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DEVELOPMENT AND VALIDATION OF A STABILITY INDICATING RP-HPLC METHOD FOR ASSAY DETERMINATION OF METHYLPARABEN, PROPYLPARABEN, POTASSIUM SORBATE AND SENNOSIDES

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ABSTRACT

A gradient, reversed phase, high pressure liquid chromatography method was developed for simultaneous assay determination of methylparaben, propylparaben, potassium sorbate and sennosides in a single analysis with good resolution between the components. The chromatographic separation is carried out in a reverse phase mode with ultraviolet (UV) detection at 258nm, Column thermo hypersil BDS C18, 250mm × 4.0mm, 5µm using a gradient program run for 40 minutes. Flow rate is 0.7mL/min with ambient column temperature and injection volume is 10.0 µL. The mobile phase consists of 0.5M ammonium acetate buffer adjusted pH to 4.5 units with glacial acetic acid and acetonitrile. The method successfully separates the three preservatives (methylparaben, propylparaben and potassium sorbate) and the active ingredient (sennosides). The developed method was validated according to the international conference on harmonization (ICH) guidelines regarding: Precision, specificity by degradation, linearity, accuracy, range and robustness. The proposed method shown good linearity (correlation coefficient and regression coefficient were not less than 0.999 and 0.998) in the range of 50 to 150% of working concentration of sennosides and 20 to 200% of working concentration of preservatives. The recovery for sennosides at 50, 100 and 150% of working concentration level was within 98 to 102%. The recovery for preservatives at 20, 50, 100, 150 and 200% of working concentration level were within the acceptance criteria. The range of the method is concluded that developed method is from 50 to 150% target concentration of sennosides and 20 to 200% target concentration of methylparaben,

propylparaben and potassium sorbate. The validated method is stability indicative, highly selective, simple, accurate, cost effective, and it is applicable for routine quality-control analysis of drug substances as well as drug product in pharmaceutical industries.

KEYWORDS: Methylparaben; Propylparaben; Potassium sorbate: Sennosides: HPLC.

INTRODUCTION

Sennosides are hydroxyanthracene glycosides derived from senna leaves. The main extracts of the senna plant contain common laxative principles of two glycosides called Sennoside A and Sennoside B are believed to be the primary laxative principles of senna. Both glycosides have the same empiric formula, differing in the manner of the linkage of glucose to the aglycone fraction of each molecule. The aglycone of the sennoside contains a carboxyl group and may be considered to be derivatives of rhein (Figure 1). They have been used as natural, safe time-tested laxatives in traditional as well as modern systems of medicine. They are mainly used to treat constipation. They may also be used to clean out the intestines before a bowel examination/surgery. They also known as stimulant laxatives and work by keeping water in the intestines, which causes movement of the intestines.[1-6]

The structure of the sennosides A and B is well known. It has a molecular weight of 862.74 g/ml, and an empiric formula of $C_{42}H_{38}O_{20}$, sennoside A is built up from the dextro-rotatory aglucon, sennidin A, and D-glucose, while sennoside B is built up from the inter-molecularly compensated mesosennidin B, and D-glucose. Sennoside A is a crystalline substance in the form of rectangular yellow plates, decomposing at 200-240C. Sennoside B is in the form of light yellow prisms, decomposing at 180-186°C. Sennosides is insoluble in water, and barely soluble in methanol; however, an aqueous mixture of 30% water and 70% methanol can dissolve sennosides. They are soluble in sodium bicarbonate as it neutralizes their acidity.[7-8]
The preservation of the formulated product is very important, mainly to prevent bacterial growth during the storage of the formulation. Three commonly used preservatives such as methylparaben (Figure 2), propylparaben (Figure 3) and potassium sorbate (Figure 4), which are used routinely for antimicrobial preservation. An assay method for determination of the sennosides, methylparaben, prorylparaben and potassium sorbate are useful for the batch release and stability studies of the drug product, as well as for the drug substances. The aim of the present work was to have one method to assay the active and the preservatives simultaneously.

There are various methods are reported in the literature for determination of the sennosides.[9-13] and preservatives,[14-18] as per literature survey reveals none of them were reported for simultaneous determination of sennosides, methylparaben, propylparaben and potassium sorbate in a single method by RP-HPLC.

High pressure liquid chromatography is well-known from traditional low pressure liquid chromatography because very small quantity of sample is sufficient to separate, the components present in sample mixture. This gives HPLC superior resolving power when separating mixture of components, which is a popular chromatographic technique. Reversed phase high pressure liquid chromatogram (RP-HPLC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase which is economically viable.

In the present work, we are focused on to achieve the optimum chromatographic conditions for the simultaneous determination of sennosides (sennoside A and sennoside B), methylparaben, propylparaben and potassium sorbate in the bulk and formulated syrup solution. For assay calculation of formulated syrup was completed using the determined density of the syrup in mg per mL. The developed method can be applied successfully to quality control and stability studies purposes. To access the reproducibility and wide applicability of the developed method, it was validated as per ICH guidelines.

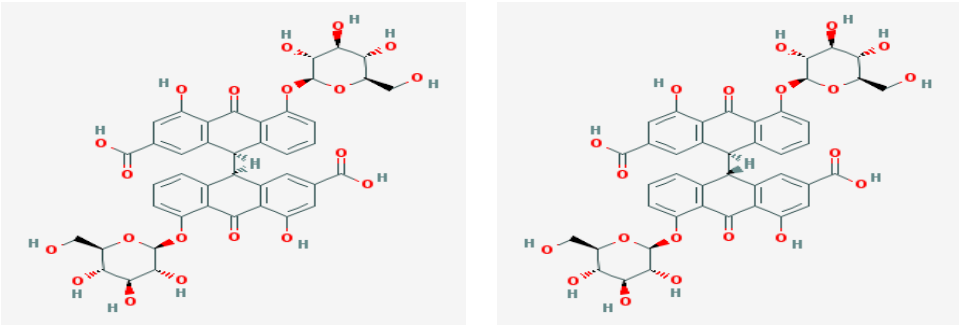

Fig.1: Sennosides (Sennosides A and Sennosides B)

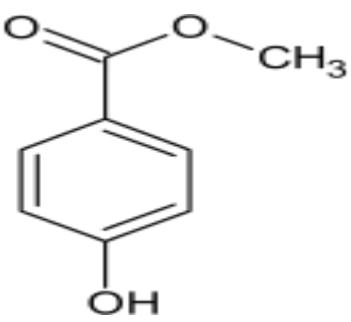

Fig.2: Methylparaben (Methyl *p*-hydroxybenzoate; Methyl parahydroxybenzoate;)

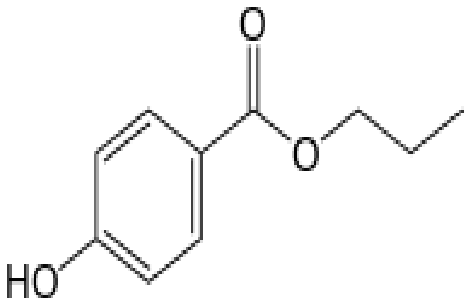

Fig.3: Propylparaben (Propyl *p*-hydroxybenzoate; Propyl parahydroxybenzoate;)

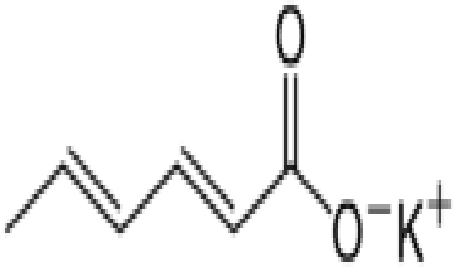

Fig.4: Potassium sorbate (Potassium (2E, 4E)-hexa-2,4-dienoate)

EXPERIMENTAL

Materials and Reagents

Sennosides drug substances, Methylparaben, Propylparaben and Potassium sorbate preservatives were gifted by local Pharmaceutical industry, Acetonitrile (HPLC grade) was purchased from Rankem Lab, Ammonium acetate (AR Grade) from Spectrochem (India), Glacial Acetic acid (AR Grade) from Sd-fine chemicals, HPLC grade water (Millipore), Hydrochloric acid (AR Grade) from Sd-fine chemicals, Hydrogen peroxide (AR Grade) from CDH Fine Chemicals, Methanol (HPLC grade) was purchased from Rankem Lab, Sodium bi-carbonate (AR Grade) from Sd-fine chemicals and Sodium Hydroxide (AR Grade) from SDFCL.

Instrumentation

A UV-Visible spectrophotometer PerkinElmer's LAMDA 25. For Chromatography an Agilent 1100 series Quaternary pump with Diode array detector/UV Detector. Separations and assay determination of the components was performed with Thermo Hypersil BDS, C18 column (250 mm x 4.6 mm, 5 µ) maintained ambient temperature. Weigh the samples and chemicals done on Sartorius analytical balance with sensitivity of 0.01mg. pH adjustments performed for mobile phase using digital pH Meter Elico.

Chromatographic conditions

The Reversed phase High Pressure Liquid Chromatography quantitative analysis was carried out at ambient temperature. The components were eluted by a gradient programme method (Table 1) with mobile phase-A (weigh 3.85gm of ammonium Acetate, dissolve in to 1000 ml of water, and mix well. Adjust the pH 4.5 with glacial acetic acid and mix well. Filter through 0.45µ membrane filter and degas) and mobile phase-B (Acetonitrile.). Flow rate is 0.7mL/min and the injection volume for standard and sample was 10.0 µL. The eluents were measured at a wavelength of 258nm and run time for the chromatographic method is 40 minutes.

Table 1: Gradient method programme

Time in minutes	Mobile Phase A (In %)	Mobile Phase B (In %)
0	90	10
8	90	10
15	75	25
20	80	20
25	10	90
30	10	90
35	90	10
40	90	10

Standard Preparation

Solution A (Potassium Sorbate, Methylparaben and Propylparaben): Weighed 50.0 mg of Potassium sorbate standard and 90.0 mg of Methylparaben Standard and 10.0 mg of Propylparaben Standard, transferred in to 200.0 mL volumetric flask, added about 40.0 mL of methanol, sonicate for 5 min with intermittent shaking and made up to the volume with water, mixed well.

Solution B (Sennosides): Weighed 20.0 mg of Sennosides Standard, transferred in to 50.0 mL volumetric flask, added about 10.0 mL of 0.2 M Sodium bi-carbonate solution, sonicate for 5 min with intermittent shaking and made up to the volume with water and mixed well. Transfer 10.0 mL of solution A and 10.0mL of solution B into 50 mL volumetric flask, make up the solution up to the mark with water and mix well.

Sample Preparation

Weight 16.0 mg of sennosides, 10.0 mg of potassium sorbate, 18.0 mg of methylparaben and 2.0 mg of propylparaben into a 200.0 mL volumetric flask, added 8.0 mL of 0.2 M Sodium

bi-carbonate solution and 100.0 mL of water, sonicate for 5 min with intermittent shaking to dissolve and made up to the mark with water, mix it well.

RESULTS AND DISCUSSION

Chromatographic method development

The previous reported HPLC methods.[19–22] were undertaken with reversed-phase chromatography, few of which used the ion-pair mode. But all these methods are difficult to carry out the simultaneous determination of preservative contents along with active compound. More over stabilization of the ion-pair chromatography is a time consuming process and expensive. Hence, multy-components detection with RP-HPLC was implemented in the proposed work.

To develop a simple, new reversed-phase chromatography method the standard compounds and sample solubility was checked in water and methanol. Methylparaben, Propylparaben and Potassium sorbate were freely soluble with methanol and water but the sennosides are soluble in water by adding the 0.2M Sodium bi-carbonate solution as it neutralizes its acidity. Therefore water, methanol and 0.2M Sodium bi-carbonate solution were kept as diluent. Then standard and sample solutions were scanned on UV Spectrophotometer to identify the wavelength at maximum absorbance. Maxima absorbance at UV region observed at wavelength 258nm. Hence this wavelength has been chosen for assay determination of the target samples by reverse phase high pressure liquid chromatography. Gradient mobile phase elution programme was finalized for better resolution between the components and quick retentions. Equilibrated the column with initial present composition of the mobile phase A and mobile phase B in the gradient programme method. Injected Blank (Diluent: Mixture of water, methanol and sodium bi-carbonate solution in the ratio 1:1:1) one injection, Standard preparation solution five injections and checked the system suitability, the relative standard deviation not more than 2.0%, Tailing factor not more than 2 and Theoretical plates not less than 2000 for the each analyte (sennosides (sennoside A + sennoside B), methylparaben, propylparaben and potassium sorbate) peak from standard preparation solution. The system suitability parameters pass and then injected the sample solution in duplicate to RP-HPLC, recorded each analyte peak response from standard and sample chromatogram and calculated assay for the each component. The degradation samples were run using different columns with same stationary phase like C18 and mobile phases containing buffers like Acetate and phosphate with different pH.

The Gradient mode method has been worked out to separate very closely eluting peaks. To improve response, peak shape and resolution between the peaks the method was tried at different column temperatures. But the separation, response of peaks and peak shapes were satisfactory in the adopted chromatographic conditions only. The retention time for sennoside B, sennoside A, potassium sorbate methylparaben and propylparaben is 11.3min, 14.8min, 19.8min 24min and 28min. It indicated that the gradient mode method with ammonium acetate buffer and acetonitrile as an organic modifier in mobile phase was successful in separating the all components and other chromatographic degradation products (Figure 5A-5H).

This method was optimized to separate every injected component from each other and also degradation product peaks formed under different stress conditions. The main target of the chromatographic assay method is to find specific, simple and well resolved elution of the four compounds (sennosides (sennoside A and sennoside B), methylparaben, propylparaben and potassium sorbate in a single analysis was achieved with a stability indicative method.

Column Selection

Based on the better peak shape and retention of the components, good column life on usage and easy availability with low cost, Thermo Hypersil BDS, C18 column (250 mm x 4.6 mm, 5 µ particle size) column was selected as suitable column for analysis of finalized RP-HPLC gradient method.

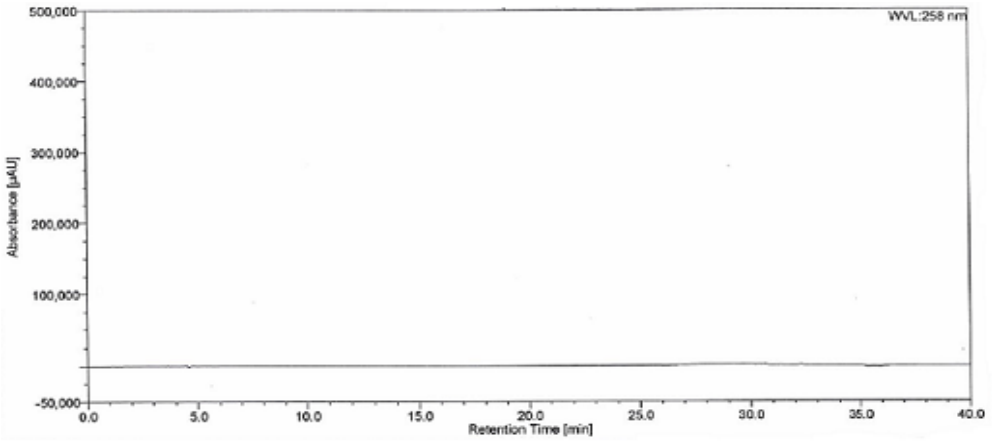

Figure 5A: A typical chromatogram of Blank.

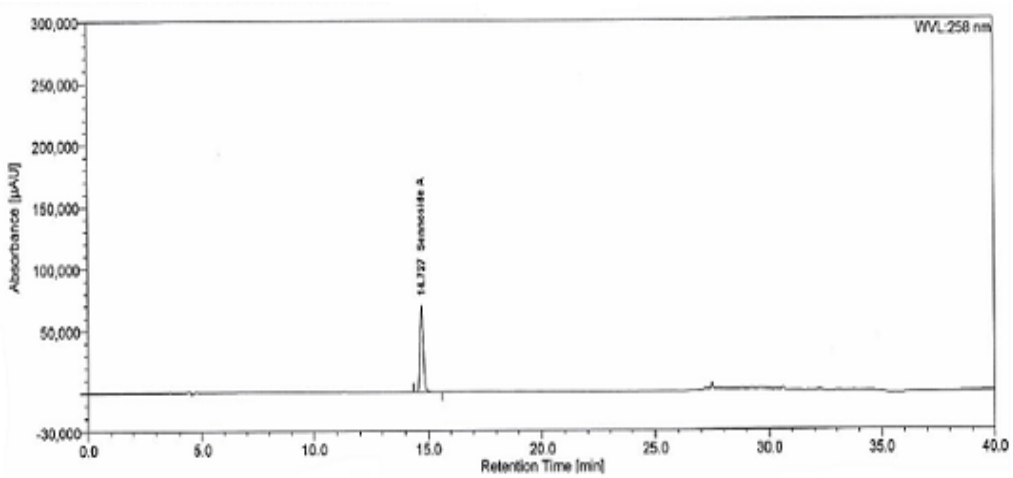

Figure 5B: A typical chromatogram of Sennoside A.

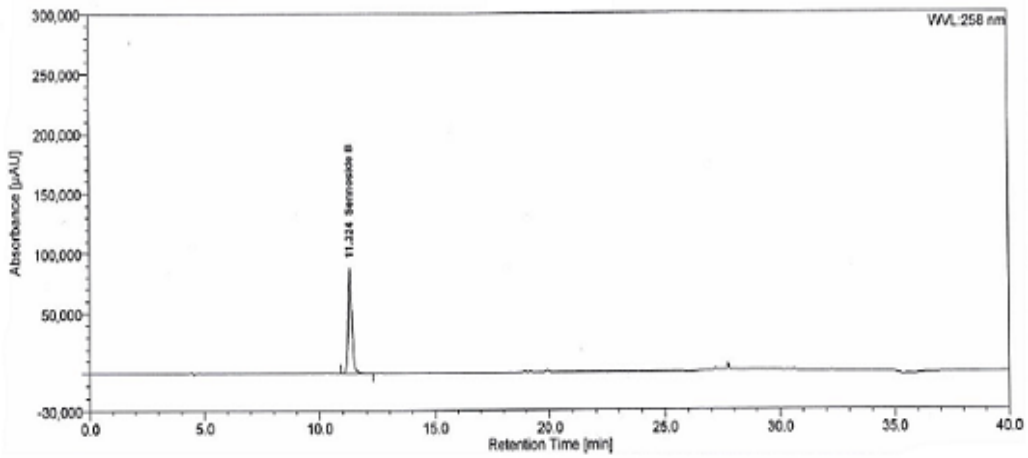

Figure 5C: A typical chromatogram of Sennoside B.

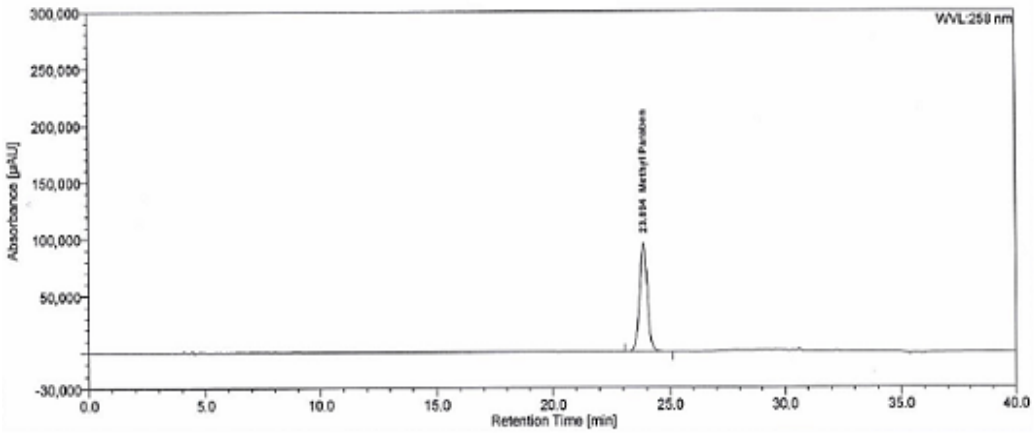

Figure 5D: A typical chromatogram of Methylparaben.

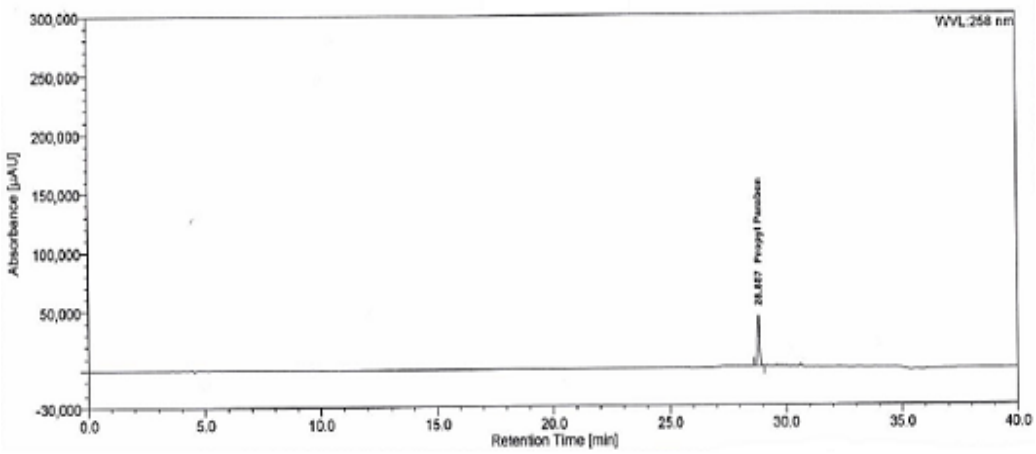

Figure 5E: A typical chromatogram of Propylparaben.

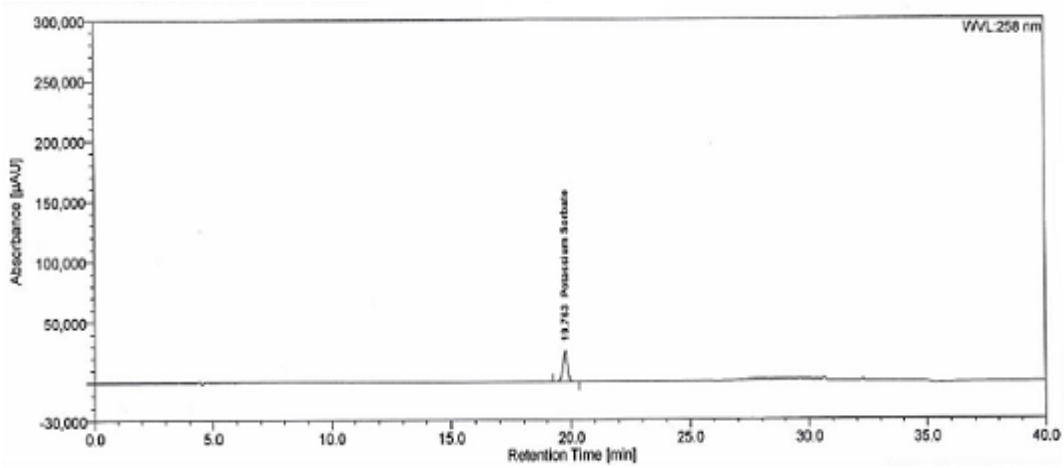

Figure 5F: A typical chromatogram of Potassium sorbate.

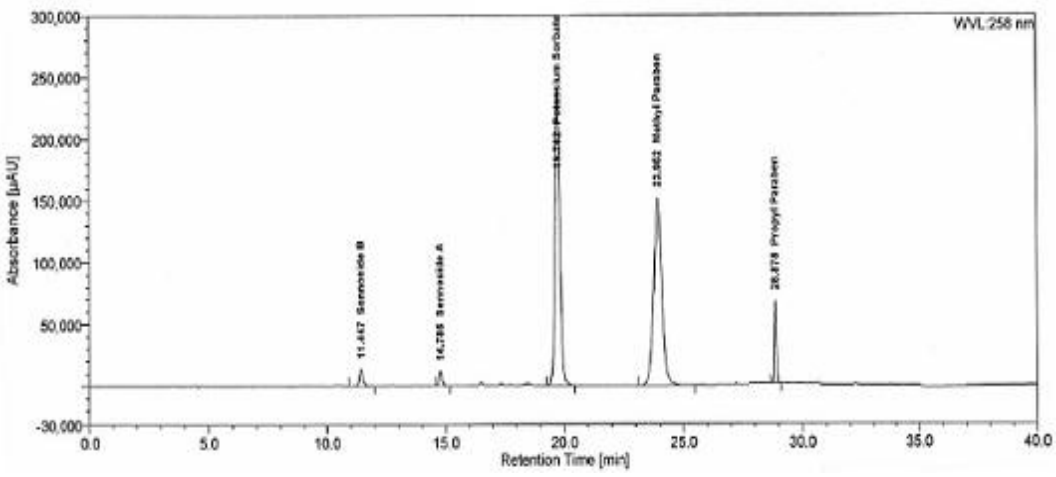

Figure 5G: A typical chromatogram of Sample solution

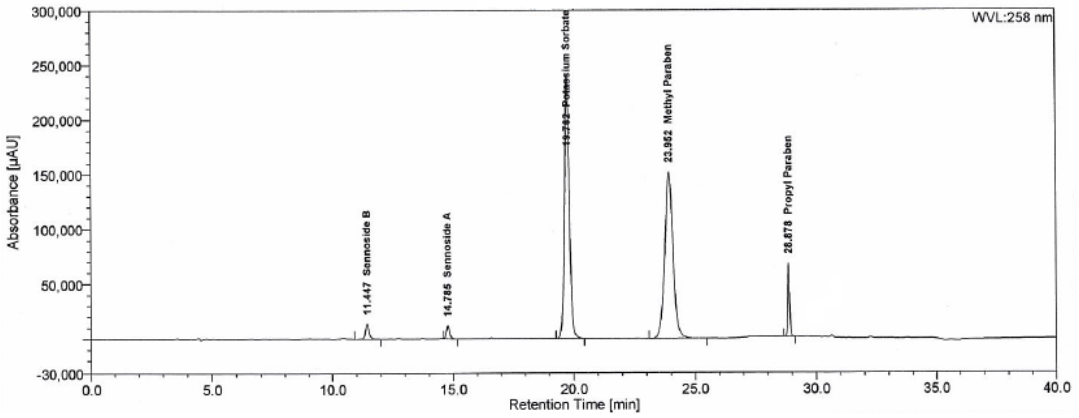

Figure 5H: A typical chromatogram of Standard solution

Method Validation.[23-25]

Validation is a documenting or process of proving that, Analytical Method provides analytical data, for the intended use. The developed RP-HPLC assay method was extensively validated as per ICH guidelines using the following parameters.

System Suitability

To verify that the analytical system is working properly and can give accurate and precise results, the system suitability parameters are to be check.

System suitability performed by injecting a gradient run, one injection of blank solution and five injections of standard solution to chromatographic system. Recorded chromatograms, the relative standard deviation of the each analyte (sum of sennosides (sennoside A + sennoside B), methylparaben, propylparaben and Potassium Sorbate) peak from the five injections of standard solution are 0.4%, 0.1%, 0.1% and 0.1%. Tailing factor for the each analyte peak are 1.15, 1.15, 0.95, 0.98 and 1.06. Theoretical plates for the each analyte peak are 30739, 66595, 51107, 25371 and 698961. From these results, it concluded that the system is suitable for analytical method validation.

Specificity

Specificity is the ability of analytical method to assess unequivocally the analyte in the presence of component that may be expected to be present, such as impurities, degradation products and matrix components. Performed the specificity parameter of the method by injecting Diluent (Blank), each analyte standard and sample solution into the HPLC System. Recorded the retention times of sennoside B, sennoside A, potassium sorbate, methylparaben

and propylparaben are about 11.0min, 15.0min, 20.0min 24.0min and 29.0min. Observed no interferences at Diluent peak and also peak of each analyte was pure.

Stress Studies

Stress study of substances can help to identify the likely degradation products, which can in tern help to establish the type of degradation pathway (whether oxidative, alkali hydrolysis, acid hydrolysis, water hydrolysis, photolytic and dry heat) for each of the degradants and the intrinsic stability of the molecule.

All stress degradation studies were performed for sample contain 80ppm concentration of sennosides, 90ppm concentration of methylparaben, 10ppm concentration of propylparaben and 50ppm concentration of potassium sorbate. Acid hydrolysis was performed in 1.0N and 0.1N Hydrochloric acid at room temperature for 8 hours. The study in basic condition was carried out in 1.0N and 0.1N sodium hydroxide solution at room temperature for 8 hours. For Neutral stressed condition, drug substances dissolved in water and refluxed at 25°C for 8 hours, Oxidation stressed study were carried out on water bath at 25°C for 8 hours in 3% hydrogen peroxide solution. Sample exposed to sun light for 8 hours, Photo degradation studies were carried out by exposing the sample to UV light for 8 hours (254nm and 356nm), The drug substances was exposed to dry heat by keeping the sample in oven at 80°C for 4 hours. Sample were withdrawn at appropriate time and subjected to RP-HPLC analysis after suitable dilution. Diluent peaks were not interfering with each analyte peak. Sum of Sennosides was found to be degraded more in Acid and Alkali Stressed condition. The degradation products generated are well separated from Sum of Sennosides. The Potassium Sorbate Peak was found to be degraded more in Neutral and Sunlight Stressed condition. The degradation products generated are well separated from Potassium Sorbate. The Methyl Paraben, Propyl Paraben Peak was found to be degraded more in Alkali Stressed condition. The degradation products generated are well separated from methylparaben, propylparaben. Peak of Sum of Sennosides, Methyl Paraben, Propyl Paraben and Potassium Sorbate Peaks is Pure. All unknown Degradation products were well separated from Sennosides, Methyl Paraben, Propyl Paraben and Potassium Sorbate Peaks and all stressed blank solution peaks are not interfered at the retention time of each analyte Peak (Table 2 and Table 3).

Table 2: The stress study results table for sennosides.

Stress condition	Peak purity*	Purity Match		%Assay
		Sennoside A	Sennoside B	
Sample as Such	P	999	1000	100.3
0.1 N HCl	P	999	1000	92.1
1.0 N HCl	P	998	1000	81.2
0.1 N NaOH	P	999	999	98.8
1.0 N NaOH	P	999	999	95.0
3.0%w/v H2O2	P	999	1000	99.8
Neutral	P	999	1000	98.9
Thermal	P	999	1000	104.3
Sun Light	P	999	1000	99.4
UV Light	P	999	1000	100.5

Table 3: The stress study results table for methylparaben, propylparaben and potassium sorbate.

Stress condition	Peak purity*	Purity Match			%Assay		
		Potassium Sorbate	Methyl Paraben	Propyl Paraben	Potassium Sorbate	Methyl Paraben	Propyl Paraben
Sample as Such	P	1000	1000	992	99.8	100.2	99.4
0.1 N HCl	P	1000	1000	990	96.2	99.7	98.4
1.0 N HCl	P	1000	1000	990	95.1	99.0	98.8
0.1 N NaOH	P	1000	1000	991	95.5	94.5	97.4
1.0 N NaOH	P	1000	1000	998	93.3	14.7	54.7
3.0%w/v H2O2	P	1000	1000	991	96.2	99.7	98.5
Neutral	P	1000	1000	992	74.1	98.6	97.2
Thermal	P	1000	1000	1000	95.5	97.0	96.3
Sun Light	P	1000	1000	991	73.6	97.7	98.9
UV Light	P	1000	1000	992	90.4	96.1	97.3

Peak purity *: ‘P’ indicates sum of sennosides, methylparaben, propylparaben and potassium sorbate peaks was pure which confirmed by diode array detector and agilent chromeleon software.

Precision of Test Method

The precision of an analytical method procedure express the closeness of agreement among individual test results when the method is applied repeatedly to multiple sampling of same homogeneous sample.

System Precision

The system precision is checked by using standard chemical substance to ensure that the analytical system is working properly. Checked by inject a gradient, blank solution and five

injections of standard solution to HPLC system. Recorded chromatograms, Calculated the relative standard deviation of the retention time for sennoside B peak at 11.0min is 0.1%, sennoside A peak at 14.8min is 0.1%, potassium sorbate peak at 19.8min is 0.1%, methylparaben peak at 24min is 0.1% and propylparaben peak at28.5min is 0.1%. Relative standard deviation for the area response of sum of sennosides is 0.4%, methylparaben is 0.1%, propylparaben is 0.1% and potassium sorbate is 0.1%. Hence it was concluded that the system precision parameter meets the requirement of validation.

Intra-day and Inter day precision

The precision of the assay method was performed by carrying out six times of same sample as per analytical procedure against qualified standard. Determine the assay for the samples of sennosides, methylparaben, propylparaben and potassium sorbate six times of same batch. Calculated the % assay for sum of sennosides (sennoside A + sennoside B), Methyl Paraben, Propyl Paraben and Potassium Sorbate with respect to area of Standard solution preparation. Evaluated the intermediate precision of the method as per analytical method procedure by different analyst from the same laboratory, calculated percentage of relative standard deviation for obtained six assay values and compared both intra-day and inter day results (Table 4).

Table 4: The results table for precision study.

Precision study	S.No	%Assay			
		Sennoside A+ Sennoside B	Methyl Paraben	Propyl Paraben	Potassium Sorbate
Intra-day precision	1	99.4	100.8	98.5	98.8
	2	99.6	100.7	98.6	98.7
	3	99.5	100.9	98.7	98.6
	4	99.6	100.9	98.6	98.6
	5	99.3	100.6	98.4	98.3
	6	98.9	100.5	98.5	98.2
Inter-day precision	7	98.8	100.8	99.1	98.6
	8	99.0	100.7	98.9	98.7
	9	98.9	100.8	99.2	98.7
	10	99.1	100.8	98.7	98.6
	11	98.7	100.6	98.8	98.3
	12	98.5	100.5	98.6	98.4
Average of 12 determinations		99.1	100.7	98.7	98.5
% RSD		0.4	0.1	0.2	0.2

Stability in Analytical Solution

Evaluated the stability of analytical solution by inject the standard preparation and sample preparation at regular interval. The standard solution is stable up to 40 hours at room temperature (25°C) with the % difference 0.4% and sample solution is stable up to 37 hours at room temperature(25°C) with the % difference 0.2%.

Linearity

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range.

Linearity test solution for the assay method were prepared from stock solution at ten different concentration levels (20%, 50%, 70%, 80%, 90%, 100%, 110%, 120%, 150% and 200%) of the target assay concentration of sennosides (80ppm), methylparaben (90ppm), propylparaben (10ppm) and potassium sorbate (50ppm). 10µL of each level solution was injected into the high pressure liquid chromatograph system and recorded the peak area response from the chromatogram, also precision study for linearity solution has been performed at lower and higher concentrations levels. A graph was plotted to concentration in ppm on X-axis verses Peak area response on Y-axis. Calculated slope, correlation coefficient, regression coefficient (R square) and intercept. Tested intercept with statistical equivalence to zero. From the statistical treatment of the linearity data sennosides, methylparaben, propylparaben and potassium sorbate, it is clear that the response of sennosides, methyl paraben, propylparaben and potassium sorbate is linear from 20% to 200% of the working concentration for sennosides. The correlation coefficient and regression coefficients are more than 0.998. In addition, the analysis of residuals show that the values are randomly scattered around zero, which shows good fit to the linear model. To evaluate whether the y-intercepts were significantly different from zero, the p–value was determined. The p value is > 0.05 then intercept is statistically equal to zero. For Sennosides, methylparaben, propylparaben and potassium sorbate p value is greater than 0.05. Hence it is statistically equal to zero. In addition, the origin is within the lower and the upper limit of the 95 % of Confidence interval, that gives high degree of confidence to the value obtained for intercept. Moreover, the value of the intercept is within the ± 2.0 % of the area response at 100 % level. Linearity results of the method are presented in Table 5 and Table 6 and Linearity graphs were shown in Figure-6 to Figure-9.

Table 5: The linearity results for Sennosides.

% Levels	Concentration of Sennosides in ppm	Area response Sennosides (Sennoside A +Sennoside B)
20	16.27	79962.040
50	40.68	204404.959
70	56.95	276222.886
80	65.08	315889.912
90	73.22	357282.030
100	81.35	392737.573
110	91.11	439518.479
120	97.62	478844.488
150	123.65	612073.424
200	162.70	804128.623
Correlation coefficient		1.000
Regression coefficient		1.000
Slope		4942.938
Intercept		3588.903
% Intercept		-0.9%

Table 6: The linearity results for Methylparaben, Propylparaben and Potassium sorbate.

% Level	Concentration in ppm			Area response		
	Methylparaben	Propylparaben	Potassium sorbate	Methyl paraben	Propyl paraben	Potassium sorbate
20	18.14	2.02	10.06	926886.766	59965.712	926886.766
50	45.34	5.05	25.14	2386746.136	173358.822	2386746.136
70	63.48	7.07	35.19	3301221.332	246991.599	3301221.332
80	72.55	8.08	40.22	3722588.506	280883.409	3722588.506
90	81.62	9.09	45.25	4178134.191	317588.440	4178134.191
100	90.69	10.09	50.28	4654160.312	356122.024	4654160.312
110	101.57	11.51	56.31	5231093.113	403528.942	5231093.113
120	105.92	12.11	58.72	5451114.328	421531.471	5451114.328
150	137.85	16.15	76.42	7237561.340	567742.465	7237561.340
200	181.38	21.40	100.55	9353626.815	742927.486	9353626.815
Correlation coefficient				1.000	1.000	1.000
Regression coefficient				1.000	1.000	1.000
Slope				51789329	35191.525	105051.664
Intercept				9123.137	3788.545	38407.319
% Intercept				-0.2%	-1.1%	0.7%

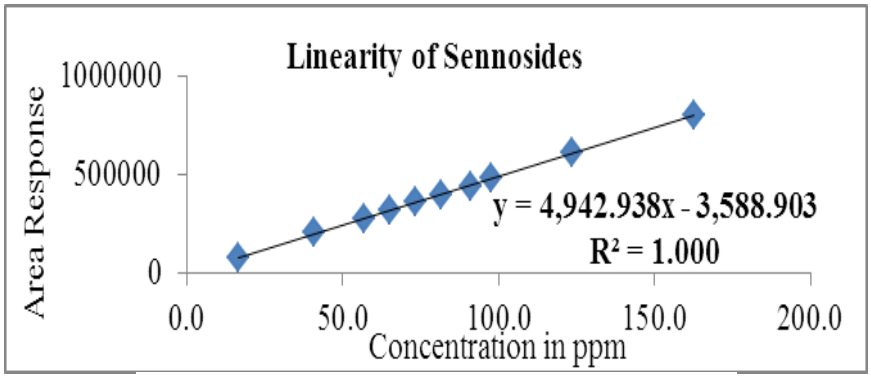

Figure 6: The linearity graph of Sennosides.

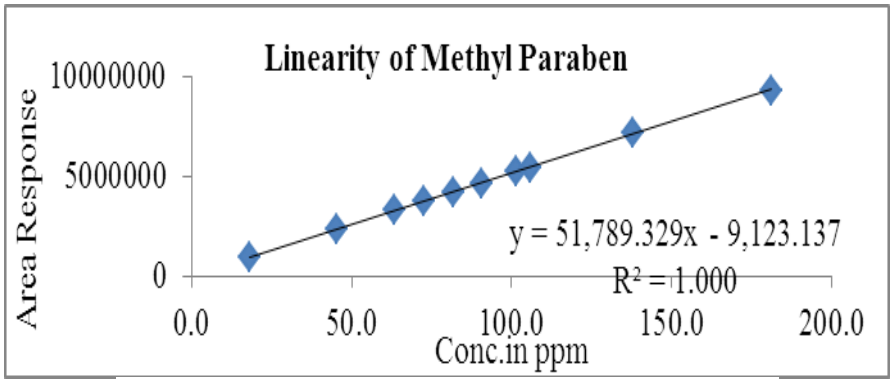

Figure 7: The linearity graph of Methylparaben.

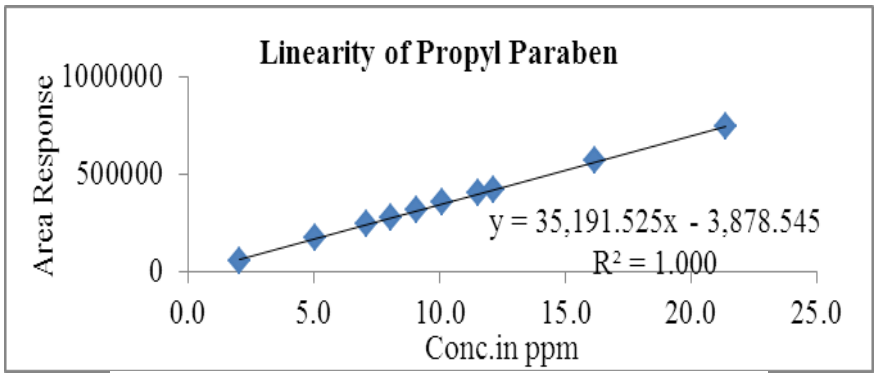

Figure 8: The linearity graph of Propylparaben.

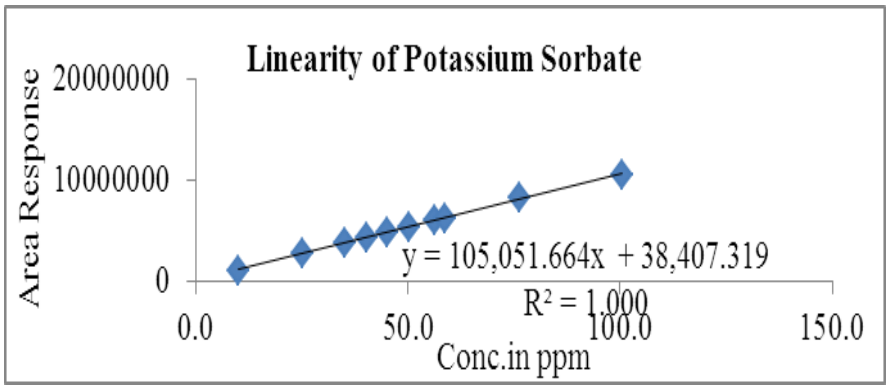

Figure 9: The linearity graph of Potassium sorbate.

Accuracy

Verified the closeness of obtain analytical method test results to the standard results from the method. The accuracy study for sennosides was evaluated in triplicate at three concentration levels, that is 50%, 100% and 150% of working concentration (80 ppm) of the sample, for methylparaben, propylparaben and potassium sorbate were in triplicate at 20%, 50% 100%, 150% and 200% of working concentration of each analyte, The percentage of recoveries were calculated and found it be as within the limits (Table 7).

Table 7: The accuracy study results for Sennosides, Methylparaben, Propylparaben and Potassium sorbate.

Sennosides				
Levels of Recovery	**Mean mg Added**	**Mean mg Recovered**	**Mean % Recovery**	**% RSD**
50%	0.8033	0.8115	101.0	0.20
100%	1.6065	1.5847	98.7	0.06
150%	2.4098	2.3706	98.4	0.47
Methylparaben				
20%	0.3611	0.3587	99.4	0.25
50%	0.9026	0.8914	98.8	0.10
100%	1.8053	1.8016	99.8	0.0
150%	2.7079	2.7331	100.9	0.6
200%	3.6105	3.6177	100.2	0.3
Propylparaben				
20%	0.0412	0.0415	100.8	0.2
50%	0.1030	0.1050	101.9	0.3
100%	0.2060	0.2066	100.3	0.2
150%	0.3090	0.3067	99.3	0.6
200%	0.4058	0.4007	98.8	0.4
Potassium sorbate				
20%	0.2004	0.1978	98.7	1.0
50%	0.5078	0.5134	101.1	0.6
100%	1.0019	0.9836	98.2	1.1
150%	1.5233	1.5105	99.1	0.6
200%	2.0310	2.0181	99.4	0.2

Range

The range of Analytical Method is the interval between the upper and lower levels of analyte that has been demonstrated to be determined with a suitable Accuracy and Linearity.

Determined the specified ranges from accuracy and linearity studies, the obtained results are presented in Table 8 and Table 9. Accuracy range graph and linearity range graph is shown in Figure 10 to Figure 17.

Table 8: The results of Accuracy range.

Accuracy range				
Level (Concentration in %)	**Mean area response of Sennosides**	**Mean area response of Methylparaben**	**Mean area response of Propylparaben**	**Mean area response of Potassium sorbate**
20		900195.583	89000.952	1020431.348
50	194647.376	2236882.607	225121.171	2648538.696
100	380104.159	4521000.787	442885.563	5073927.435
150	568596.170	6858354.255	657620.909	7791770.431
200		9078303.604	859190.477	10410300.525
Correlation Coefficient	1.000	1.000	1.000	1.000
RSD	1.3%	0.8%	1.7%	1.7%

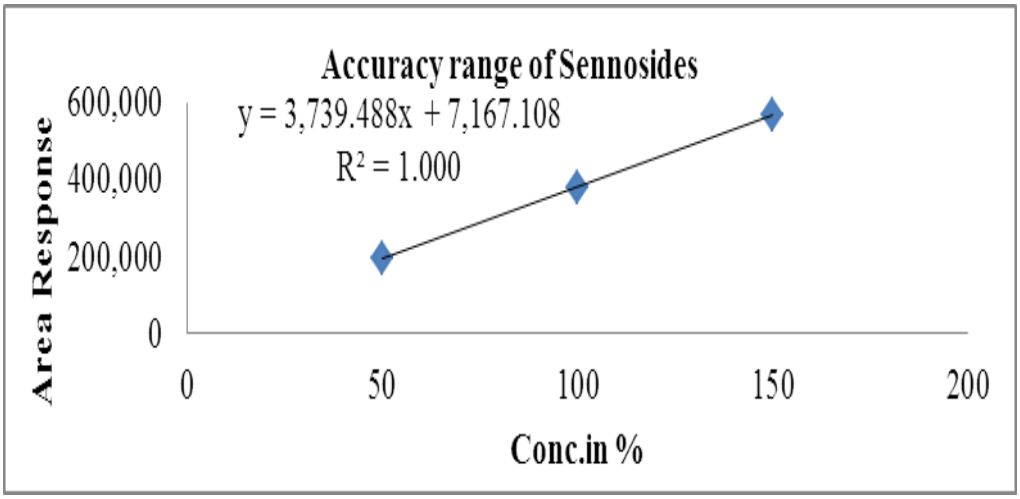

Figure 10: Accuracy range graph for Sennosides.

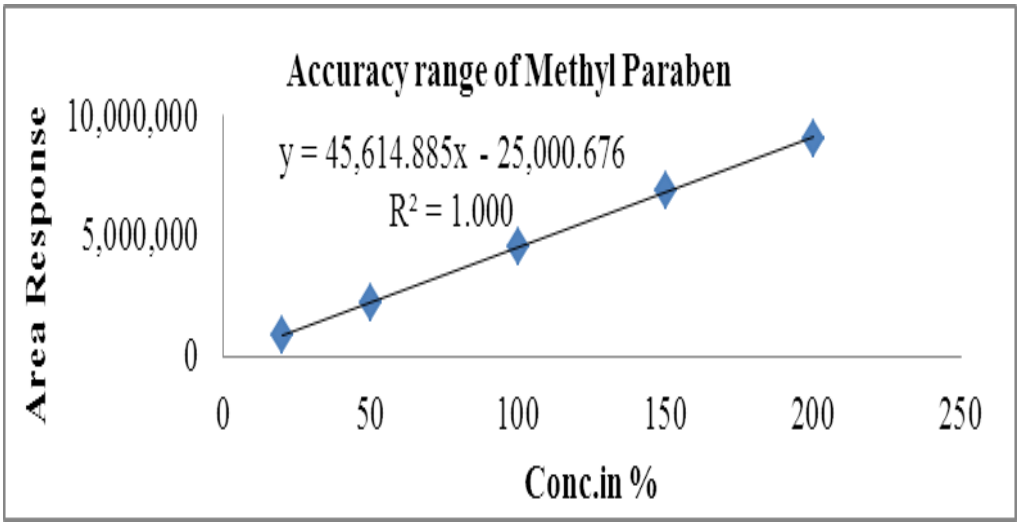

Figure 11: Accuracy range graph for Methylparaben

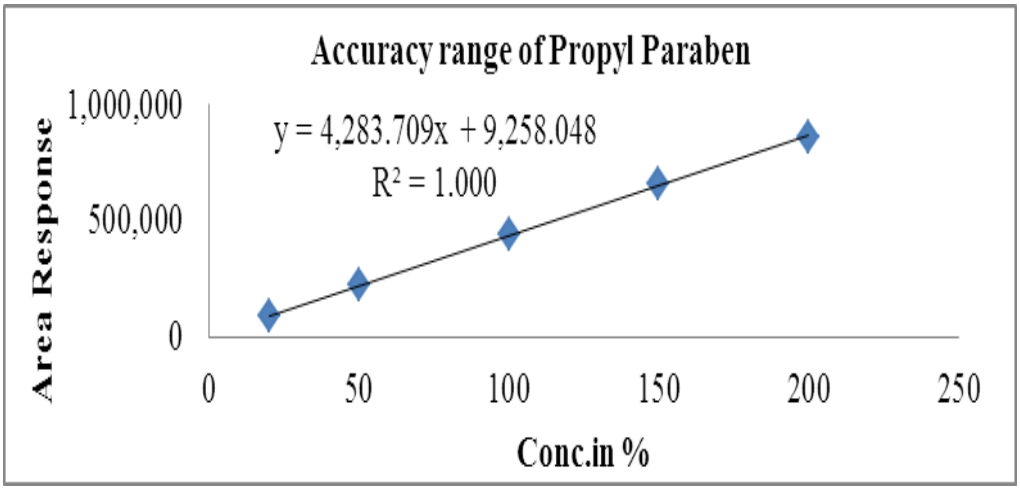

Figure 12: Accuracy range graph for Propylparaben

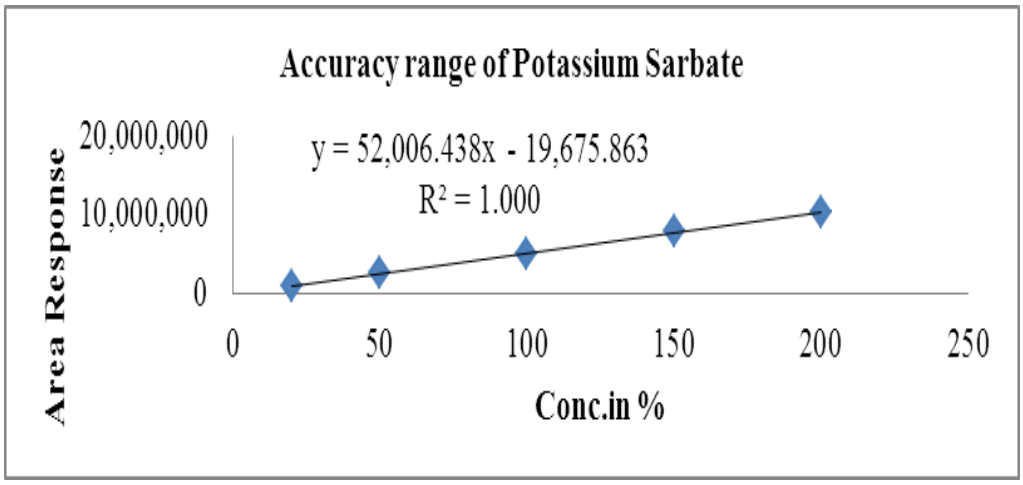

Figure 13: Accuracy range graph for Potassium sorbate.

Table 9: The results of Linearity range

Linearity range				
Level (Concentration in %)	Mean area response of Sennosides	Mean area response Methylparaben	Mean area response Propylparaben	Mean area response Potassium sorbate
20		926886.766	59965.712	1069314.759
50	204404.959	2386746.136	173358.822	2732232.838
100	392737.573	4654160.312	356122.024	5278245.481
150	612073.424	7237561.340	567742.465	8186499.622
200		9353626.815	742927.486	10557299.54
Correlation Coefficient	0.999	0.999	0.999	1.000

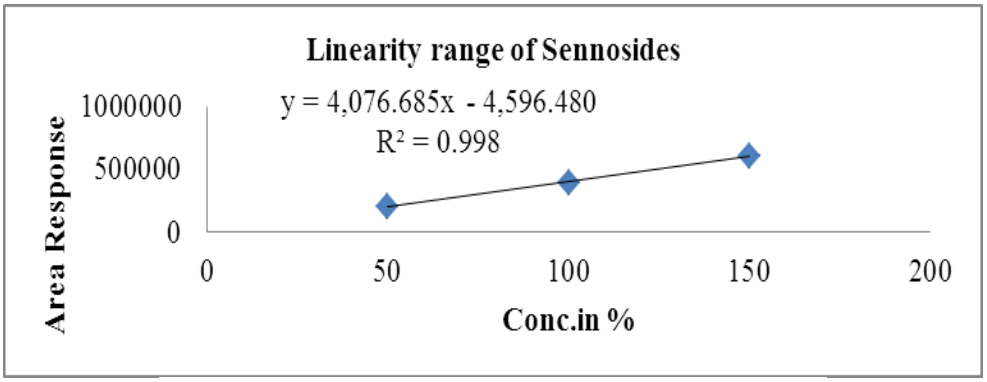

Figure 14: Linearity range graph for Sennosides

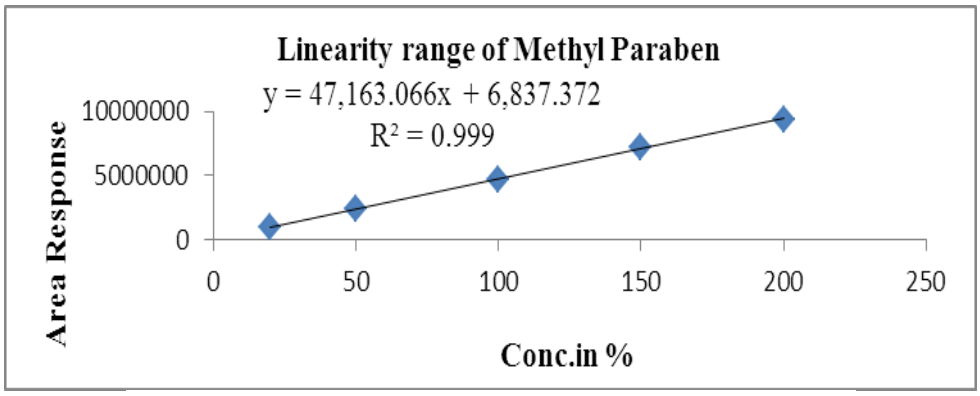

Figure 15: Linearity range graph for Methylparaben

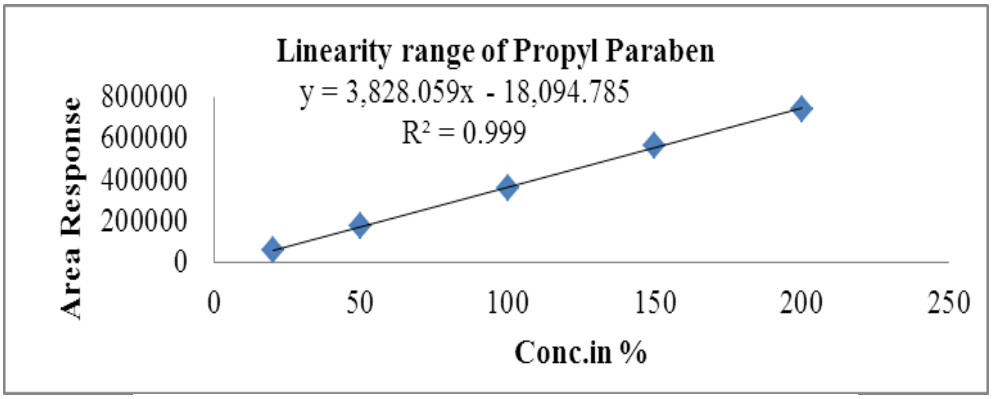

Figure 16: Linearity range graph for Propylparaben

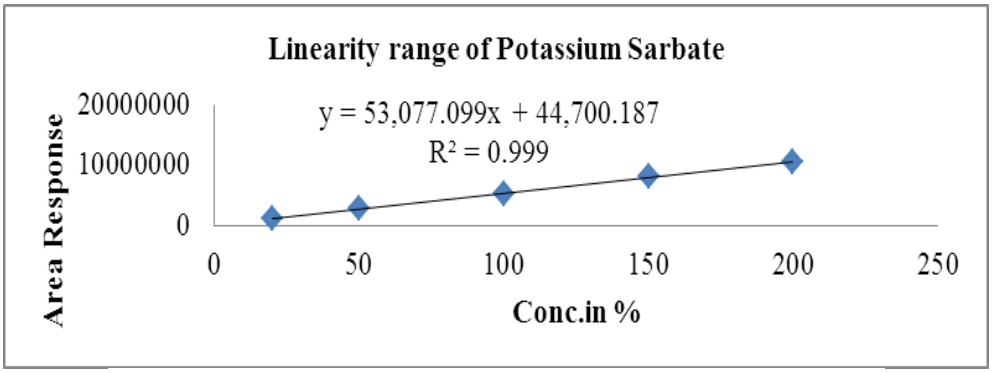

Figure 17: Linearity range graph for Potassium sorbate.

ROBUSTNESS

The robustness of analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Evaluated the robustness of the analytical method by the experimental conditions were deliberately altered with change in column temperature ± 5°C, change in flow rate ± 0.2mL/min and change in mobile phase pH ± 0.2 units. The system suitability criteria are within the acceptance limit for all the above changed conditions, the obtained data is given in Table.6.

Table.5: The results of Robustness study.

Altered conditions of the method for Robustness Study	Sennoside A	Sennoside B	Methyl paraben	Propyl paraben	Potassium sorbate
	Tailing factor for the each analyte (sennoside A+sennoside B, methylparaben, propylparaben and potassium sorbate) peak from the standard solution preparation should be NMT 2.0.				
Original Condition	1.410	1.430	1.260	1.300	1.310
Increase in Flow	1.364	1.388	1.306	1.302	1.279
Decrease in Flow	1.544	1.540	1.348	1.516	1.369
Increase in Temperature	1.528	1.457	1.377	1.372	1.367
Decrease in Temperature	1.262	1.476	1.395	1.462	1.337
Increase in pH (+0.2 Units)	1.520	1.420	0.950	0.970	0.940
Decrease in pH (-0.2 Units)	1.510	1.380	0.950	0.850	0.930
	% RSD of the each analyte (sum of sennosides (sennoside A+sennoside B), methylparaben, and propylparaben and potassium sorbate) peak area from the 5 replicate injections of standard solution preparation should be NMT 2.0%				
Original Condition	0.4%	0.1%	0.1%	0.1%	0.4%
Increase in Flow	0.2%	0.0%	0.2%	0.0%	0.2%
Decrease in Flow	0.6%	0.0%	0.2%	0.2%	0.6%
Increase in Temperature	0.1%	0.1%	0.1%	0.0%	0.1%
Decrease in Temperature	0.1%	0.0%	0.1%	0.0%	0.1%
Increase in pH (+0.2 Units)	0.3%	0.2%	0.1%	0.2%	0.3%
Decrease in pH (-0.2 Units)	0.2%	0.1%	0.1%	0.1%	0.2%
	Theoretical plates for the each analyte (Sennoside A+ Sennoside B, Methyl Paraben, and Propyl Paraben and Potassium Sorbate) peak from the Standard Preparation should be NLT 2000.				
Original Condition	68150	41967	39795	849803	88467
Increase in Flow	45686	26002	55370	775294	50193
Decrease in Flow	58166	30864	241406	221408	50059

Increase in Temperature	43329	26921	35291	526393	51497
Decrease in Temperature	56000	30220	21807	479135	58250
Increase in pH (+0.2 Units)	27434	21576	32498	852493	48670
Decrease in pH (-0.2 Units)	48487	25314	34978	812837	54161

CONCLUSION

The proposed Reverse Phase High Pressure Liquid Chromatographic method with a novel gradient mode was found to be a simple, specific, linear, precise, robust and accurate. The method was completely validated showing satisfactory data for all the method validation parameters tested. The developed method is stability indicative and can be used for quantitative determinations of the Ciprofloxacin hydrochloride in presence of degradation products in stability by the Pharmaceutical industries.

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