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Simultaneous Estimation of Guggulsterone E, Guggulsterone Z, KBA, AKBA, Withaferin A and 6-Gingerol By Using HPLC-DAD Method From Ariflex Tablet Formulation

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ABSTRACT
Simple, accurate, precise, sensitive and validated HPLC method was developed for simultaneous determination of Guggulsterone E (GE), Guggulsterone Z (GZ), 11-keto-β-boswellic acid (KBA), 3- Acetyl 11-keto-β-boswellic acid (AKBA), Withaferin A (WA) and 6-Gingerol (GNRL) in Ariflex tablet. The gradient RP-HPLC analysis was performed on Inertsil ODS-3V C_{18} column (250 × 4.6 mm id), using a mobile phase consisting of 0.1% Ortho-phosphoric acid and mixture of acetonitrile: Methanol (50: 50 v/v) in solvent gradient elution for 60 min at a flow rate of 1.0 mL min^{-1}. Quantification was carried out using a photodiode array detector at 225nm. The employment of diode array detector allowed selectivity confirmation by peak purity evaluation. The method was validated according to International Conference on Harmonization guidelines. No chromatographic interference from tablet excipients was found and hence this method is applicable for simultaneous determination of GE, GZ, KBA, AKBA, WA and GNRL in Ariflex Tablet.

Keywords: 6-Gingerol, Boswelic acid, Ariflex Tablet, Guggulsterone, Withaferine-A, Osteoarthritis.

INTRODUCTION

Osteoarthritis (OA) is a chronic, degenerative joint disorder characterized by joint pain, tenderness, limitation of movements, crepitus, occasional effusion, and variable degrees of local inflammation, without systemic manifestations. The high frequency, economic burden, and adverse implications on the quality of life make OA a major public health issue. The hypoxic conditions, elevation in the activities of proteolytic enzymes, biochemical stress, genetic factors, and trauma are main causes of OA. Obesity is a major risk factor for the disease affecting the knee. [1-6]

Currently, though pharmacological, mechanical and surgical interventions are used, there is no known cure for OA. Herbal remedies are widely used all over the world to treat the OA. Anti-arthritic herbs described in the Indian system of medicine are being used effectively in the management of OA. Many permutations and combinations of the herbs mentioned in the classical texts of Indian system of medicine have been made and the formulations are being promoted as effective and safe remedies for the management of OA. [7-9] Most of the formulations present in the market are not standardized. There is a need to have standardized anti-arthritic formulation so as to maintain batch to batch consistency in terms of efficacy and safety.

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We (R & D Center, Ari Healthcare Pvt. Ltd.) have conceptualized and developed an anti-arthritic formulation i.e. Ariflex Tablet. It contains Guggul (*Commophora mukul*), Shallaki (*Boswellia serrate*), Ashwagandha (*Withania somnifera*), and Shunthi (*Zingiber officinale*) along with other active ingredients. These extracts are reported in the Ayurvedic book to have efficacy on antiarthritic and other pain full conditions.

The ingredients of the Ariflex tablet possess anti-inflammatory, analgesic, antipyretic, chondoprotective, anti-osteoporotic, immunomodulator, anti-oxidant, rejuvenating and anti-stress activities. These multiple activities of the ingredients present in the formulation help to reduce inflammation, pain in rheumatologic conditions. Besides this, the ingredients also help to protect articular cartilage from damage and help reduce osteoporosis.

In order to standardize Ariflex Tablet, the attempt has been made in the present study to develop simultaneous determination of six different markers by using HPLC.

MATERIALS AND METHODS

Chemicals

HPLC-grade solvents such as acetonitrile, methanol, orthophosphoric acid and water were obtained from Merck Ltd. Bangalore India. Standards were purchased from Natural Remedies Ltd. Bangalore India.

Preparation of mixture of standard solution

The six standards namely Guggulsterone E (GE), Guggulsterone (GZ), 11-keto-β-boswellic acid (KBA), 3-Acetyl 11-keto-β-boswellic acid (AKBA), Withaferin A (WA) and 6-Gingerol (GNRL) were taken for preparation of standard solution. 7.5 mg of WA was dissolved into 20 mL, 10 mg of GNRL was dissolved in 20 mL, and 4.2 mg of GE was dissolved in 25 mL, 4.5 mg GZ was dissolved in 25 mL, 6.1 mg of KBA was dissolved in 25 mL and 4.9 mg of AKBA was dissolved in 25 mL of Methanol in separate volumetric flasks. From above solutions, 0.8 mL of WA, 3.2 mL GNRL, and 4 mL each of sGE, GZ, KBA and AKBA was transfer in 20 mL volumetric flask and mixed well. This solution was used as standard stock solution.

Further 2.5 mL of standard stock solution was diluted up to 5 mL with methanol. This solution was used as standard working solution. The solution was filtered through a 0.45μm syringe filter and the resulting solution was used as standard solution.

Preparation of the test solution

20 tablets of Ariflex formulation were weighed and crushed into the powder form. 2 g powder formulation was taken into 20 mL volumetric flask. About 15 mL of diluent was added and the solution was sonicated in ultrasonic water bath for 30 minutes. The solution was allowed to cool and the volume was made up to the mark with diluent. The resulting solution was filtered with Whatman No. 41 filter paper and further it was filtered through 0.45μ syringe filter. The resulting solution was used as test solution.

Chromatographic conditions for HPLC

HPLC was performed using a Waters 2695 Alliance system with e2996 photodiode array detector (PDA). The standards as WA, GNRL, GE, GZ, KBA and AKBA were resolved on a reverse-phase 250 × 4.6 mm, 5μm, Inertsil ODS-3V column (LCGC, India). The mobile phase was prepared from 0.1% orthophosphoric acid in water of pH 2.5 (solvent-A) and Acetonitrile: Methanol (50:50 v/v) (solvent-B). The mobile phase was degassed and filtered through a 0.45-μm filter before use. The gradient program used is given in Table 2.

The mobile phase flow rate was kept at 1 ml/min. Before the first injection, the column was saturated for 30 min with the initial mobile phase. Temperature was maintained at 30°C. Injection volume was decided to maintain at 10μL. The PDA was set by optimizing wavelength to give best response for all four peaks at 225 nm to acquire the chromatogram. The standard WA, GNRL, GE, GZ, KBA and AKBA were identified by comparing the retention time and spectra obtained from the sample and standard solutions. The present work was performed in an air-conditioned room maintained at 25°C.

Preparation of Calibration Graph

Seven different concentrations were prepared by diluting the standard stock solution. The dilutions were given in Table 3.

The calibration graph of each standard was constructed by plotting concentrations against peak area for the respective standards.

Table 1: List of Standards

S. No	Name of Standard	Batch No.	Potency (%)
1	Withaferin A (WA)	T12F047	98.7
2	6- Gingerol (GNRL)	T12H044	97.9
3	Guggulsterone- E (GE)	T13D025	99.5
4	Guggulsterone- Z (GZ)	T13D026	97.5
5	Keto beta boswellic acid (KBA)	T12K033	99.9
6	Acetyl keto beta boswellic acid (AKBA)	T13E006	99.0

Table 2: Details of Gradient program

Time (minute)	Flow (mL/minute)	% solvent A	% solvent B
0	1.0	70	30
50	1.0	05	95
55	1.0	05	95
56	1.0	70	30
60	1.0	70	30

Table 3: Dilutions for Linearity

S. No.	% Level	Dilution (mL)	
1	50	2.5	10
2	60	1.5	5
3	80	2	5
4	100	2.5	5
5	120	3	5
6	140	3.5	5
7	150	3.8	5

Validation of HPLC Method

The proposed HPLC method was validated in terms of specificity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ),

standard solution stability, sample solution stability and robustness as per the International Conference on Harmonization (ICH) guidelines. [10]

Specificity

The specificity of the method was studied by assessment of peak purity of WA, GNRL, GE, GZ, KBA and AKBA using the Waters empower software and diode array detector and represented in terms of purity angle, purity threshold, and purity flag.

Precision

Precision was studied in terms of system precision, method precision, and intermediate precision.

System precision

System precision was carried out by six replicate injections from the same vial of standard and was expressed in terms of percent relative standard deviation (% RSD) tailing, plate count, and resolution.

Method precision

The sample was analyzed for six times using above mentioned procedure. The % assay for each analyte was expressed in terms of % RSD.

Intermediate precision

Intermediate precision was performed on different systems, i.e. Waters e2695 Alliance system with a 2996 PDA and 2489 ultraviolet (UV) detector by different analysts by analyzing six different sample of extract and was expressed in terms of % RSD.

Recovery studies

The accuracy of the method was determined from recovery studies by adding known amount of each standard at 80%, 100%, and 120% levels to the pre-analyzed sample followed by replicate quantitative analysis using proposed method.

Analytical solution stability

The standard solution and sample solution were prepared as per the proposed method and subjected to stability study at room temperature for 12 h. The sample solution was analyzed at initial and at different time intervals of 4 h up to 12 h. Change in the response of WA, GNRL, GE, GZ, KBA and AKBA in the sample solution with respect to time was calculated as absolute percent difference against initial response.

Robustness

Robustness of the method was determined by slight deviation in the method parameters. The parameters selected were deviation in column chemistry, wavelength, column temperature, flow rate, and mobile phase gradient. The retention time of WA, GNRL, GE, GZ, KBA and AKBA, respectively, was determined and % RSD using system suitability parameters was observed.

LOD and LOQ

LOD and LOQ were determined based on the standard deviation of the response and the slope of the calibration curve at low concentration levels according to ICH guidelines. It was determined by plotting a calibration graph of the respective standard solution at low concentration and calculated by using the following equations:

$$LOD = 3.3\ \sigma / S$$

$$LOQ = 10\ \sigma / S;$$

σ = standard deviation of response; S = slope of calibration curve

% Assay of each analyte from Ariflex tablet

Ariflex tablet was analyzed to determine the contents of WA, GNRL, GE, GZ, KBA and AKBA as per method described under chromatographic conditions using HPLC. Every single analysis was repeated three times and results were expressed in mean ± SD.

RESULTS AND DISCUSSION

The composition of the mobile phase in the HPLC method was optimized by testing different solvent compositions of varying polarity, column chemistry, column temperature, and pH of mobile phase, and the best results were obtained by using the present method, which produces highly symmetrical peaks showing good resolution between each standard and other peaks (Figure 3 and 4). The scanning wavelength selected was 225 nm to provide comparable results and at this wavelength all analyte showed optimum response.

WA, GNRL, GE, GZ, KBA and AKBA were satisfactorily resolved with retention time about 25, 28, 35, 37, 48 and 53 mins, respectively.

Table 4: Specificity Parameters

S. No.	Standard	Purity angle	Purity threshold	Purity flag
1	Withaferin A	0.322	0.565	No flag found*
2	6- Gingerol	0.100	0.266	No flag found*
3	Guggulsterone- E	0.060	0.233	No flag found*
4	Guggulsterone- Z	0.086	0.240	No flag found*
5	KBA	0.124	0.281	No flag found*
6	AKBA	0.171	0.349	No flag found*

Table 5: System precision parameters

S. No	Name of analyte	% RSD	USP Tailing	USP Plate count
1	Withaferin A	0.98	1.02	21649
2	6- Gingerol	0.64	1.12	116689
3	Guggulsterone- E	0.52	1.02	21542
4	Guggulsterone - Z	0.74	1.13	200561
5	KBA	1.49	1.20	382601
6	AKBA	0.55	1.11	559900

Table 6: Method precision parameters

S. No	Name of Analyte	% RSD
1	Withaferine A	1.86
2	6- Gingerol	1.45
3	Guggulsterone- E	1.31
4	Guggulsterone- Z	1.50
5	KBA	1.82
6	AKBA	1.92

Table 7: Intermediate precision parameters

S. No	Name of Analyte	% RSD for System-1	% RSD for System-2	Overall % RSD
1	Withaferine A	1.86	0.98	1.65
2	6- Gingerol	1.45	1.45	1.39
3	Guggulsterone- E	1.31	1.23	1.29
4	Guggulsterone- Z	1.50	0.68	1.62
5	KBA	1.82	1.92	1.92
6	AKBA	1.92	1.91	1.34

The calibration graph was linear in the working range of 50-150 μg/ml, with acceptable correlation coefficients 0.9981, 0.9988, 0.9986, and 0.9989, 09980 and 0.9983 for WA (7.5-22.5μg/ml), GNRL (40-120μg/ml), GE (17-51μg/ml), GZ (18-54μg/ml), KBA (25-75μg/ml), and AKBA (20-60μg/ml), respectively (Table 10). The graph for each standard is given in Figure 5.

Table 8: Recovery studies

Analyte	Recovery level	% Recovery	Average % Recovery
Withaferine A	80% - 1	101.46	101.37
	80% - 2	100.46	
	80% - 3	102.18	
	100% - 1	100.18	101.68
	100% - 2	102.51	
	100% - 3	102.36	
	120% - 1	100.43	98.09
	120% - 2	96.09	
	120% - 3	97.75	
6- Gingerol	80% - 1	102.94	99.19
	80% - 2	99.265	
	80% - 3	98.701	
	100% - 1	100.06	101.98
	100% - 2	101.07	
	100% - 3	101.44	
	120% - 1	97.62	99.21
	120% - 2	98.32	
	120% - 3	99.94	
Guggulsterone E	80% - 1	100.97	100.30
	80% - 2	98.26	
	80% - 3	101.67	
	100% - 1	102.55	101.65
	100% - 2	99.26	
	100% - 3	103.14	
	120% - 1	101.99	101.41
	120% - 2	102.47	
	120% - 3	99.76	
Guggulsterone Z	80% - 1	97.86	98.60
	80% - 2	98.36	
	80% - 3	99.58	
	100% - 1	100.15	99.18
	100% - 2	100.79	
	100% - 3	96.60	
	120% - 1	98.99	98.03
	120% - 2	97.48	
	120% - 3	97.61	
KBA	80% - 1	98.89	99.47
	80% - 2	100.12	
	80% - 3	99.42	
	100% - 1	99.69	98.91
	100% - 2	99.54	
	100% - 3	97.49	
	120% - 1	98.64	99.13
	120% - 2	98.39	
	120% - 3	100.35	
AKBA	80% - 1	99.43	99.19
	80% - 2	97.99	
	80% - 3	100.15	
	100% - 1	101.02	101.98
	100% - 2	101.93	
	100% - 3	102.98	
	120% - 1	100.33	99.21
	120% - 2	98.89	
	120% - 3	98.40	

The values of system precision, method precision, and intermediate precision are given against sample application and scanning of peak area and are expressed in terms of % RSD.

For system precision % RSD values were found to be 0.98 %, 0.64%, 0.52%, and 0.74%, 1.49% and 0.52% for WA, GNRL, GE, GZ, KBA and AKBA respectively (Table 5).

Method precision was done and % RSD value were found to be 1.86 %, 1.45%, 1.31%, 1.50% 1.82 % and 1.92% for WA, GNRL, GE, GZ, KBA and AKBA respectively (Table 6).

Table 9: Robustness parameter

Robustness parameter	% RSD	Peak tailing	Theoretical plates	Remark
Withaferine A				
Wavelength: - 5nm	0.64	1.02	115373	Pass
Wavelength: + 5nm	0.68	1.03	115622	Pass
Temperature: - 2°C	0.26	1.02	91941	Pass
Temperature: + 2°C	0.51	1.02	97252	Pass
6 Gingerol				
Wavelength: - 5nm	0.61	1.03	151991	Pass
Wavelength: + 5nm	0.51	1.03	152261	Pass
Temperature: - 2°C	0.09	1.03	118404	Pass
Temperature: + 2°C	0.34	1.03	125757	Pass
Guggulsterone E				
Wavelength: - 5nm	0.40	1.03	213120	Pass
Wavelength: + 5nm	0.47	1.03	212477	Pass
Temperature: - 2°C	0.17	0.99	166712	Pass
Temperature: + 2°C	0.57	0.99	174998	Pass
Guggulsterone Z				
Wavelength: - 5nm	0.53	1.02	253437	Pass
Wavelength: + 5nm	0.54	1.03	252081	Pass
Temperature: - 2°C	0.32	1.03	199286	Pass
Temperature: + 2°C	0.35	1.02	209465	Pass
KBA				
Wavelength: - 5nm	0.34	0.96	492947	Pass
Wavelength: + 5nm	0.32	1.01	491228	Pass
Temperature: - 2°C	1.75	0.96	384226	Pass
Temperature: + 2°C	0.73	1.01	403766	Pass
AKBA				
Wavelength: - 5nm	0.24	1.02	523627	Pass
Wavelength: + 5nm	0.65	1.02	505158	Pass
Temperature: - 2°C	0.18	1.07	381664	Pass
Temperature: + 2°C	0.37	1.02	438987	Pass

Fig. 1: Purity spectra for specificity

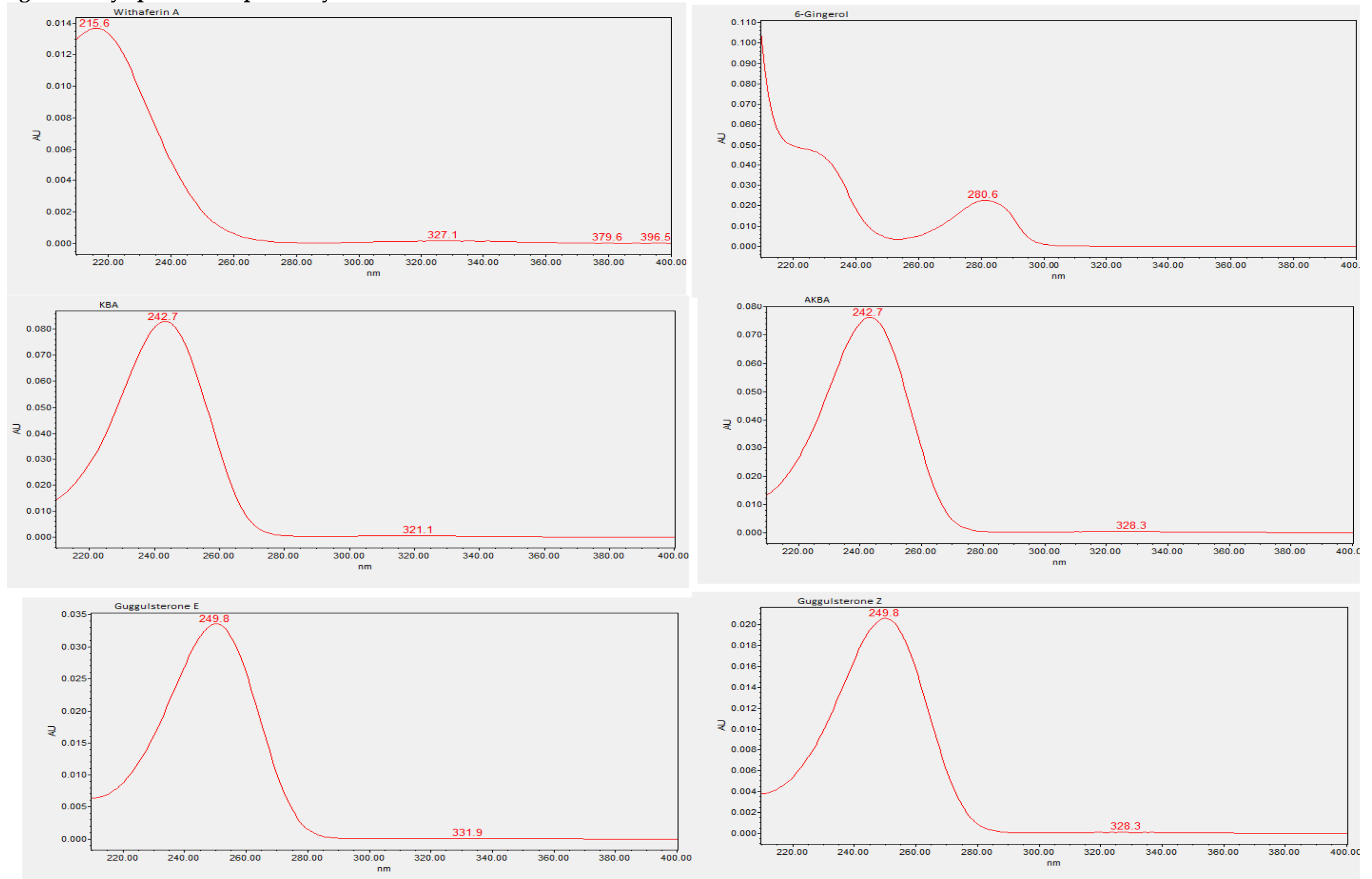

Fig. 2: Purity spectra for Standards

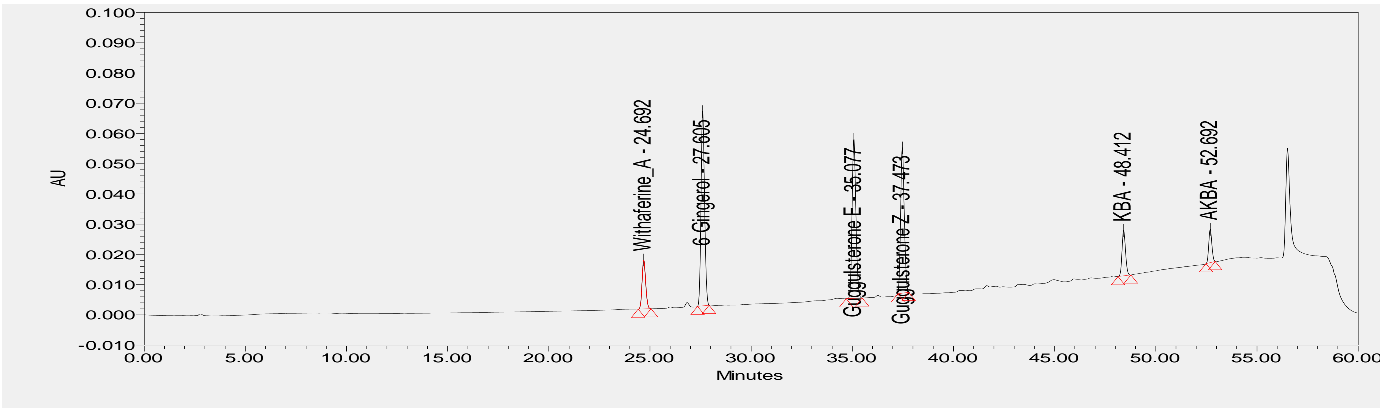

Fig. 3: Standard chromatogram of mixed standard

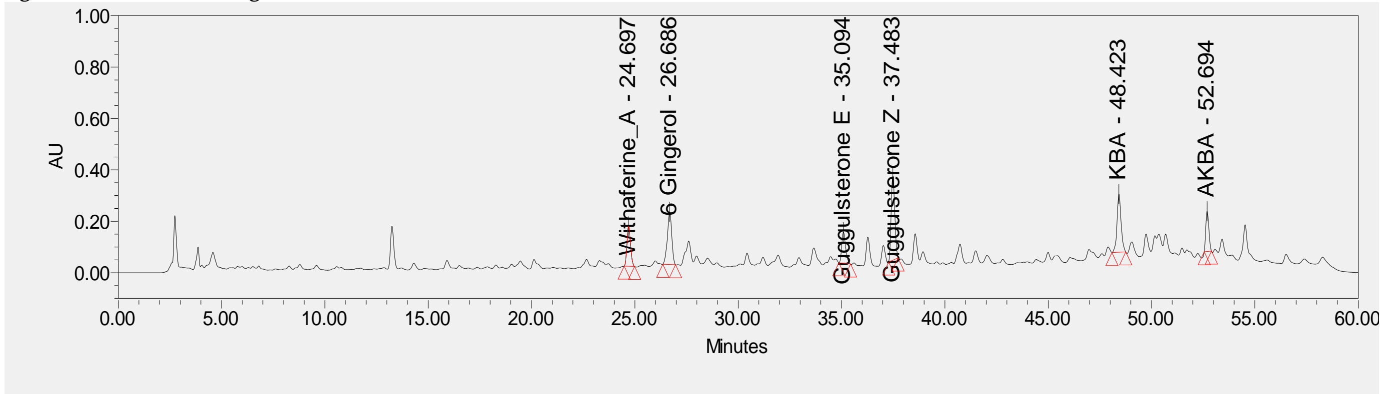

Fig. 4: Chromatogram of tablet formulation

Linearity of Withaferine A
y = 17588x - 4022.3
R² = 0.9981
Area (AU)
Concentration (ppm)

Linearity of 6-Gingerol
y = 65468x - 16690
R² = 0.9988
Area (AU)
Concentration (ppm)

Linearity of Guggulsterone E
y = 27071x - 13726
R² = 0.9986
Area (AU)
Concentration (ppm)

Linearity of Guggulsterone Z
y = 22628x - 12207
R² = 0.9989
Area (AU)
Concentration (ppm)

Linearity of KBA
y = 4813.8x - 1775
R² = 0.998
Area (AU)
Concentration (ppm)

Linearity of AKBA
y = 3973.1x - 1496.9
R² = 0.9983
Area (AU)
Concentration (ppm)

Fig. 5: Linearity graphs for standard

For intermediate precision % RSD values between the two analysts were found to be 1.65%, 1.39%, 1.29%, 1.62% 1.92 % and 1.34 % for WA, GNRL, GE, GZ, KBA and AKBA respectively (Table 7).

For the values of system precision, method precision, and intermediate precision, the % RSD values showed that the proposed method provides an acceptable level of system precision, method precision, and intermediate precision.

The peak purity of for each analyte was assessed by comparing their respective spectra at peak start, peak apex, and peak end positions of the spot from standard and extracts (Figures 1 and 2).

The purity angle and purity threshold values are given in table (Table 4).

It was confirmed that the given method is robust. The peak area for each analyte was calculated for each parameter and % RSD was found to be less than 2%. The values of % RSD as shown in Table 9 indicate better robustness of the method.

The recovery study was carried out by spiking known amount of standards into placebo solution at 80%, 100% and 120% of working concentration. The overall recovery percent were found to be 100.38 % for WA, 100.13 % for GNRL, 101.12% for GE, 98.60% for GZ, 99.17% for KBA and 100.13% for AKBA respectively (Table 8).

LOD and LOQ were determined based on the standard deviation of the response and the slope of the calibration curve at low concentration levels of each analyte separately. The LOD and LOQ values for each analyte are given in Table 10. The values for LOD and LOQ were found to be 0.42μg/ml and 1.42μg/ml, for WA, 0.36μg/ml, and 1.20μg/ml for GNRL, 0.81μg/ml and 2.71μg/ml for GE, 0.77μg/ml and 2.58μg/ml for GZ, 1.39μg/ml and 4.65μg/ml for KBA and 1.05μg/ml and 3.51μg/ml for AKBA respectively.

Table 10: LOD, LOQ and Linearity Study

S. No.	Analyte	LOD (μg/ml)	LOQ (μg/ml)	Linearity (correlation coefficient = r^2)
1	Withaferine A	0.42	1.42	0.9981
2	6- Gingerol	0.36	1.20	0.9988
3	Guggulsterone- E	0.81	2.71	0.9986
4	Guggulsterone- Z	0.77	2.58	0.9989
5	KBA	1.39	4.65	0.9980
6	AKBA	1.05	3.51	0.9983

Table 11: % Assay of each analyte from Ariflex tablet

S. No	Name of Analyte	(% assay w/w) (mean ± SD)
1	Withaferine A	1.40 ±0.026
2	6- Gingerol	1.99 ±0.029
3	Guggulsterone- E	0.50 ±0.005
4	Guggulsterone- Z	1.91 ±0.027
5	KBA	5.37 ±0.108
6	AKBA	3.41 ±0.145

Analytical solution stability was done on standard solution and sample solution. The standard and test solutions were analyzed at initial and at different time intervals for 12 hours. Both the solutions were found to be stable up to 12 hours.

% Assay of WA, GNRL, GE, GZ, KBA and AKBA from Ariflex tablet

The % Assay of WA, GNRL, GE, GZ, KBA and AKBA were expressed in (mean ± SD) and were found to be 1.40 ± 0.026, 1.99 ± 0.029, 0.50 ± 0.005, 1.91 ± 0.027, 5.37 ± 0.108 and 3.41 ± 0.145 respectively from Ariflex tablet (Table 11).

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