RESEARCH ARTICLE

RP-UHPLC and HPTLC Method Development and Validation for Analysis of Andrographolide from Herbal Hepatoprotective Formulation

Sufiyan Ahmad[1]*, Tabrej Mujawar[2], Bhushan Batewal[1], Priyanka More[3], Asmita Gaikwad[4], Deshraj Chumbhale[5], Harshal Tare[6]

*[1]*Department of Quality Assurance, Gangamai College of Pharmacy, Nagaon, Affiliated to Kavayitri Bahinabai Chaudhari North Maharashtra University, Jalgaon, Maharashtra, India.*

[2]*Department of Pharmacology, Gangamai College of Pharmacy, Nagaon, Affiliated to Kavayitri Bahinabai Chaudhari North Maharashtra University, Jalgaon, Maharashtra, India.*

[3]*Department of Pharmacology, Gourishankar Education Society's Satara College of Pharmacy, Satara, Affiliated to Dr. Babasaheb Ambedkar Technological University, Lonere, Maharashtra, India*

[4]*Department of Pharmaceutical Chemistry, Sharadchandra Pawar College of Pharmacy, Otur, Affiliated to Savitribai Phule Pune University, Pune, Maharashtra, India.*

[5]*Department of Pharmacognosy, Amrutvahini College of Pharmacy, Sangamner, Affiliated to Savitribai Phule Pune University, Pune, Maharashtra, India.*

[6]*Department of Pharmacognosy, Sharadchandra Pawar College of Pharmacy, Otur, Affiliated to Savitribai Phule Pune University, Pune, Maharashtra, India.*

Received: 08th Janurary, 2023; Revised: 10th February, 2023; Accepted: 04th March, 2023; Available Online: 25th March, 2023

ABSTRACT

This work aimed to create a speedy, precise, and selective HPTLC and RP-UHPLC method for analysing andrographolide in finished products and raw materials. Asian medicines are using *Andrographis paniculata* since long. It's used to treat skin eruptions, boils, scabies, and chronic, unexplained fevers, all of which are caused by blood "abnormalities." Liquid chromatographic methods were developed to separate andrographolide and its herbal dosage form. HPTLC chromatography employed a 10 by 10 cm aluminum plate coated with 0.2 mm of silica gel 60 F254 (E. Merck, Germany). Camag Linomat 5 applicator with 100 µL syringe was used to apply samples in 6 mm bands (Hamilton, Switzerland). 14 mm separated the two bands, and 150 nL sec^{-1} was applied. Mobile phase was dichloromethane, toluene, ethyl acetate, and formic acid (6:4:1:0.5). This chromatogram runs 80 mm. Camag TLC scanner measured density at 254 nm. Mean RF=0.69. The linear calibration curve covers 500–3000 ng/spot and has a 0.996 correlation coefficient. Limit of Detection: 31.5 ng; Limit of Quantitation: 95.48 ng. A validated RP-UHPLC method for quantifying andrographolide in extract and formulation has been established. UHPLC analysis was performed in isocratic mode, at room temperature, using acetonitrile: Water (0.2% acetic acid) (85:15, v/v) as mobile phase on a 250 mm 4.6 mm i.d., 5 µm Cosmosil C18 column. Detection was at 230 nm. Andrographolide has a 4.1 minute half-life. Between 10 and 60 g/mL, andrographolide was linear. The approach met or exceeded ICH's linearity, precision, accuracy, and robustness.

Keywords: Andrographolide, HPTLC, RP-UHPLC, ICH Guidelines.

International Journal of Pharmaceutical Quality Assurance (2023); DOI: 10.25258/ijpqa.14.1.17

How to cite this article: Ahmad S, Mujawar T, Batewal B, More P, Gaikwad A, Chumbhale D, Tare H. RP-UHPLC and HPTLC Method Development and Validation for Analysis of Andrographolide from Herbal Hepatoprotective Formulation. International Journal of Pharmaceutical Quality Assurance. 2023;14(1):96-104.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

The medicinal plant has lactones, diterpenoids, diterpene glycosides, flavonoids, and flavonoid glycosides in its aerial part. Clinical testing has shown that it is safe and effective for treating respiratory tract infections.[1] *Andrographis paniculata* are often used to treat a wide variety of diseases, it's important to understand what those diseases are. Although panic attacks are thought to resolve on their own in conventional medicine, the advantages traditionally attributed to them have not been adequately examined. *A. paniculata's* effects on bacteria, fungi, viruses, bile flow, blood sugar, cholesterol, and adaptogens have all been documented.[2] In the Unani medicinal tradition,

**Author for Correspondence: harshaltare51@gmail.com*

it is employed as an aperient, anti-inflammatory, emollient, astringent, diuretic, emmenagogue, stomach and liver tonic, carminative, antihelmintic, and antipyretic. Recommended for leprosy, gonorrhea, scabies, boils, skin eruptions, chronic and seasonal fevers, and other ailments requiring "blood purifying" characteristics. Juice or an infusion of fresh leaves may be used to treat an infant's colic, diarrhea, and loss of appetite.[3] Dyspepsia due to gastric distension, severe cases of dysentery, and general weakness are all improved using leaves and root.[4] *A. paniculata* is mostly used now to treat and prevent the common cold. Its antithrombotic effects suggest possible benefits in cardiovascular disease. There may be favourable benefits in conditions, including cancer and HIV infections, according to pharmacological and clinical studies. One of the world's most extensively used medicinal plants is *A. paniculata* (Burm. f.) Wall. Ex Nees (AP). To the genus Acantha and family Acanthaceae it is a member.[5] High-performance liquid chromatography (HPLC) separates, identifies, and quantitates individual components of a mixture. HPLC has a number of benefits, including its quick processing time, high resolution, sensitivity, precision, and repeatability. C18 column stationary phase is widely used in high-performance liquid chromatography.[6] Toluene:MeOH was the initial mobile phase used for HPTLC analysis to try and get the best separation and resolution of andrographolide. Multiple mixtures of ethyl acetate and acetic acid, methanol and ethyl acetate, toluene and ethyl acetate and ortho-phosphoric acid, toluene and ethyl acetate and formic acid, and dichloromethane and ethyl acetate and acetic acid. The wavelength of 254 nm was chosen as the detection wavelength for andrographolide and its herbal dose form due to its high absorption at this wavelength.[7] We're utilizing HPTLC and RP-UHPLC to determine how much andrographolide is in *A. paniculata*. Chromatographic condition optimization, preliminary validation, and final method validation were all parts of the study.[8]

MATERIALS AND METHODS

Chemical and Reagents

Produced by Zandu pharmaceuticals and distributed by Sunpure herbal Pvt Ltd., Delhi, Zandu Kalmegha Capsule contains an extract of *A. paniculata*. We used dichloromethane, toluene, ethyl acetate, formic acid, acetonitrile, acetic acid, and methanol as mobile phase solvents. All chemicals used were of HPLC quality (Finar Ltd., Mumbai) and did not necessitate any further purification.

Instrumentation

A CAMAG HPTLC system with a Linomat 5 Applicator and Scanner 3 with a Win CATS processor and a Thermo Fisher ScientificsVanquish UHPLC system with a quaternary pump incorporating a UV-detector. Both of these systems are equipped with UV detectors.

Selections of Solvent

It was determined whether or not andrographolide could be dissolved in a variety of solvents by taking into account the qualities of both the medication and the solvent. In the end, methanol was chosen to act as the solvent during the process of dissolving the medication.

METHOD DEVELOPMENT

For HPTLC

Preparation of Standard Stock Solution

After careful weighing, one mg of andrographolide was transferred to a volumetric flask containing 10 mL of methanol and then dissolved in the methanol to get the desired concentration of 100 mg per mL.

Linearity Study of Andrographolide

With the use of a microlitre syringe and a Linomat 5 sample applicator, we spotted varying concentrations of andrographolide on a TLC plate, ranging from 500 to 3000 ng/spot.Following the aforesaid chromatographic procedures, the plate was developed and scanned. Calibration curves were generated by plotting peak area versus drug concentration.

Analysis of Extract

One gramme of *A. paniculata* extract was carefully weighed and then added to a 100 mL volumetric flask. It was diluted with methanol until the volume was exactly right. This was then diluted to a concentration of 100 mg per mL by transferring 1-mL to a volumetric flask and filling it up to the mark with the same solvent. The solution was spotted (10 µL, 1000 ng). When a regression equation was used to determine the concentration, the relative standard deviation of the results should be less than 2.0%.

Analysis of Formulation

Twenty capsules' worth of contents (Zandu Kalmegha Capsule; Manufactured by Zandu Ayurveda) were weighed, the mean weight was calculated, and the contents were coarsely crushed and reweighed to estimate the concentration of andrographolide. Ten mg of andrographolide powder was measured. Methanol was used to separate the andrographolide from the powder. The andrographolide was completely extracted by sonicating the mixture for 30 minutes before bringing the volume to 100 mL. This solution was then filtered over a 0.45 m membrane (Millifilter, Milford, MA). Ten microliters of the aforementioned solution (1,000 nm per spot) were spotted, developed, and scanned onto a thin-layer chromatography (TLC) plate. The experiment was performed three times. Regression equations were used to find the concentration, and the relative standard deviation was restricted to be less than 2.0%.

For RP-UHPLC

Selection of Mobile Phase

By analysing previously published works, After examining the solubility of drug in different solvents as well on the basis of literature survey; Acetonitrile: Water (0.2% Acetic acid) (85:15) was selected.

Optimization of Detection Wavelength

The UV-detector was chosen since it was simple to calibrate and provide accurate results. Different wavelengths were used to measure a constant analyte concentration. Andrographolide spectral data were recorded at 230 nm.

Selection of Chromatographic Method

Andrographolide was separated using a Cosmosil C_{18} column (250 mm x 4.6 mm i.d., 5 m), which provides adequate resolution and run time. The mobile phase consists of acetonitrile (85%) and water (15%) diluted with acetic acid (0.2%). A mobile phase flow rate of 1.2 mL/min was achieved. For andrographolide, the best chromatographic conditions yielded a retention duration of 4.1 minutes, with detection at a wavelength of 230 nm.

Preparation of Standard Stock Solutions

Add 7 mL of the solvent combination to a 10 mL volumetric flask containing 10 mg of andrographolide, and sonicate the mixture for 15 minutes. Fill in any missing space with the solvent combination and stir thoroughly to combine. The concentration of andrographolide was 1-g/mL.

Sample Preparation

- *A. paniculata* Extract

After transferring 10 mg of *A. paniculata* extract to a 5 mL volumetric flask: adding 5 mL of the solvent combination, sonicating the mixture for 25–30 minutes, and finally filtering it through a 0.2 membrane. The filtered 1-mL sample is added to a 10 mL volumetric flask with the appropriate diluent and thoroughly mixed. (100 µg/mL Concentrate).

- Herbal Formulation

Took 10 capsules of a herbal supplement (Zandu Kalmegha Capsule; Zandu Ayurveda) (Equivalent to Andrographolide). Shake the 50 mg herbal formulation in a sonicator for 25–30 minutes in a volumetric flask containing 25 mL of solvent mixture before filtering through a 0.2 membrane. The filtered 1-mL sample was placed in a 10 mL volumetric flask with the appropriate amount of diluent added. (100 µg/mL Concentrate)

- Linearity Studies

To achieve a final concentration in the 10–60 g/mL range, aliquots were taken from the stock standard solution and diluted to the mark with mobile phase in a series of 10 mL volumetric flasks.

- Analysis of *A. paniculata* extract

The extract of *A. paniculata* was weighed out at 10 mg, and then poured into a 100 mL volumetric flask. Mobile phase was used to bring the volume up to standard. The final extract concentration was 10.0 µg/mL, after further diluting. The peak area was measured after a constant volume injection of 20 L into the column. The linearity curve was used to calculate the concentration. Six times during the process, we found that the relative standard deviation was not higher than 2.0%.

- Analysis of formulation

Twenty capsules were carefully weighed and powdered to determine the Andrographolide content of the formulation (Label claim: 250 mg). We weighed 100 mg of formulation in powder form and poured that amount into a 100 mL volumetric flask containing roughly 100 mL of the solvent mixture. Membrane filter paper with a pore size of 0.45 microns was used to purify the solution. A filtered sample of 1-mL was placed in a volumetric flask holding 10 mL of diluent and thoroughly mixed. Six separate times, sample solutions were injected into the column. The linearity curve was used to determine the concentrations. There shouldn't be more than a 2.0% gap between the mean and the standard deviation of relative percentages.

METHOD VALIDATION

For HPTLC

ICH standards were used to verify the accuracy of the procedure.

Accuracy

Three recovery stages were tested: 80, 100, and 120%. Andrographolide standard drug solutions were added at three different concentrations to the sample solutions before they were analyzed. We scanned and developed the chromatogram.

Precision (Intra- day and Inter- day Precision)

The method's accuracy was measured by comparing the range of results obtained on separate days. The intra-day fluctuations of andrographolide were calculated by testing three different concentrations (1.5, 2.0, and 2.5 mg/mL) of the reference solution on the same day. Over the course of a week, three separate analyses were performed on 1500, 2000, and 2500 ng/spot of andrographolide reference solution to assess the intra-day precision.

Repeatability

To test the reproducibility of sample application, 15 L containing 1500 ng/spot of standard andrographolide for was spotted on a TLC plate in triplicate, developed, and scanned. No plate movements occurred during the six scans of the isolated spot. No more than a 2% RSD is acceptable.

Sensitivity

Using the concepts of Limit of Detection (LoD) and Limit of Quantitation (LoQ), we assessed the sensitivity of andrographolide readings using the proposed approach (LoQ). Standard deviation of drug peak areas (n = 3), used as a measure of noise, and slope of the matching calibration curve, B, were used to determine the LoD and LoQ, respectively.

Ruggedness

Two researchers, replicating the experiment setup and environmental factors, examined the robustness of the proposed approach. Andrographolide was spotted onto HPTLC plates at a 1500 ng/band concentration. The procedure for creating and scanning bands was carried out as previously stated. Triplicates were performed because the allowable range for RSD is 2%.

Robustness

The method's robustness was investigated by purposefully altering a small number of parameters, such as the mobile

phase's composition and the stock solution's stability. Andrographolide at a concentration of 1000.0 ng/band was used in the study, with just one variable at a time being altered to determine its impact.

For RP-UHPLC

Accuracy

The proposed UHPLC method was put to the test on samples that had already been analysed for quality control (10.0 µg/mL of andrographolide) and on samples that had been spiked with known amounts of standard andrographolide at the 80, 100, and 120% levels. The expected range for the recovery percentage was 98–102%.

Precision

Studies of the repeatability and intermediate precision of the approach confirmed its accuracy. Accuracy within a single day was investigated by doing three separate analyses of andrographolide at 10, 20, and 30 ng/mL on the same day. During a week, we analyzed the same concentration on three separate days to ensure consistency across analyses. Analyzing 10 g/mL of andrographolide six times allowed us to determine its repeatability. A 3% RSD is considered unacceptable.

Ruggedness

The same 10 µg/mL Andrographolide sample solution was prepared from stock solutions and analyzed by two separate analysts under controlled conditions. Six measurements were taken of the peak area for solutions of the same concentration, and the results should have a relative standard deviation of no more than 2.0%.

Robustness

Modifying the mobile phase composition, acid concentration (pH modifiers), and flow rate were among the variables tested to determine the method's robustness. Injections of 10 µg/mL of andrographolide were used to examine the effects; just one variable was altered at a time in order to calculate the impact.

Sensitivity

For the measurement of contaminants and/or degradation products, the quantitation limit is a quantitative assay parameter for low amounts of chemicals in sample matrices. The following formulas were used to calculate the LoD and the LoQ. Where SD is the standard deviation of the measured response and S is the slope of the calibration curve, LoD is defined as SD/3.3 and LoQ as SD/10.

Specificity and Selectivity Study for Andrographolide

The analytes should be clearly separated from any background noise and other impurities. Quantitative detection of the analyte in the presence of components that might be present in the sample matrix is referred to as specificity, whereas qualitative detection of the analyte in the presence of components that might be present in the sample matrix is referred to as selectivity.

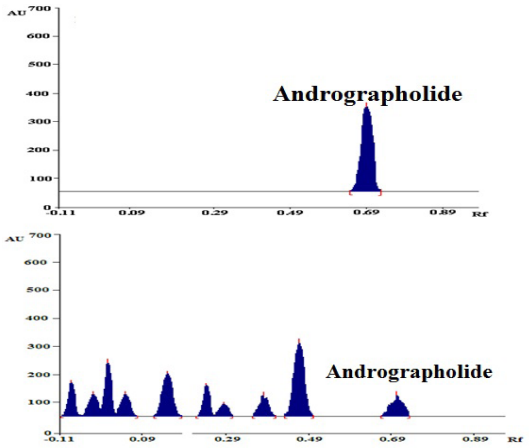

Figure 1:HPTLC chromatogram of Standard Andrographolide and formulation.

The procedure is highly discriminating. Andrographolide's retention time was unconcerned by any other peaks, and the baseline showed no appreciable noise.

RESULTS AND DISCUSSION

For HPTLC

Andrographolide and its commercially available formulations were tested for solubility in a variety of solvents to ensure they were compatible with the medication and the solvent. As can be seen in Figure 1, methanol was ultimately chosen as the solvent to dissolve the medication.

Linearity Study

With the use of a mL syringe and a Linomat 5 sample applicator, we spotted varying concentrations of andrographolide on a TLC plate, ranging from 500 to 3000 ng/spot. Following the aforesaid chromatographic procedures, the plate was developed and scanned. Calibration curves (Figure 2), and observations are summarised (Table 1).

The correlation coefficient must be lower than or equal to 0.996 in order to be considered acceptable.

In summary, a correlation coefficient of 0.996 may be found. As a result, andrographolide separations using HPTLC exhibit linearity.

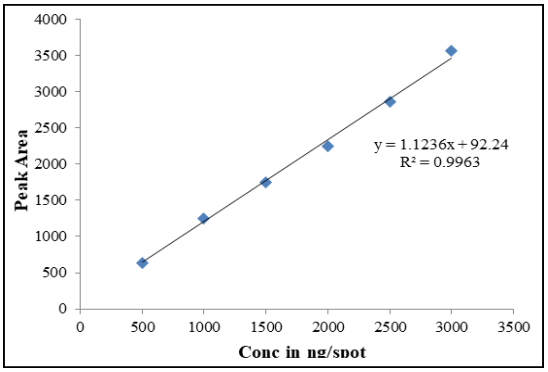

Figure 2: HPTLC Calibration Curve for Andrographolide.

Table 1: Linearity study of Andrographolide by HPTLC

S. No.	*Concentration of Andrographolide in [ng/spot]*	*Peak area mean ± S.D. [n = 3]*	*% R.S.D.*
1	500	635 ± 7	5.715
2	1000	1246 ± 22	17.962
3	1500	1742 ± 9.1	7.483
4	2000	2251.33 ± 14.	11.585
5	2500	2858.66 ± 4.5	3.681
6	3000	3568.33 ± 7.5	6.128

Recovery Study

Three recovery stages were tested: 80, 100, and 120%. Andrographolide standard medication solutions were added to the sample solutions at three concentrations before they were analyzed. We scanned and developed the chromatogram. Table 2 displays the outcomes in terms of the percentage of recovery.

Precision (Intra- day and Inter- day Precision)

The method's accuracy was measured by comparing the range of results obtained on separate days. Analyzing 1500, 2000, and 2500 /spot of andrographolide standard solution three times on the same day allowed us to calculate intraday variations. Over the course of a week, three separate analyses were performed on 1500, 2000, and 2500 ng/spot of andrographolide reference solution to assess the intra-day precision. Table 3 and 4 display the outcomes.

Repeatability

Repeatability of sample application was evaluated by applying three replicate spots of 20 µL (each containing 2000 ng of standard andrographolide) to a TLC plate, and then developing, scanning, and analysing the results. No plate movements occurred during the six scans of the isolated spot. No more than a 2% RSD is acceptable (Table 5).

Sensitivity

We evaluated the sensitivity of andrographolide readings by applying the concepts of LoD and LoQ to the suggested method (LoQ). LoD and quantification (LoQ) were calculated using the standard deviation of drug peak areas (n = 3), a measure of noise, and the slope of the corresponding calibration curve (B). The linearity equation is Y= 1.1236X + 92.24, which was found. Both the detection and quantification limits for andrographolide were determined to be 31.5 and 95.48 ng, respectively as shown in Table 6.

Ruggedness

Replicating the experiment and surroundings, examined the suggested method for its robustness. Andrographolide at a concentration of 1500 ng/band was spotted on HPTLC plates. The procedures for creating and scanning bands were executed as mentioned above. The process was carried out three times to ensure accuracy, and the findings are displayed in Table 7. The allowable range for the RSD (% RSD) is 2%.

Robustness

The method's robustness was investigated by purposefully altering a small number of parameters. Table 8 displays the results of a study in which 1000.0 ng/band of andrographolide was applied and the effects of varying a single component were estimated.

For RP-UHPLC

Preparation of Standard Stock Solutions

Added 7 mL of the solvent combination to a 10 mL flask (volumetric) containing 10 mg of andrographolide, and sonicate the mixture for 15 minutes. Fill in any missing space with the solvent combination and stir thoroughly to combine. The concentration of andrographolide was 1 g/mL. Use the mobile phase you prefer from Figure 3 to create a chromatogram.

Table 2: Results of recovery studies for andrographolide

Drug	*Initial Amount [ng]*	*Amount added [ng]*	*Amount recovered ± S.D.*	*%Recovered*	*%R.S.D.*
	1500	1200	2693.13±4.01	99.74	3.29
Andrographolide	1500	1500	2995.46±3.5	99.84	2.86
	1500	1800	3292.09±3.3	99.76	2.72

Table 3: Results of precision studies for Andrographolide (Inter–day)

Drug	*Concentratio [ng/spot]*	*Inter-day*			
		Peak are Mean ± S.D.	*%R.S.D.[n = 3]*	*Amount found*	*%Amount Found*
	1500	1744.14 ±8.07	1.59	1498.60 ± 0.97	99.90
Andrographolide	2000	2246.84 ±5.51	1.50	1998.69 ± 0.76	99.93
	2500	2860.32 ±5.59	1.56	2498.38 ± 0.41	99.93

Table 4: Results of precision studies for Andrographolide (Intra – day)

Drug	*Concentration [ng/spot]*	*Intraday*			
		Peak area Mean ± S.D.	*%R.S.D.[n = 3]*	*Amount found*	*%Amount Found*
	1500	1754.20 ±5.50	1.49	1498.66±1.1	99.91
Andrographolide	2000	2247.96 ±3.47	1.83	1998.3±0.72	99.91
	2500	2861.78 ±2.49	2.03	2496.16±3.30	99.84

Table 5: Results of repeatability studies of andrographolide

S. no.	*Application volume [µL]*	*Area of Andrographolide*
1	20	2245.6
2	20	2248.9
3	20	2254.98
4	20	2251.7
5	20	2259.3
6	20	2254.81
Mean		2252.54
S.D.		4.87
%R.S.D.		1.45

Table 6: Results of sensitivity studies of andrographolide

S. no	*Application volume [ng/spot]*	*Area of Andrographolide Mean ± SD*	*%RSD*
1	500	635 ± 7	5.71
2	1000	1246 ± 22	17.96
3	1500	1742 ± 9.16	7.48
4	2000	2251.33 ± 14.18	11.58
5	2500	2858.66 ± 4.5	3.68
LOD = (3.3* Average St.D)/ Slope		31.5	
LOQ = (10* Average St.D)/ Slope		95.48	

Table 7: Results of Ruggedness of Andrographolide

Analyst	*Amount found of andrographolide (%)*	*%RSD [n=3]*
I	99.75	0.089
II	99.71	0.187

Sample Preparation

- *A. paniculata* extract

After placing 10 mg of *A. paniculata* extract in 5 mL flask, adding 5mL solvent combination, and shaking vigorously for 25–30 minutes, the liquid was filtered through a 0.2 membrane. Filtered Sample of 1ml is added to a 10 mL flask (Volumetric) with the appropriate diluent and thoroughly mixed. Concentration: 100 g/mL (Figure 4 shows the same chromatogram).

- Herbal formulation

Taking 10 capsules of a herbal preparation (*A. paniculata* extract) (Equivalent to andrographolide). Shake the 50 mg herbal formulation in a sonicator for 25–30 minutes in a volumetric flask containing 25 mL of solvent mixture before filtering through a 0.2 membrane. The filtered 1-mL sample was placed in a 10 mL volumetric flask with the appropriate amount of diluent added. concentration of 100 g/mL (Figure. 5).

Linearity Study

To achieve a final concentration in the range of 10–60 g/mL, aliquots were taken from the stock standard solution and diluted to the mark with mobile phase in a series of 10 mL volumetric flasks. Each sample was injected at the same volume. The peak area was plotted against the Andrographolide concentration to create a calibration curve, and the experiment was performed three times for each concentration. Table 9 displays the collected data, Figures 6 and 7 display the calibration curves and calibration peaks, respectively.

The minimum required value for the correlation coefficient to meet the acceptance criteria is 0.995.

This leads us to the final conclusion that the correlation coefficient is 0.9989. As a result, the andrographolide U-HPLC technique is linear.

Analysis of A. paniculata Extract

The extract of *A. paniculata* was weighed out at 10 mg, and then poured into a 100 mL volumetric flask. The mobile phase

Table 8: Results of robustness studies for andrographolide

Parameters	*Andrographolide*	
	SD of peak area	*% RSD*
Mobile phase composition		
A Dichloromethane: Toluene : Ethyl acetate: Formic acid (6:4:1:1)	21.89	0.12
B Dichloromethane: Toluene : Ethyl acetate: Formic acid (6:4.5:1:1)	13.41	0.24
Mobile phase volume (mL)		
4.7	13.38	0.16
9.4	12.37	0.12
Development distance (mm)		
70	15.84	0.29
75	05.05	0.11
80	08.85	0.19
Relative humidity (%)		
55	11.73	0.12
65	08.80	0.09
Duration of saturation (min)		
20	15.31	0.15
25	14.11	0.11
30	08.17	0.09
Activation of prewashed TLC Plates (min)		
08	08.57	0.10
10	4.22	0.05
12	6.19	0.08
Spotting to chromatography Time	6.22	0.5
Chromatography to scanning Time	08.18	0.80

Table 9: Linearity Study of Andrographolide (RP-UHPLC)

Concentration	*[Mean ± SD; n = 3] Area of Peak (mAU per min)*	*%RSD*
10 µg/mL	3.37 ± 0.22	00.18
20 µg/mL	7.44 ± 0.26	00.21
30 µg/mL	10.53 ± 0.40	00.33
40 µg/mL	14.76 ± 0.11	00.08
50 µg/mL	18.49 ± 0.15	00.12
60 µg/mL	21.93 ± 0.55	00.45

Table 10: Analysis of Andrographolide extract by RP-UHPLC

Drug	*Amount Taken (µg/mL)*	*Amount Found [µg/mL]*	*Amount Found %*
A. paniculata extract	20	19.15	95.75
	20	19.65	98.25
	20	19.85	99.25
	20	19.64	98.2
	20	19.78	98.9
	20	19.97	99.85
	Mean ± SD	19.67 ± 0.28	98.36 ± 1.42
	%RSD	0.26017	1.3

Table 11: Analysis of formulation by RP-UHPLC

Formulation	Label Claim (mg)	Amount Found mg ± SD [n = 6]	Percentage Amount found	% RSD
A. paniculata extract capsule	250 mg capsule contains Andrographolide	249.43 ± 0.31	99.77 ± 0.12	0.12

was used to bring the volume up to standard. The final extract concentration was 10.0 g/mL, after further diluting. The peak area was measured after a constant volume injection of 20 L into the column. The linearity curve was used to calculate the concentration. The method was carried out six times, with the results presented in Table 10. The relative standard deviation was not higher than 2.0%.

Analysis of Formulation

Twenty capsules were carefully weighed and powdered to determine the andrographolide content of the formulation (Label claim: 250 mg). We weighed 100 mg of formulation in powder form and poured that amount into a 100 mL volumetric flask containing roughly 100 mL of the solvent combination. Membrane filter paper with a pore size of 0.45 microns was used to purify the solution. A filtered sample of 1-mL was placed in a volumetric flask holding 10 mL of diluent and thoroughly mixed. Six separate times, sample solutions were injected into the column. The linearity curve was used to determine the concentrations. There shouldn't be more than a 2.0% gap between the mean and the standard deviation of relative percentages. Table 11 displays the results.

Accuracy

Table 12 displays the findings of a recovery investigation in which standard andrographolide was introduced to a previously examined sample (10.0 g/mL of andrographolide) at three different concentrations. The percentage recovery was found to be between 98 and 102%.

Precision

Studies of the repeatability and intermediate precision of the approach confirmed its accuracy. Accuracy within a single day was investigated by doing three separate analyses of andrographolide at 10, 20, and 30 ng/mL on the same day. During a week, we analysed the same concentration on three separate days to ensure consistency across analyses. Table 13 displays the outcomes, and Table 13 displays the repeatability.

Repeatability Studies

Analyzing 10 g/mL of Andrographolide six times allowed us to determine its repeatability. No more than a 2% RSD is acceptable Table 14 shows.

Ruggedness

The same 10 g/mL Andrographolide sample solution was produced from stock solutions and examined by two separate analysts under controlled conditions. Six measurements of peak area were taken with solutions of the same concentration, and There should have been no more than a 2% relative standard deviation between the actual and predicted values. Table 15 displays the findings.

Table 12: Recovery studies for andrographolide

Drug	*Initial amount [µg/mL]*	*Excess drug added to the analyte [%]*	*Amount recovered ± S.D. [µg/mL]*	*Recovery [%]*	*%RSD [n = 3]*
Andrographolide	10	80	17.87 ± 0.15	99.29	0.12
	10	100	19.86 ± 0.18	99.31	0.15
	10	120	21.75 ± 0.36	98.89	0.29

Table 13: Precision study for andrographolide

Drug	*Conc. [µg/mL]*	*Intra –day Amount Found [µg/mL] [n = 3]*		*Inter- day Amount Found µg/mL] [n = 3]*	
		Mean	*% RSD*	*Mean*	*% RSD*
Andrographolide	10	9.75 ± 0.16	0.13	9.78 ± 0.2	0.16
	20	19.47 ±0.16	0.13	19.42 ± 0.37	0.30
	30	29.79 ±0.13	0.11	29.82 ±0.17	0.14

Table 14: Ruggedness study Andrographolide

Drug	*Amount in µg/mL*	*Amount Found [n = 6] Mean ± SD*		*% Amount Found [n = 6]*		*% RSD*	
		Analyst 1	*Analyst 2*	*Analyst 1*	*Analyst 2*	*Analyst 1*	*Analyst 2*
Andrographolide	10	9.7 ± 0.17	9.56±0.3	97.05	95.68	1.62	2.82

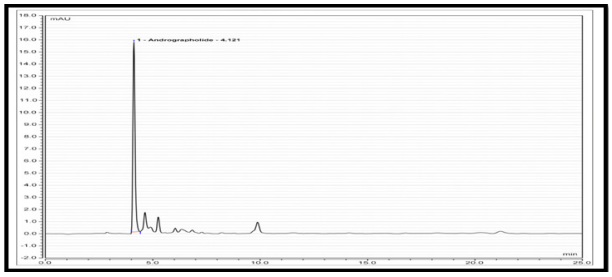

Figure 3: RP-UHPLC graph of Standard Andrographolide.

Figure 4: RP-UHPLC graph of Andrographolide Extract.

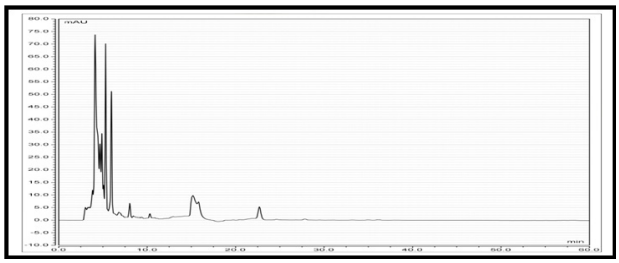

Figure 5: RP-UHPLC graph of Andrographolide formulation

Table 16: Robustness Studies Andrographolide

Parameters	*Andrographolide Rt*
Acid Concentration Shift for pH Regulation	
00.1 %	4.9
00.2 %	4.1
00.3 %	3.7
Flow Rate Changes	
00.8 milliliters/min	6.1
01.2 milliliters/min	4.1
01.5 milliliters/min	3.9
Mobile Phase Composition Changes	
(60 : 40 v/v)	10.6
(65 : 35 v/v)	9.5
(70 : 30 v/v)	6.3
(80 : 20 v/v)	5.8

Robustness

The method's stability was investigated by varying conditions like mobile phase composition, acid concentration (pH modifiers), and flow rate. Andrographolide (10 g/mL) was injected intravenously, and the effects on outcomes were analysed by varying a single independent variable at a time, with the results summarised in Table 16.

Table 15: Repeatability study for andrographolide

Conc.(10 µg/mL)	*Amount Found*
10µg/mL Each	9.98
	9.87
	9.93
	9.89
	9.85
	9.93
Mean	9.9
Standard Deviation	0.04
Percent R.S.D.	0.043

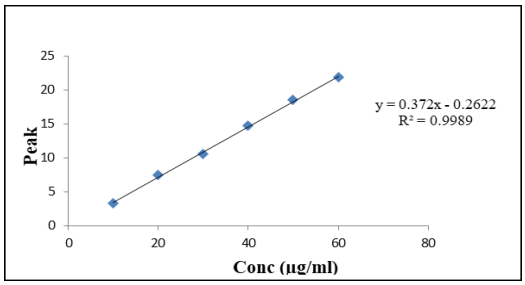

Figure 6: Calibration Curve of Andrographolide.

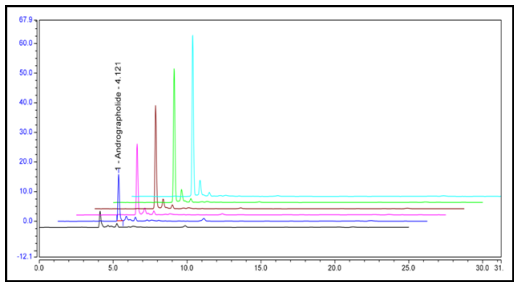

Figure 7: RP-UHPLC calibration graph of Andrographolide.

Sensitivity

The quantitation limit is a quantitative assay parameter for measuring trace levels of compounds in sample matrices, such as pollutants and/or degradation products. LoD and LoQ were determined using the following formulas (LoQ). LoD is defined as SD/3.3 and LoQ as SD/10, where SD is the standard deviation of the observed response and S is the slope of the calibration curve. The limits of detection and quantification for andrographolide were calculated to be 2.54 and 7.71 mg, respectively.

Selectivity and Specificity

Samples must be clearly separated and no any background noise, other impurities. Analyte selectivity refers to the process of detecting analytes qualitatively, whereas analyte specificity refers to the process of detecting analytes quantitatively in the presence of such components. The procedure is highly discriminating. Additionally, neither the retention duration of andrographolide nor the baseline showed any appreciable noise.

CONCLUSION

The Andrographolide sample used in the development of the mobile phase exhibited clearly distinct peaks. Andrographolide in extract and formulation was estimated with high accuracy, precision, and robustness. It was verified for accuracy, precision, and linearity range to ensure the created method fulfilled ICH standards. The devised method for the assay of andrographolide in dosage form proved robust, linear, specific. A validated RP-UHPLC technique was shown to be straightforward and accurate. As an added bonus, the created approach was sturdy, reliable, and responsive. It was revealed that the validated HPTLC method for determining andrographolide in extract and in the formulation was straightforward and accurate. In addition, the devised approach was sturdy, reliable, and sensitive. Every single one of these techniques is suitable for regular analysis of andrographolide in its pharmaceutical form.

REFERENCES

1. Jamaludin, R., Mohd, N., Safazliana, R., Sulong, R., Yaakob, H., 2021. *A. paniculata* -loaded niosome for wound healing application : Characterisation and *in-vivo* analyses. J. Drug Deliv. Sci. Technol. 63, 1–9.
2. Li, W., Fitzloff, J.F., 2004. HPLC – PDA Determination of Bioactive Diterpenoids from Plant Materials and Commercial Products of *Andrographis paniculata* Diterpenoids from Plant Materials. J. Liq. Chromatogr. Relat. Technol. 27, 2407–2420.
3. Patarapanich, C., Laungcholatan, S., Mahaverawat, N., 2007. HPLC determination of active diterpene lactones from *Andrographis paniculata* Nees planted in various seasons and regions in Thailand. Thai J. Pharm. Sci. 31, 91–99.
4. Patil, R., Jain, V., 2020. Andrographolide: A Review of Analytical Methods. J. ofChromatographic Sci. 8, 1–13.
5. Srivastava, A., Misra, H., Verma, R.K., Gupta, M.M., 2004. Chemical Fingerprinting of *Andrographis paniculata* Using HPLC , HPTLC and Densitometry. Phytochem. Anal. 285, 280–285.
6. Zhao, Y., Kao, C., Wu, K., Liao, C., Ho, Y., Chang, Y., 2014. Chemical Compositions, Chromatographic Fingerprints and Antioxidant Activities of Andrographis Herba. Molecules 19, 18332–18350.
7. International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use, Validation of analytical procedures: Text and Methodology, ICH-Q2(R1), Geneva (2005).
8. International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use, Validation of analytical procedures, ICH-Q2A, Geneva (1995).