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Development and Validation of a Novel HPLC Method for the Analysis of Andrographolide in Marketed Herbal Formulation

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KEYWORDS

Andrographolide, Andrographis paniculata, HPLC, Method development, Method validation

ABSTRACT:

A specific and straightforward HPLC method is developed and validated for the analysis of andrographolide in crude material. The method was performed utilizing Poroshell 120 EC C18 column (3.0 x 50mm i.d, 2.7μm molecule size), Column temperature was kept 25ºC, isocratic elution with mobile phase of methanol and water (53:47) observed at 223nm wavelength with infusion volume of 20μl. The purposed technique is particular to isolate the peak of andrographolide from different segments, at retention time of 6.6 min with r2 value of 0.9992 in the range of 230 to 1000ppm. The LOD and LOQ were found to be 0.013 ppm and 0.041 ppm respectively. Correlation coefficient was valued 0.9992 in the 230 to 1000 ppm concentration range, hence calibration plots showed good linear relationship. The recovery of the method was found to be between 109.06-116.23%. The purposed technique is appropriate for the quality control of the raw material of Andrographis paniculata.

1. Introduction

Medicinal plants have been the important part of human life to cure illness and suffering since ancient civilization. These medicinal plants are commonly used in basic health care system due to accessibility, affordability, and compatibility.

Andrographis paniculata belonging to the family Acanthaceae, is originated from Taiwan and present in certain parts of China, South India, and Sri Lanka. In India it is mainly found in Kerala, Karnataka, Madhya Pradesh, and Andhra Pradesh. Morphological characteristics include smooth leaves with lanceolate arrangement, yellowish brown seeds of subquadrate shape, flowers with rose purple spots on petals and dark green quadrangular stem of the length of 30 – 100 cm with wings on the young and enlarged parts near the nodes [1-5]. The plant consists of flavonoids, diterpenoids and polyphenols [6-7]. It is widely used as analgesic, antibacterial, hepatoprotective, antimalarial, antithrombotic, antiviral and hypoglycaemic. Major chemical constituents present in this plant are Andrographolide [8-9]. Andrographolide is a labdane diterpenoids with molecular formula $C_{20}H_{30}O_5$ and molecular weight of 350.5g/mole [10-13].

2. Objectives

The main aim of study is to develop and validate a simple, isocratic and linear reverse phase HPLC method that is suitable for quantitative analysis of andrographolide in active pharmaceutical ingredients. As there are many marketed formulations containing andrographolide are already present, hence there is a risk of adulteration. Therefore, a novel HPLC method is

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developed and validated to ensure the quality and purity of the compound. This novel method is simple to use and gives accurate results.

3. Materials & Method

Chemicals

Methanol and Water of HPLC Grade were used as solvents, purchased from Merck (Germany). These solvents were filtered by 0.45 micrometre pore size membrane filters which were purchased from Millipore.

Samples

Anushan Herbal Kalmegh Powder Marketed Formulation is used as sample.

Sample preparation

1 gm of sample was weighed and dissolved in 10ml methanol in a beaker and covered with foil. It was placed on a water bath at a temperature of 40°C for 30 to 40 minutes. Sample was filtered and the filtrate was then diluted upto 100 ml with methanol. 0.2 µl of the above diluted sample was then dissolved in 0.2 µl of methanol [13-16].

HPLC analysis

HPLC analysis was accomplished using Shimadzu scientific technologies I series provided with manual injector, UV detector and column oven. C18 R.P column (3. 0×50mmi.d, 2.7µm particle size) was used. The temperature was kept at 25ºC with flow rate of 0.7ml/min and injection volume of 20µl. The solution of andrographolide sample was prepared in methanol and the concentration of the sample was kept 1mg/ml. It was further diluted to obtain a concentration range of 1000µg/ml. Stock solution of andrographolide standard was prepared and the concentration of the stock was kept 1000ppm. 500 ppm and 200ppm standard solution were prepared by stock solution using methanol as the solvent for dilution. Firstly, the HPLC instrument was started by switching on the Pump, Column, Detector, Controller and then purge. Then Class VP software was opened in computer. Chromatographic conditions were changed in the sample setting. A blank run was done. It takes 20 minutes after giving the command for complete washing. The standard drug solution of Andrographolide was prepared and injected in the HPLC using 20µl rheodyne injector.

Chromatographic Conditions

Mobile phase	Methanol and water (53:47)
Wavelength	223 nm
Flow rate	0.7ml/min
Retention time	6.64
Area	14037080
Pressure (Pump A & B)	157kgf - 161kgf

Validation of HPLC method

The developed HPLC method was evaluated by the validation parameters as per the ICH guidelines. Linearity of the method was determined by taking the blank and standard solution of concentration 230, 550 and 1000 ppm. Method recovery was done using 550 ppm standard and test sample in four different concentrations. Sensitivity of the method was determined by calculating limit of detection (LOD) and limit of quantification (LOQ) [17].

Syringe Washing and filtration

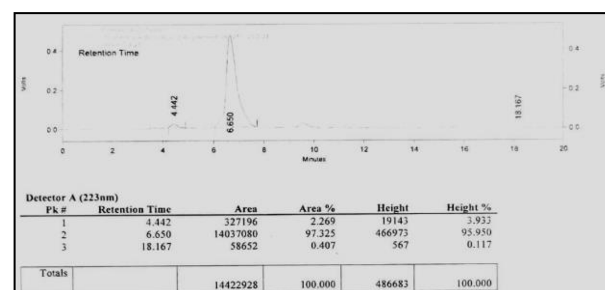
Sample and standard were filtered through 0.45 micrometre filter using syringe filtration technique.

Solvent Filtration using vacuum filter

Solvent should be filtered using vacuum filtration technique (0.2 micrometre filter).

4. Results and Discussion

Figure 1, 2 and 3 revealed the chromatograms of standard andrographolide solution. Solvents used were methanol and water in ratio of 53:47 and they were separated within 12 minutes in isocratic set-up. The peak of marker compound was identified by the retention time in which the peak is obtained.



Detector A (223nm)

Pk #	Retention Time	Area	Area %	Height	Height %
1	4.442	327196	2.269	19143	3.933
2	6.650	14037080	97.325	466973	95.950
3	18.167	58652	0.407	567	0.117
Totals		14422928	100.000	486683	100.000

Figure 1- Chromatogram of 200 ppm Andrographolide Standard

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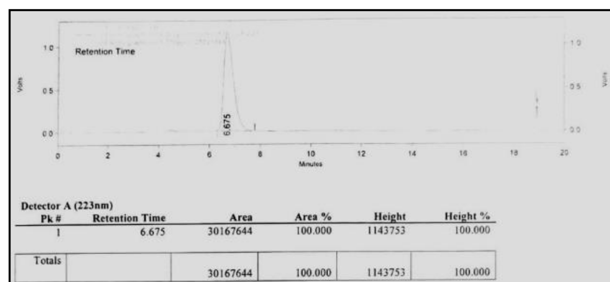

Figure 2- Chromatogram of 500 ppm Andrographolide Standard

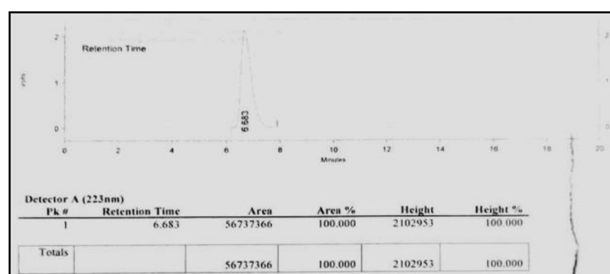

Figure 3- Chromatogram of 1000 ppm Andrographolide Standard

Andrographolide Analysis by HPLC

Claim Andrographolide - 500 ppm

Standard weight - 10.3mg

Dilution- 10ml methanol

Standard stock- 1000ppm

Table 1– Slope and correlation coefficient values of marker compound by HPLC

Sample	Concentration (ppm)	ml of stock	Dilution in methanol	Area by HPLC
Blank	0	0	0	0
Standard 1	230	2.3	10	14495719
Standard 2	550	5.5	10	33223254
Standard 3	1000	10	10	56990452
			Slope	56810.87
			Intercept	896519.20
			Corr. Coefficient	0.9992

Table 2- Recovery values of marker compound by HPLC

Sample	Weight(gm)	Area	Concentration	Recovery (%)
Sample 1	Std 550 ppm	33223254.0	569.024	113.8
Sample 2	Std 550 ppm	33910939.0	581.129	116.23
Sample 3	Std 550 ppm	35121090.0	602.43	120.49
Sample 4	Std 550 ppm	3187670g1.0	545.321	109.06
		Average	574.48	
		SD	23.84272673	
		RSD	4.150343397	

Validation Parameters

1. Limit of detection (LOD)

Limit of detection is the smallest amount of analyte that can be detected by the instrument. It was obtained by administering lowest amount of standard which was detectable. LOD was determined by multiplying the standard deviation with 3.3 and dividing it by the slope [18-19]. The value of LOD was found to be 0.0014 ppm.

2. Limit of Quantification (LOQ)

Limit of quantification is the amount of analyte that can be detected and estimated by the instrument. It is obtained by multiplying standard deviation with 10 and dividing the whole value by slope. The value of LOQ was found to be 0.0040 ppm

3. Recovery

The recoveries of different samples were determined by adding 550 ppm standard solution of andrographolide to the all sample solutions of different concentration. Recovery was found to be in the range of 109.06 - 120.49 percent (Table 2), which shows good accuracy of the developed method.

4. Linearity

Linearity of the method indicates the capacity of the method to give proper results as it is directly proportional to the amount of analyte present in the sample. Figure 4 shows the linearity graph of standard solutions of andrographolide whose concentrations were 230, 550 and 1000ppm along with a blank solution. The graph obtained is linear which shows that the method developed gives proper results. Table 1 shows the correlation coefficient and slope of the graph which is 0.9992 and 57810.87.

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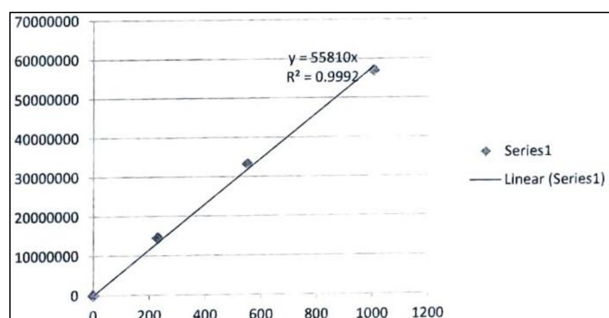
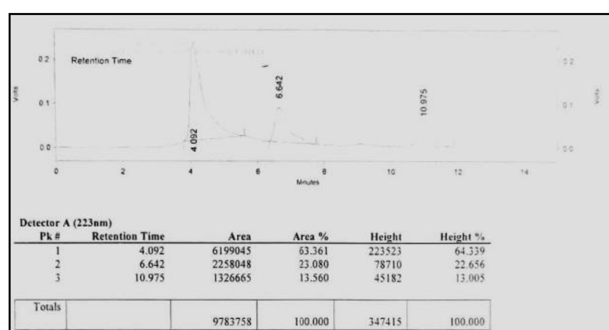


Figure 4-Linearity graph



Pk #	Retention Time	Area	Area %	Height	Height %
1	4.092	6199045	63.361	223523	64.339
2	6.642	2258048	23.080	78710	22.656
3	10.975	1326665	13.560	45182	13.005
Totals		9783758	100.000	347415	100.000

Figure 5-Chromatogram of andrographolide sample

Table3- Area, % area and retention time of standard and sample of andrographolide

Sample Name	Peak Area	% Area	Retention Time
Standard 500ppm	30167644	100 %	6.675 min
Kalmegh Powder Sample	6199045	63.361	4.092
	2258048	23.080	6.642
	1326665	13.560	10.975

Figure 5 shows the chromatogram of andrographolide sample solution and solvent used for the preparation of the sample was methanol. Anushan Herbal Kalmegh powder was used as sample. The marker compound (Andrographolide) was identified and analysed by developed method in which the solvent were methanol and water in ratio of 53:47 and separated in 18minutes. The peak of the Marker compound was identified by comparing the retention time of reference andrographolide. Table 3 shows the retention time of sample and standard solution.

Simple, rapid and validated HPLC method has been adopted for quantification of andrographolide in marketed powder formulation. Previously used methods were complex and time consuming and utilized high proportion of organic solvents. Therefore, a simple and validated method has been proposed. This method required cheaper solvents which are easily available to keep the method less expensive. Methanol is used to prepare sample and reference solutions of andrographolide.C18 R.P column (3. 0×50mmi.d, 2.7μm particle size) was utilized. ƛ max of andrographolide was obtained by UV visible spectroscopy which was found to be 223nm and this wavelength was used to in the method along with the mobile phase of methanol and water in the ratio of 53:47 so that prominent peak would be obtained, and separation of the marker compound could be achieved. The linearity and recovery data of the assay showed that the HPLC method for determination of andrographolide is reliable in various marketed formulations of andrographolide. This method can be used to check the quality and purity of andrographolide. LOD and LOQ values of the method were found to be 0.0014 and 0.0040 ppm respectively. Therefore, the method is highly sensitive. Linearity of the method was determined by 230,550 and 1000 ppm standard solution of andrographolide. Figure 13 reveals the linearity graph of andrographolide, and it revealed that the method gives correct results. The recovery of the method was found in the range of 109.06 - 120.49 ppm which reveals the good accuracy of the developed method. Figure 18, 19 and 20 shows the chromatogram of andrographolide standard solution of 200, 500 and 1000 ppm. The peak of marker compound was identified by the retention time which was found to be 6.7 min. The developed method was able to give a prominent peak of andrographolide. Figure 22 illustrate the chromatogram of andrographolide sample (Anushan Herbal Kalmegh powder). Peak of the andrographolide in sample was identified by comparing the retention time of the peak of marker compound in standard solution.

5. Conclusion

In this research we drew conclusions on the current state of spectroscopic and chromatographic methods for the quantification of Andrographolide in a marketed formulation. In the literatures, significant number of methods particularly UV visible detection in combination with mass spectroscopy are applied for quantification of Andrographolide in pharmaceutical formulation. All these methods are complex and expensive. Therefore, a simple, raid, precise, accurate

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and validated HPLC method has been developed for quantitative and qualitative analysis of Andrographolide. Hit and trial method is used for method development. ICH guidelines were used to validate the developed method. Use of less expensive and easily available high polarity solvents, easy and rapid preparation of mobile phase, linearity over wide range of concentration, all these factors make the developed method more economical and useful that can be utilized in various fields such as quality management, test facilities, approved testing laboratories and biopharmaceuticals.

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