance (cm)	6	4134.77	14.26	0.34	0.44
		7	4146.87	16.35	0.39	
		8	4144.03	24.28	0.59	
	Saturation Time (minute)	19	4118.17	18.32	0.44	0.47
		20	4145.10	20.70	0.50	
		21	4173.03	19.68	0.47	
	Mobile Phase Composition (Change in Chloroform composition)	6.8	4074.93	21.26	0.52	0.55
		7.0	4145.77	18.17	0.44	
		7.2	4157.57	28.47	0.68	

Table 6: Robustness evaluation of the method for WA.

Std. Conc. (ng/band)	Robustness Parameter		Average Peak Area	SD	RSD (%)	Average RSD (%)
300	Development Distance (cm)	6	4107.10	53.69	1.31	1.20
		7	4016.87	51.58	1.28	
		8	4177.37	42.42	1.02	
	Saturation Time (minute)	19	4157.17	55.54	1.34	1.30
		20	4181.77	60.57	1.45	
		21	4139.70	45.65	1.10	
	Mobile Phase Composition (Change in Chloroform composition)	6.8	4141.60	37.80	0.91	1.50
		7.0	4082.10	79.51	1.95	
		7.2	4097.57	67.37	1.64	

Estimation of KBA and WA from Painquit Tablet

Estimation of KBA and WA was performed by the developed HPTLC method. The analysis was repeated in triplicate. Area was recorded. Percentage assay was determined from linearity equation for Painquit Tablet. The assay % content for KBA and WA were found to be 0.15 % and 0.09 %, respectively results are illustrated in Table 7.

Table 7: Results for simultaneous estimation of KBA and WA in Painquit Tablet.

Analyte in Painquit Tablet	Sample conc. (µg/band)	Average Peak Area	Average Amount Found (ng)	Average Amount found (%)	% RSD
11-Keto-β-Boswellic acid	1008.6	5294.10	1523.32	0.15 ±0.0018	1.20
Withaferin A	1008.6	2829.90	872.59	0.09 ±0.0008	0.88

CONCLUSION

The developed HPTLC method for simultaneous estimation of 11-Keto-β-Boswellic acid (KBA) and Withaferin A (WA) in Painquit Tablet was found to be simple, sensitive, selective and accurate. The method can be used for routine analysis in Quality Control Lab.

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