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A RP- HPLC Method Development for Analysis of Selected Synthetic Analgesic Agents in Herbal Formulation

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ABSTRACT

Objective: *Herbal formulations are popular in worldwide due to safer and not causing any toxicity. Recently there have been reported herbal medicines adulterated with undeclared synthetic drugs. A simple, specific, accurate and isocratic RP-HPLC method was developed and subsequently validated for determination of adulterant.*

Methods: *Separation was achieved with a Phenomax Luna C¬18 (250 × 4.5 mm) 5µm column with a mobile phase comprising phosphate buffer: Acetonitrile pH-4 adjusted with 0.1% ortho phosphoric acid in the volume ratio of (45:55% v/v) was developed. The detection was carried out using a photo diode array detector set at a wavelength of 254 nm.*

Results: *The method was validated as per ICH guideline demonstrating the accuracy and precision within corresponding linearity range of 10 µg/ml to 60 µg/ml and get the correlation coefficient of paracetamol, diclofenac sodium and aceclofenacwerefound to be 0.995, 0.997, and 0.995, respectively.*

Conclusion: *The developed method was validated with respect to linearity, accuracy, precision limit of detection and quantification and robustness. The method can be used for quality control of herbal formulation.*

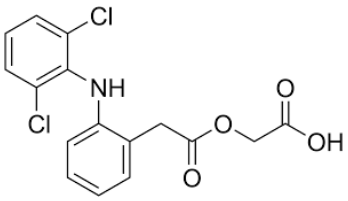
Keywords: Analgesics, Herbal formulation, Adulteration, RP-HPLC

INTRODUCTION

Traditional herbal medicines are used in different places of world by practitioner of traditional system of medicines – Indian Ayurvedic medicine, Traditional Chinese medicine, Arabic Unani medicine, Kampo medicine system in Japan, African & Latin American practices and used as primary home medicine. Herbal medicines are currently used and their popularity is increasing day by day. Today estimate that about 80% of people in developing countries still relays on traditional medicine based largely on species of plants and animals for their primary health care [1-6].

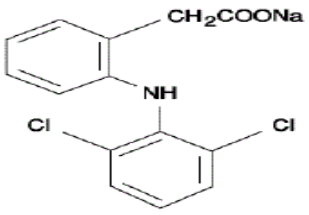
An examination of distributed herbal medicine thus becomes an important issue to prevent harmful side effects due to adulterated herbal medicine. As previously reported glibenclamide was detected in food product, the adulterant contain drugs with associated with serious toxic effects like steroids. Traditional herbal medicines are not well regulated by regulation and it easily available from health food stores, in super market and on internet. Due to poor regulation herbal product contaminated and adulterated with illegal synthetic drugs. In 1997 US market withdraw after studies the weight loss supplement and hypertension drug sold and marketed as "all natural blend of Chinese herbs" contain not only flenfuramide, stimulant which cause heart valve damage and also found three potentially harmful drugs beta blocker which can harm people with asthma and weight loss drug sibutramine which increased risk of heart attack and stroke in patients [7-11].

Herbal formulation shall mean a dosage form consisting of one or more herbs or processed herb (s) ins pecified quantities to provide specific nutritional, cosmetic benefits, and/or other benefits meant for use to diagnose, treat, and mitigate diseases of human beings or to alter the structure or physiology of human beings or animals [12-29]. Analyses of the adulterated synthetic analgesic drug in herbal formulation are carried out in two steps, First, extract the analytes of interest by extraction method and Separated, quantified and analyzed by chromatography Herbal formulation generally contains large number of chemical constituents so the extraction of the desired analyses is so difficult task. Solvent extraction is a method used for the separation of a mixture using two immiscible solvents [30]. In solvent extraction, one of the phasesis aqueous and the other is an immiscible organic solvent. The concept "like dissolves like" works in solvent extraction [31-35]. Aceclofenac (ACE), chemically [[2- (2', 6-dichlorophenyl) amino] phenylacetoxyacetic acid], is a phenylacetic acid derivative with potent analgesic and anti-inflammatory properties and an improved gastrointestinal tolerance. Paracetamol (PCM) is chemically N- (4-hydroxyphenyl) acetamide. It is used as an analgesic and antipyretic [36,37]. Diclofenac sodium (DIC) is, sodium [2- (2, 6-dichloroanilino) phenyl] acetate, used as analgesic and anti-inflammatory drug [37].



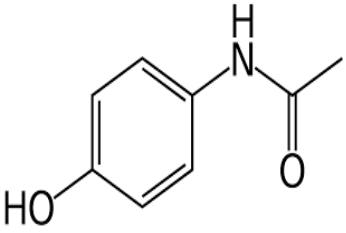
Aceclofenac

pKa – 4.7



Diclofenac sodium

pKa - 4.15



Paracetamol

pKa - 9.38

The main objective of present study was detection of synthetic analgesic drugs from herbal formulation by using high performance liquid chromatography. Reversed phase chromatography is the most popular chromatography for analytical and preparative separations of compound of interest in herbal, biological, pharmaceutical, chemical, food and biomedical sciences. The material used as non-polar packingis C_8, C_8, Phenyl, C_3, etc. Most reverse phase separations are carried out using a buffered aqueous solution as a polar mobile phase.

MATERIALS AND METHODS

Instruments

The chromatography analysis was performed using a Shimadzu LC-10ATHPLC separation module (Shimadzu, Japan) equipped with a photo diode array detector (SPD – M10A). The output signal was checked and processed LC Solution using Software. The pH of the solutions was measured by a Digital pH meter (HANNA).

Chemicals and reagents

Paracetamol, diclofenac sodium and aceclofenac were purchased by college, HPLC grade acetonitrile, ortho phosphoric acid (0.1%), Water (HPLC grade) and all other chemicals were obtained from Labchem Chemicals.

Chromatographic conditions

The method was developed by using a Phenomax Luna C_{18} (250 × 4.5 mm) 5µm column with a mobile phase comprising phosphate buffer: acetonitrile pH 4 adjusted with 0.1% OPA in the volume ratio of (45:55% v/v). Flow rate of the mobile phase was 1.0 ml/min and the eluted compounds were monitored at the wavelength of 254nm.

Preparation of mobile phase

13.6 g of potassium dihydrogen phosphate (KH_2PO_4) dissolved in 1000 ml of water adjusted pH 4.0 with ortho-phosphoric acid.

Preparation of standard solution

Preparation of Standard Stock Solution and Working Standard Solution of mixture of paracetamol, diclofenac sodium and aceclofenac

Accurately weighed 5 mg each of paracetamol, diclofenac sodium and aceclofenac than transferred into 50 ml volumetric flask, dissolved it in acetonitrile. The samples were sonicated for 10 minutes and filter using 0.45 µm nylon filters. Volume of filtrate made up to the mark with water to give a stock solution having strength 100 µg/ml of each.

Test sample preparation

Selective solvent extraction from herbal formulation for analysis

Selected synthetic analgesic agent is soluble in methanol. The extraction of selected analgesic agents paracetamol, diclofenac sodium and aceclofenac were performed at pH 2 was adjusted to aqueous by the buffer solution. Solution of Extract was taken separately in separating funnel; 30 ml of chloroform was added in each separating funnel and shake it properly. Keep separating funnel aside for five minutes allow organic layer and aqueous layer to separate, the organic layer and aqueous layer were collected separately in to two different conical flasks. Step no 2 and 3 was repeated two times with unsaturated chloroform with same aqueous layer, homogenize the all the step chloroform layer. The absorbance of all homogenize chloroform solution was measured by UV Spectrophotometer. Chloroform layer was completely evaporated at low temperature without degrading the drug. The residues after the evaporation were reconstituted by the methanol. TLC was carried out on reconstituted solution. Step No. 1 to 8 were continual by using the different buffer solution (pH 3, 4, 5, 6, 8, 9) for each solution. Compare the absorbance of the three drugs in chloroform solution which was previously extracted by the different pH solution, and subjected to UV absorbance, TLC and HPLC At pH 10, extraction of drugs found higher. The selected Analgesic agents were spiked into herbal sample (1 g) and extracted as procedure described in section 4 , 3 , 5 , 2 and subjected to TLC and RP-HPLC. Overlay Spectra of selected anti analgesic drug agents.

Validation procedure

Method validation was achieved as per ICH guidelines for determination of the paracetamol, diclofenac sodium and aceclofenac. The following validation features were addressed: linearity, detection limit, quantification limit, precision, accuracy, robustness and specificity.

Linearity

Standard solutions at six different concentration levels ranging from 10 µg/ml to 60 µg/ml were prepared and analyzed in order to demonstrate the linearity. The regression curve was obtained by plotting peak area versus concentration. The regression equation was obtained by using the regression analysis.

Accuracy

The standard addition and recovery experiments were conducted to demonstrate the accuracy of the method. The accuracy of the method evaluated in triplicate at three concentration levels, i.e., 50%, 100% and 150% of target test concentration and the percentages of recoveries were calculated.

Precision

The Precision of the method was determined by injecting a standard solution of paracetamol, diclofenac sodium and aceclofenac for six times and measured the area for all six injections in HPLC chromatographic system.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of Components which may be expected to be present. Typically these might include impurities, Degradants, matrix, etc. In these we specifically found paracetamol, aceclofenac, and diclofenac sodium in herbal preparation.

Limits of detection and quantification (LOD and LOQ)

The sensitivity of the method was measured by calculating the limit of detection and limit of quantification. The LOD and LOQ were assessed at signals to noise ratio of 3:1 and 10:1 respectively by injecting a series of dilute solutions with known concentrations.

Robustness

The robustness of a method was demonstrated by altering experimental conditions and chromatographic resolution to evaluate robustness. The deliberate changes were made in the chromatographic conditions, viz. change in flow rate by ± 0.1 ml/min and change in the buffer concentration ± 5%.

RESULTS AND DISCUSSION

Chromatograms depicting the method development

Table 1: Optimized chromatographic conditions.

Column	Enable C_{18} Phenomax Luna (250 mm × 4.5 mm) 5 µm
Mobile phase	Phosphate Buffer: Acetonitrile (45:55 v/v) pH 4 adjust with 0.1% OPA × OPA
Injection volume	20 µl
Flow rate	1.0 ml/min
Detection wavelength	254 nm
Temperature	Roomtemperature
Run time	10 minutes
Diluent	Water
Note: *OPA – Ortho Phosphoric Acid	

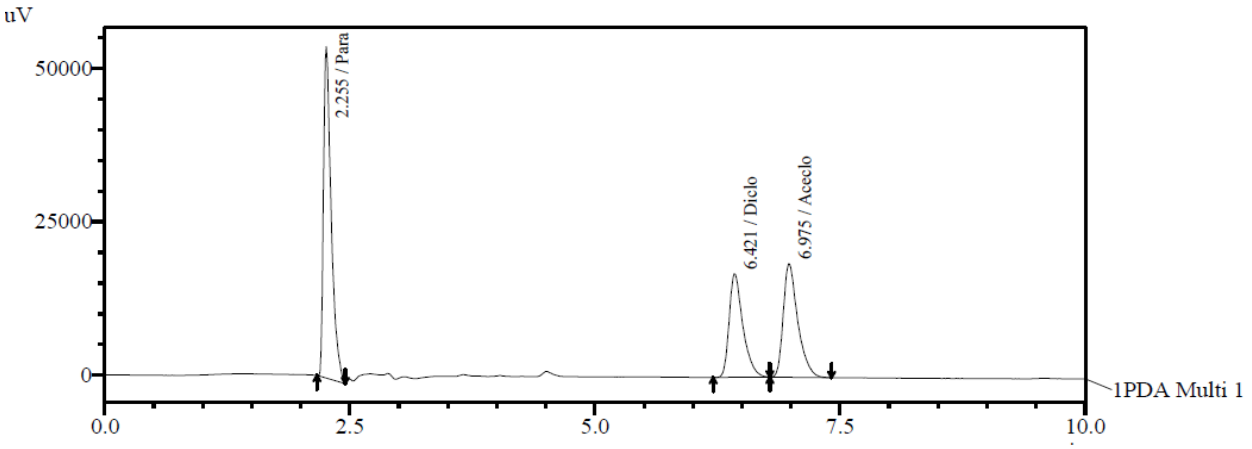


Figure 1: Optimized chromatogram.

Chromatogram of optimized mobile phase phosphate buffer: acetonitrile (45:55 v/v) pH 4 adjust with 0.1 % OPA at 1.0 ml/min flow rate at 254 nm.

Table 2: System suitability data.

Parameters	PCM	DIC	ACE
Retention time (min)	2.25 ± 0.03	6.4 ± 0.07	7.02 ± 0.17
Theoretical plates	3256 ± 112.18	9406 ± 927.67	10069 ± 1023.96
Tailingfactor	0.77 ± 0.38	1.18 ± 0.17	1.06 ± 0.01
Resolution	0.00	2.89	3.20
Note: PCM - Paracetamol, DIC – Diclofenac sodium, ACE - Aceclofenac			

System suitability criteria

Analytical method validation

Linearity: The linearity of the optimized method was determined for six concentrations (n=6) and the correlation coefficient for paracetamol, diclofenac sodium and aceclofenac was found to be 0.995, 0.997 and 0.995, respectively. It was found that Lambert-Beer's law was followed in the concentration ranges of 10-60 µg/ml for paracetamol, diclofenac sodium and aceclofenac.

Table 3: Linearity data for paracetamol, diclofenac sodium and aceclofenac.

Conc. (µg/ml)	PCM		DIC		ACE	
	Mean Area ± SD	% RSD	Mean Area ± SD	% RSD	Mean Area ± SD	% RSD
10	305346 ± 3256	1.07	164434 ± 1598	0.97	190719 ± 2689	1.41
30	698437 ± 6589	0.94	479039 ± 5632	1.18	539345 ± 5425	1.01
40	930260 ± 5568	0.60	593349 ± 6584	1.11	644433 ± 2547	0.40
50	1210499 ± 9589	0.79	776392 ± 5625	0.72	855210 ± 7845	0.92
60	1439034 ± 8589	0.60	908392 ± 5698	0.63	1018064 ± 5546	0.54

Note: PCM – Paracetamol, DIC- Diclofenac Sodium, ACE – Aceclofenac, SD – Standard Deviation, % RSD – % Relative Standard Deviation

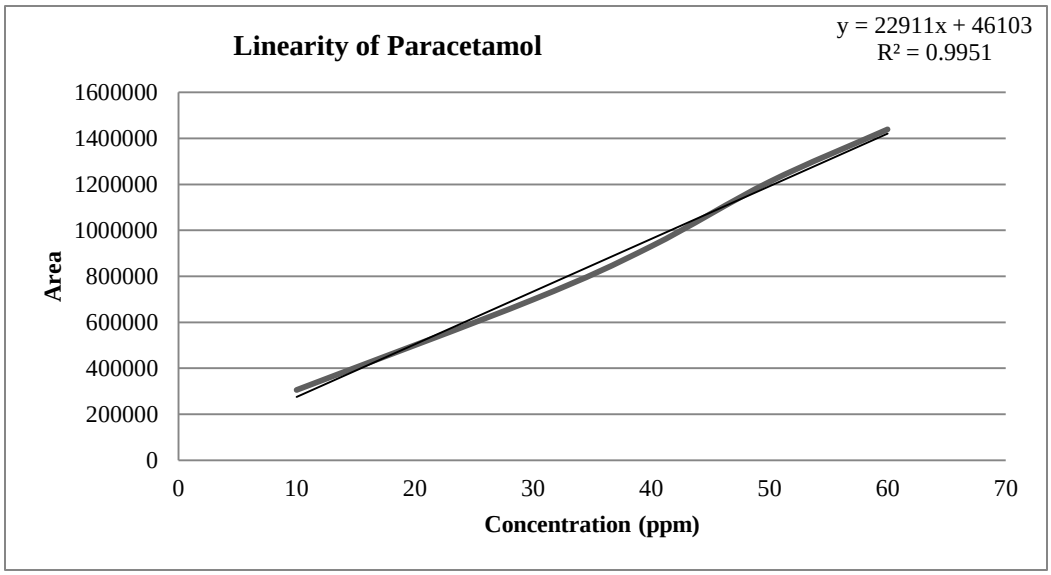


Figure 2: Calibration curve for paracetamol.

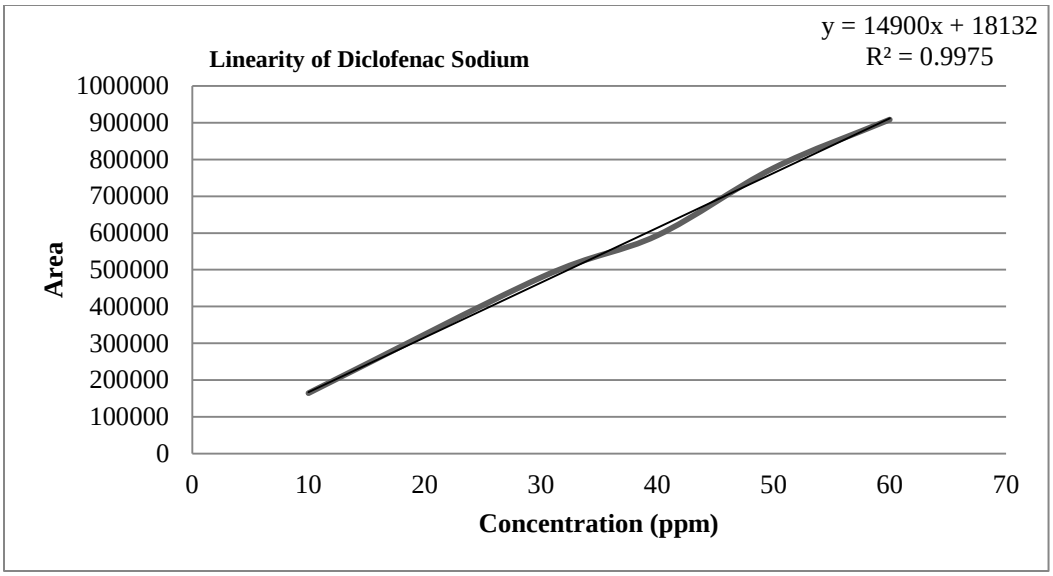


Figure 3: Calibration curve for diclofenac sodium.

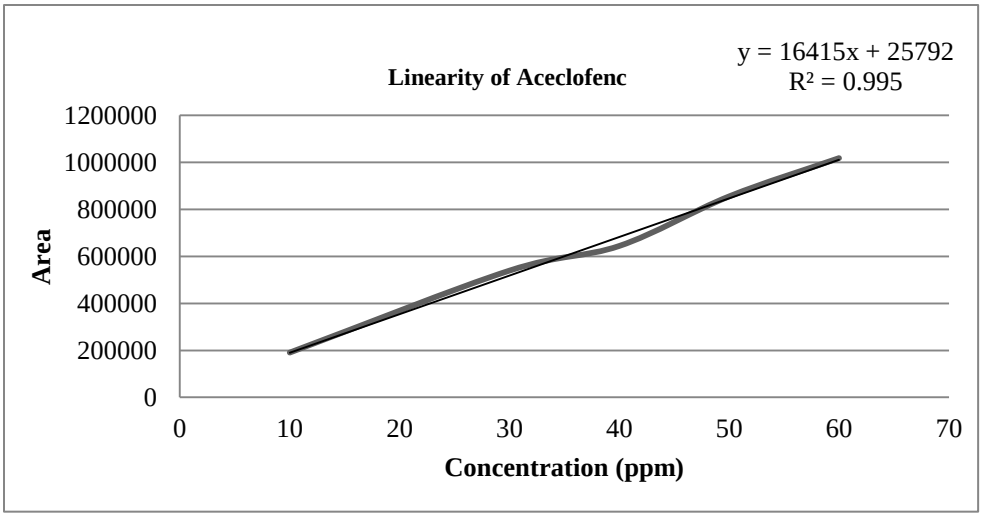


Figure 4: Calibration curve aceclofenac.

Accuracy

A known amount of paracetamol, diclofenac sodium and aceclofenac (4 mg, 8 mg, 12 mg) working standard were added to composite herbal formulations and the overall recoverywas estimated as shown in Table 4.

Table 4: Accuracy data for paracetamol, diclofenac sodium and aceclofenac.

Drug	Amount of standard added (mg)	Amount of standard found (mg)	%Recovery Mean ± SD
PCM	4	3.73	93.33 ± 0.23
	8	7.74	96.71 ± 0.58
	12	11.46	95.47 ± 0.56
DIC	4	3.76	94.00 ± 0.35
	8	7.33	91.63 ± 0.54
	12	11.47	95.58 ± 0.78
ACE	4	3.76	93.90 ± 0.35
	8	7.66	95.80 ± 0.14
	12	11.30	94.20 ± 0.65
Note: PCM – Paracetamol, DIC- Diclofenac Sodium, ACE – Aceclofenac; SD – Standard Deviation			

Precision

Repeatability: The repeatability data for paracetamol, diclofenac sodium and aceclofenac (40 µg / ml) were shown in Table 5. The% RSD for paracetamol, diclofenac sodium and aceclofenac were found to be 0.74, 1.74 and 1.05, respectively.

Table 5: Repeatability data.

S. No.	Peak area (40 µg/ml n = 6)		
	PCM	DIC	ACE
1	981583	598645	667894
2	972356	589865	685213
3	975698	575682	685642
4	980256	584572	685265
5	990256	598642	685895
6	970256	576589	678521
Mean	978401	587333	681405
SD	7270.4	10208.5	7188.6
% RSD	0.74	1.74	1.05
Note: PCM - Paracetamol, DIC – Diclofenac Sodium, ACE – Aceclofenac; N- Number of Injections =6, SD – Standard Deviation, % RSD –% Relative Standard Deviation			

Intraday precision

The data for intraday precision for paracetamol, diclofenac sodium, aceclofenac are shown in Table 6. The % RSD of paracetamol, diclofenac sodium and aceclofenac were found to be 0.41-1.22, 0.47-0.94 and 0.92-1.31, respectively.

Interday precision

The data for interday precision paracetamol, diclofenac sodium and aceclofenac were shown in Table 6. The% RSD of paracetamol, diclofenac sodium and aceclofenac were found to be 0.31-0.82, 0.32-0.72 and 0.37-1.04, respectively

Table 6: Intraday and Interday precision data for paracetamol, diclofenac sodium, aceclofenac.

Drug	Conc. (µg/ml)	Intra-day precision		Inter-day precision	
		Mean area ± SD (n=3)	% RSD	Mean area ± SD (n=3)	% RSD
PCM	10	304685.67 ± 1240.21	0.41	325610.00 ± 1005.96	0.31
	40	931135.67 ± 5927.21	0.64	936992.00 ± 7684.25	0.82
	60	1440385.00 ± 17534.58	1.22	1431317.33 ± 5111.02	0.36
DIC	10	165317.33 ± 776.9	0.47	164133.00 ± 529.68	0.32
	40	593387.33 ± 5558.6	0.94	602032.33 ± 2927.95	0.49
	60	909769.67 ± 4950.4	0.54	909959.00 ± 6517.49	0.72
ACE	10	192958.33 ± 2526.9	1.31	194634.00 ± 974.16	0.5
	40	650964.67 ± 7984.0	1.23	637908.00 ± 6605.49	1.04
	60	1026081.33 ± 9467.3	0.92	1028240.33 ± 3756.23	0.37

Note: PCM - Paracetamol, DIC – Diclofenac Sodium, ACE – Aceclofenac; N: Number of Injections =3, SD – Standard Deviation, % RSD – % Relative Standard Deviation

Specificity

The specificity was demonstrated by the comparison of the chromatograms:

- Standard solution of mixture of paracetamol, diclofenac sodium, aceclofenac (Figure 5).
- Solution of unspiked herbal sample is equivalent to the diluents (Figure 6).
- Solution of spiked herbal sample with paracetamol, diclofenac sodium, aceclofenac (Figure 7).

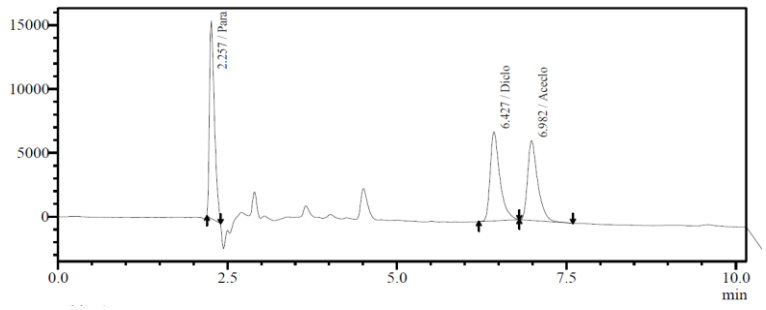


Figure 5: Chromatogram of standard solution of paracetamol, diclofenac sodium, aceclofenac.

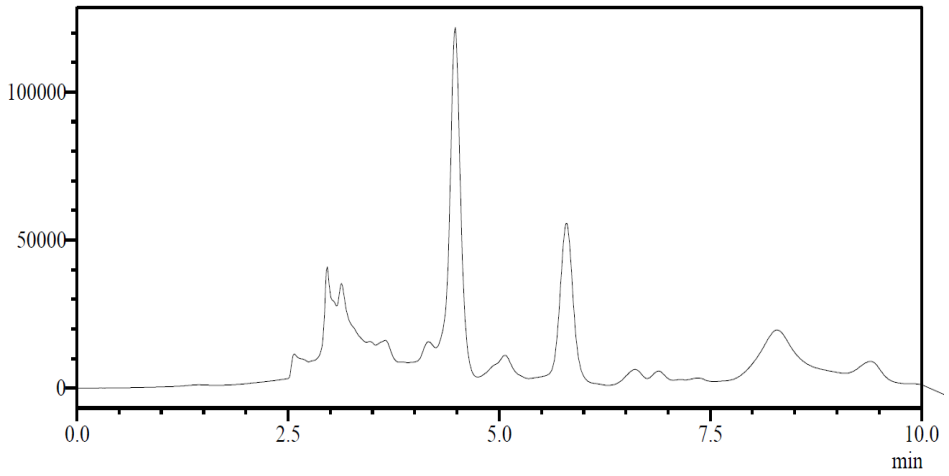


Figure 6: Chromatogram of unspiked herbal sample is equivalent to the herbal formulations.

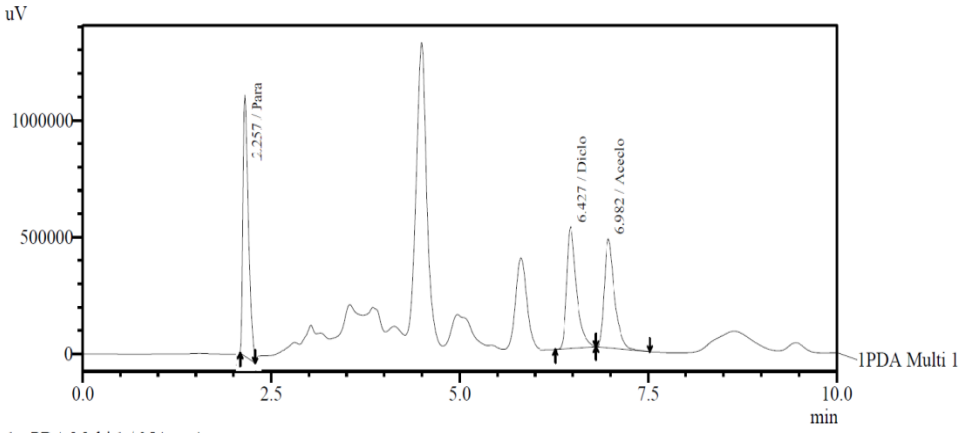


Figure 7: Chromatogram of spiked (paracetamol, diclofenac sodium, aceclofenac) Herbal sample.

Limit of detection (LOD)

LOD for paracetamol, diclofenac sodium and aceclofenac were found to be 3.61, 3.10, and 3.61, respectively. The LOD data for paracetamol, diclofenac sodium andaceclofenac were shown in Table 7.

Limitof Quantification (LOQ)

LOQ for paracetamol, diclofenac sodium, and aceclofenac were found to be 10.94, 9.39, and 10.95, respectively. The LOQ data for paracetamol, diclofenac sodium, aceclofenac were shown in Table 7.

Table 7: Data of limit of detection and limit of quantification.

Drug	LOD (µg/ml)	LOQ (µg/ml)
PCM	3.61	10.94
DIC	3.1	9.39
ACE	3.61	10.95
Note: PCM – Paracetamol, DIC- diclofenac sodium, ACE - aceclofenac		

Robustness

The variable parameters change was done inflow rate and composition of buffer. % RSD for area were calculated which found to be less than 2. The change in flow rate data and buffer composition data are shown in Table 8.

Table 8: Robustness data of variation in flow rate and buffer concentration for paracetamol, diclofenac sodium, and aceclofenac.

Parameters		PCM		DIC		ACE	
	Method condition	Mean Assay	%	Mean Assay	%	Mean Assay	%
		± S.D.	RSD	± S.D.	RSD	± S.D.	RSD
Flow rate (ml/min)	0.8	98.80 ± 0.67	0.7	99.68 ± 0.52	0.5	99.78 ± 0.17	0.2
	1	100.23 ± 0.34	0.3	100.48 ± 0.66	0.7	99.23 ± 1.15	1.2
	1.2	99.89 ± 1.15	1.2	100.34 ± 0.31	0.3	100.00 ± 0.53	0.5
Buffer composition	Increase by 5%	99.29 ± 0.56	0.6	100.46 ± 0.85	0.8	100.15 ± 0.46	0.5
	Optimized Condition	100.28 ± 0.67	0.7	99.93 ± 0.56	0.6	100.00 ± 0.52	0.5
	Decrease by 5%	99.46 ± 0.85	0.9	100.02 ± 0.49	0.5	99.99 ± 0.50	0.5
Note: PCM - Paracetamol, DIC – Diclofenac Sodium, ACE – Aceclofenac; SD – Standard Deviation, % RSD - % Relative Standard Deviation							

Table 9: Summary of validation parameters.

S. No.	Parameters	PCM	DIC	ACE
1	Linearity (n=6) (µg/ml)	10-60	10-60	10-60
2	Correlation coefficient	0.995	0.997	0.995
3	Accuracy (% recovery) (n=3)	93.3-96.7	91.6-95.6	93.9-95.8
	Precision (% RSD)			
4	Repeatability (n=6)	0.74	1.74	1.05
5	Intraday precision (n=3)	0.41-1.22	0.47-0.94	0.92-1.31
6	Interday precision (n=3)	0.31-0.82	0.32-0.72	0.37-1.04
7	LOD	3.61	3.10	3.61
8	LOQ	10.94	9.39	10.95
Note: PCM - Paracetamol, DIC – Diclofenac Sodium, ACE – Aceclofenac, N- Number Of Injections, LOD – Limit of Detection, LOQ – Limit Of Quantification, % RSD – % Relative Standard Deviation				

Compilation of validation parameters

Screening of marketed herbal formulations by RP-HPLC method

Different Herbal formulations from different manufacturer subjected to pH selective extraction and analysis of these herbal formulations were performed by RP-HPLC method. One out of six different Herbal formulations was found to be adulterated with synthetic anti-asthmatic prednisolone observed by developed selective extraction method and RP-HPLC. Chromatogram of different Herbal samples shown in Figures 8-17.

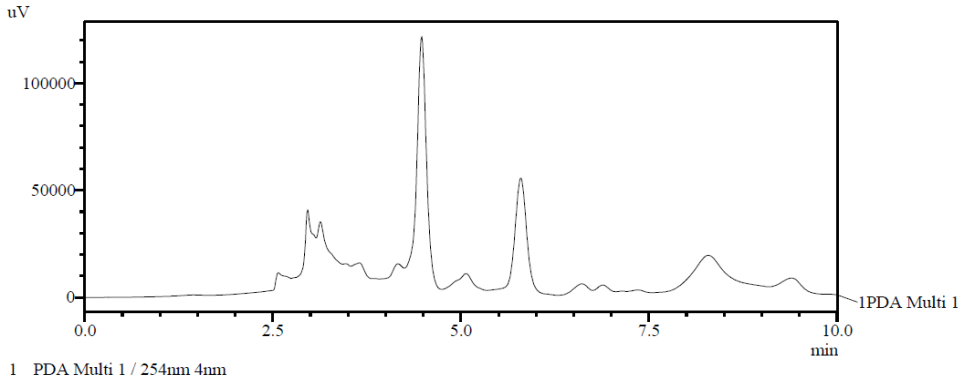


Figure 8: Chromatogram of blank.

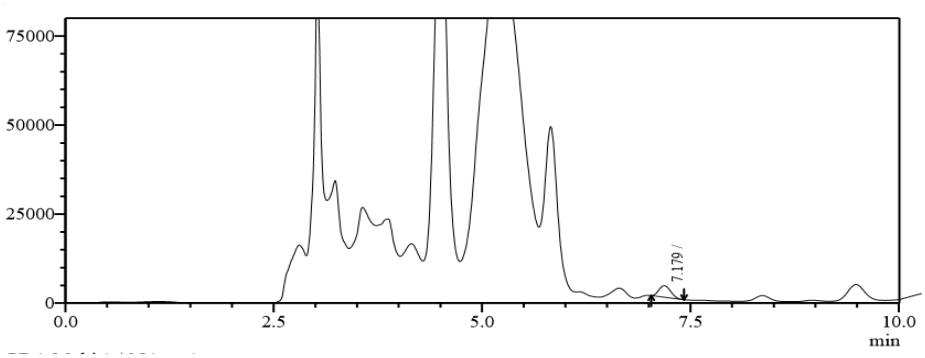


Figure 9: Chromatogram of marketed herbal sample (H-A).

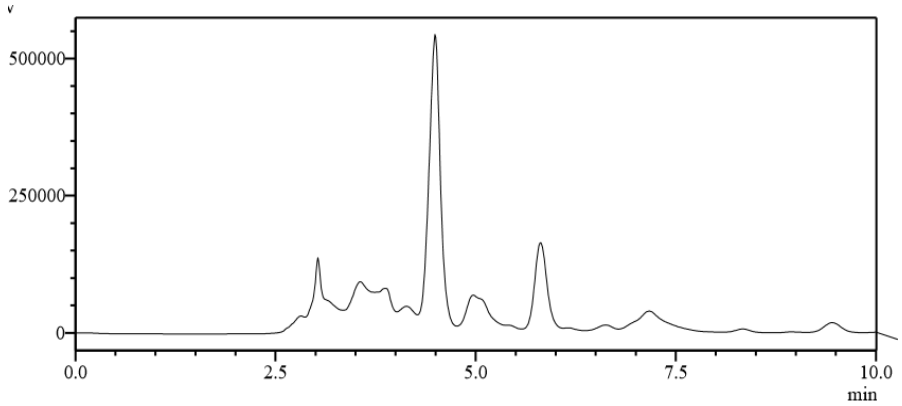


Figure 10: Chromatogram of marketed herbal sample (H-B).

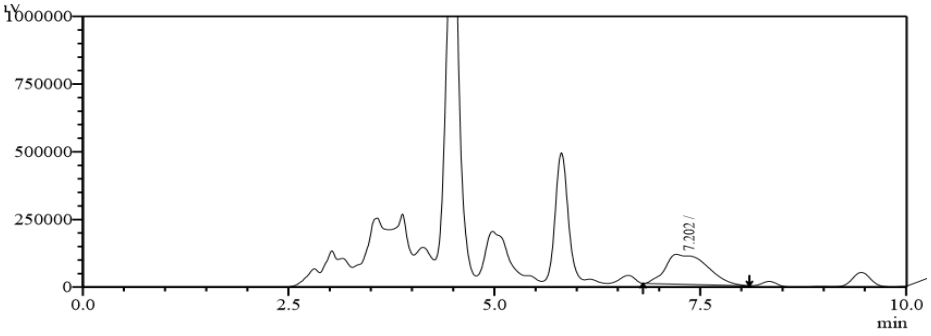


Figure 11: Chromatogram of marketed herbal sample (H-C).

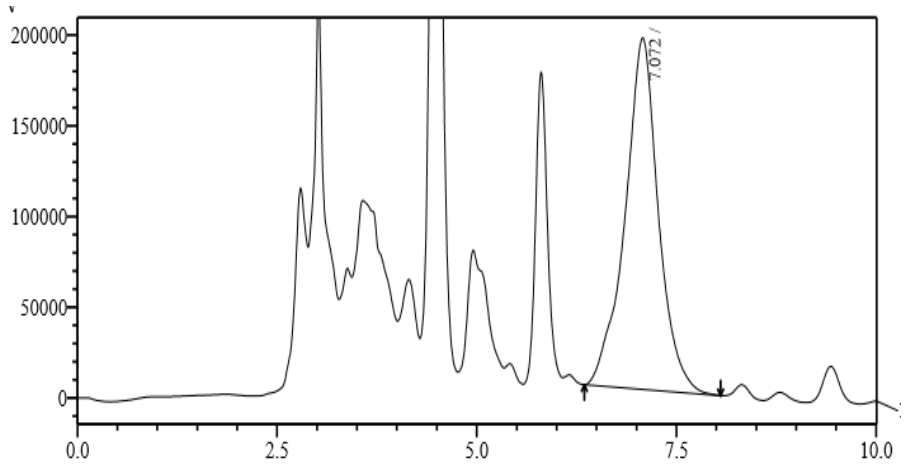


Figure 12: Chromatogram of marketed herbal sample (H-D).

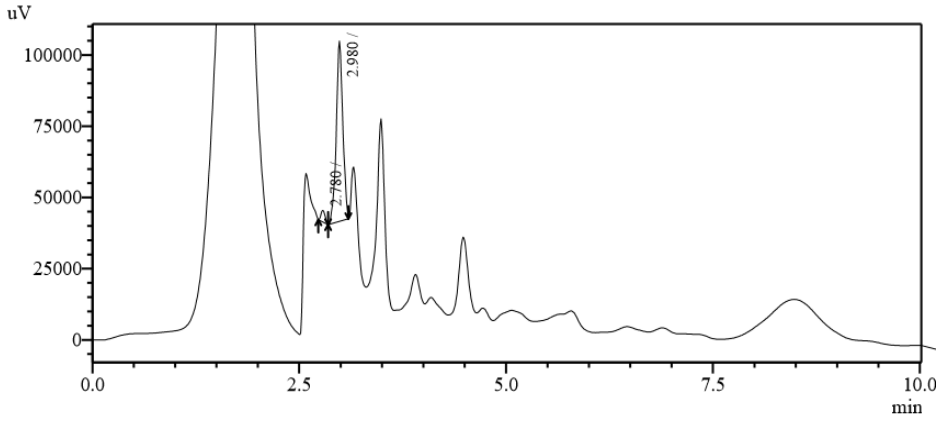


Figure 13: Chromatogram of marketed herbal sample (H-E).

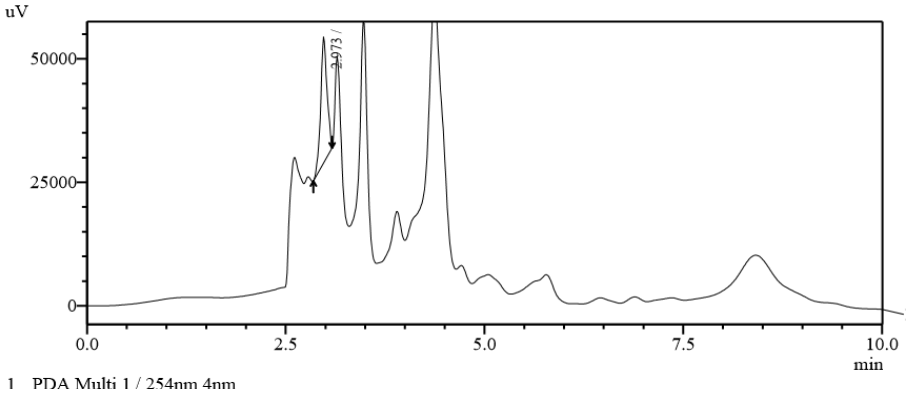


Figure 14: Chromatogram of marketed herbal sample (H-6).

Confirmation of detected adulterant by LC-MS

Extracted herbal sample

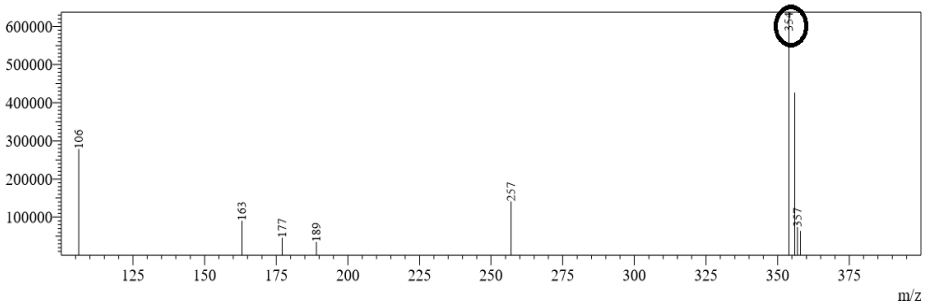


Figure 15: Confirmation of the marketed herbal formulation by mass spectrometer (for aceclofenac).

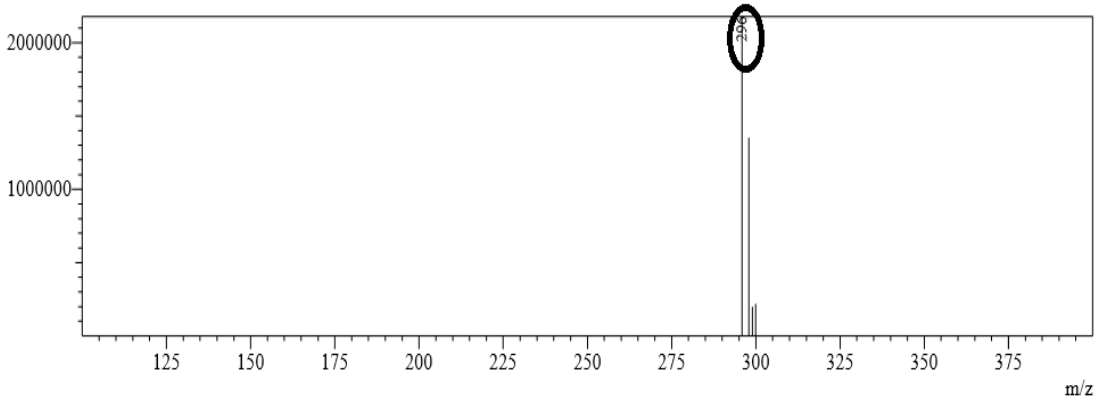


Figure 16: Confirmation of the marketed herbal formulation by mass Spectrometer (for diclofenec sodium).

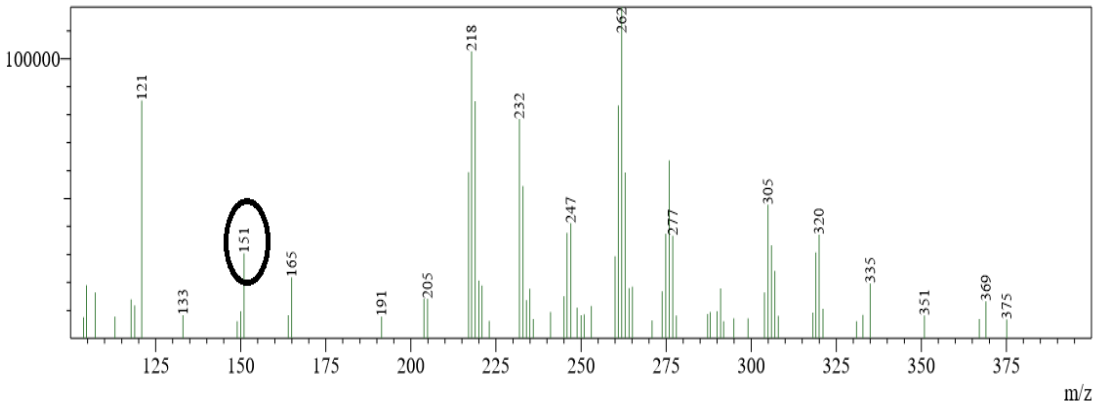


Figure 17: Confirmation of the marketed herbal formulation by mass spectrometer (Paracetamol).

Validation of the developed method was done as per ICH guidelines. The method was found to be specific. This method exhibited an excellent performance in terms of selective. Satisfactory results were obtained from validation of the method. The correlation coefficient was found to be greater than 0.98 which was within the limits specified (NLT 0.99). Hence, the results shown that an excellent correlation existed between the peak area and concentration of the analytes. The high value of the correlation coefficient showed good linearity. The standard addition and recovery experiments were conducted to demonstrate the accuracy of the method. The recovery was found to be in the range of 91-97% of three of drugs. High recovery results obtained from the proposed HPLC assay method indicates that this method can be used for quantitative routine quality control analysis of Formulation. The precision of a method determines the closeness of agreement between a series of measurements of the same sample. The % RSD values were found to be 0.74, 1.74, 1.05 of PCM, DIC, ACE, Respectively, These values were well within the generally acceptable limit of <2%. Hence, confirming the good precision of the assay method. The limit of detection (LOD) of a compound is defined as the lowest concentration that can be detected. The limit of quantification (LOQ) is the lowest concentration of a compound that can be quantified. The sensitivity of the method was measured by calculating the limit of detection and limit of quantification [38]. It was observed that the LOD was 3.61 µg/ml, 3.10 µg/mland 3.61 µg/ml and LOQ was 10.94 µg/ml, 9.39 µg/ml, 10.95 µg/ml of PAR, DIC, ACE, Respectively. This proves the sensitivity of the method and its effectiveness. The ability of this method to separate and accurately measure the peak of interest indicates the specificity of the method [36]. The robustness of an analytical procedure is the measure of its ability to remain unaffected by small, but deliberate, variations in method parameters and provides sign of its reliability during normal usage. In all the deliberate varied chromatographic conditions the tailing factor of three of the drug was less than 2.0. There was a very slight variation in the resolution and tailing factor results observed in all the robustness conditions illustrating the robustness of the method.

CONCLUSION

From the present work, good separation of compounds were obtained using HPLC conditions as described in above procedure. By comparing the retention time of herbal sample with that of the pure crystals of paracetamol, diclofenac sodium and aceclofenac the similarity was observed which was further confirmed by LC-MS. LC-MS data showed characteristic peaks of all the functional group of paracetamol, diclofenac sodium and aceclofenac as said above the molecular mass was also confirmed by mass spectroscopy. The results of above studies revealed that there is a presence of paracetamol, diclofenac sodium and aceclofenac in so called pure herbal formulation.

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