RESEARCH ARTICLE

Development and Validation of an Analytical Method for the Simultaneous Estimation of Azadirachtin and Camphor in a New Herbal Formulation

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ABSTRACT

Background: High-performance liquid chromatography (HPLC) is an analytical tool for determining the quality of a drug product. Separating, identifying, and quantifying the various drugs should be able to HPLC methods. To quantify azadirachtin and camphor, a novel HPLC technique was created that is sensitive and selective.

Objective: The analysis of azadirachtin and camphor has been designed and verified using a simple, efficient, selective, and precise HPLC technique.

Material and Method: With the help of an HPLC C18 column (4.6 x 250 mm, 5 m), a mobile phase made up of buffer (pH adjusted to 3.0 with orthophosphoric acid) and acetonitrile in the ratio 15:85, flow rate of 1.0 mL/min, and column temperature kept at 35°C, good chromatographic separation was achieved in the current study. The resulting effluents were seen at 294 nm using a UV-visible detector.

Result and Discussion: Sharp peaks for azadirachtin and camphor were found at retention times of 3.5 and 4.8 minutes, respectively, during the detection process at 294 nm. Over the concentration ranges of 0.1–0.9 g/mL for azadirachtin and 20–180 g/mL for camphor, the calibration curve was linear. According to International Council on Harmonisation (ICH) guidelines, the method was verified for accuracy, precision, repeatability, specificity, robustness, and detection and quantification limits.

Conclusion: The proposed method has specificity, accuracy, and sensitivity, with an excellent linear.

Keywords: Azadirachtin, Camphor, Reversed-phase high-performance liquid chromatography, Formulation, Validation.

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INTRODUCTION

The neem tree (*Azadirachta indica*), a native of India, produces azadirachtin (AZD), a tetranortriterpenoid typical of the Meliaceae family. In particular, bark, leaves, fruits, and seeds contain it.[1-3] It has long been acknowledged for its benefits to human health and against insects. Neem oil is currently available in northeast India and is highly helpful in creating tablets that repel mosquitoes. Neem has a number of medical applications as well, and medicines made from it are used to treat acquired immunodeficiency syndrome (AIDS), gastrointestinal problems, cancer, and skin conditions. It is traditionally used in Oman to treat diabetes and fever.[4] The primary compound responsible for both the antifeedant and poisonous effects on insects is AZA, a complex tetranortriterpenoid limonoid from the neem seeds. Other limonoid and sulfur-containing compounds, such as those in the tree's leaves, blossoms, bark, and roots, have repellant, antiseptic, contraceptive, antipyretic, and antiparasitic qualities.[5] Neem has a wide range of triterpenoids, in addition to AZA, including nimbin, salannin, azadirachtol, nimbidinin, and gedunin.[6] Figure 1 depicts the structure of AZA.

The wood of the camphor laurel (*Cinnamomum camphora*) and other similar plants of the laurel family is the source of camphor, a white, crystalline chemical with a strong aroma and bitter taste. Wood, twigs, and tree bark are processed through steam distillation, filtration, and sublimation to produce camphor. Camphor (CAM) has a wide range of medical uses, including as a topical analgesic, antibacterial, antispasmodic, antipruritic, anti-inflammatory, anti-infective, rubefacient, contraceptive, moderate expectorant, nasal decongestant, and

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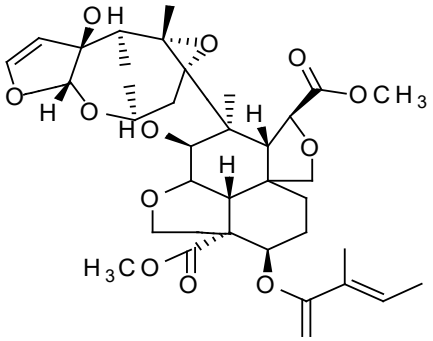

Figure 1: Chemical structure of azadirachtin

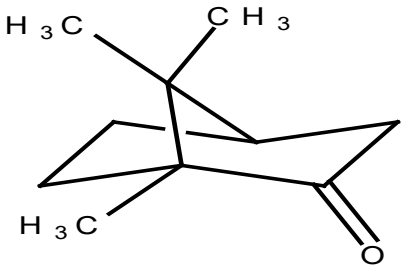

Figure 2: Chemical structure of camphor

cough suppressant.[5] Figure 2 depicts the camphor's structural makeup.

We chose a herbal formulation for the current investigation that has an impact on termites. *A. indica* and *C. camphora* are two of the herbs used in the composition. A single chemical manufacturer is chosen for quantification. The review of the literature reveals that different analytical techniques for estimating azadirachtin and camphor have been described both alone and in combination with other medications. However, to the best of our knowledge, no such high-performance liquid chromatography (HPLC) analysis approach has been published for the simultaneous measurement of azadirachtin and camphor.

In the current study, we created a simple, efficient, and validated HPLC approach to standardize a novel formulation employing AZA and CAM. According to the requirements of the International Council on Harmonization (ICH) Q2(R1) guideline, the approach was validated.[6]

Chemical and Reagents

Azadirachtin and camphor was procured Sigma Aldrich. HPLC grade solvents: Water, methanol, acetonitrile, and orthophosphoric acid were obtained from Merck India Ltd., Mumbai. . This approach made use of the HPLC system from Shimadzu, Japan, the SPD-20A detector, and the LC-20AT pump software. Shim-pack HPLC C18 (250 X 4.6 mm, 5 m) analytical column was utilized for analyte separation.[7]

METHOD DEVELOPMENT

HPLC Instrument and Conditions

HPLC analysis was carried out using a Shimadzu Japan Deyector: SPD-20A Pump: LC-20A. The method was developed using HPLC C18 column (250 X 4.6 mm, 5 µm). The run time was of 10 minutes. Degassed and filtered using a Millipore vacuum filter system equipped with a 0.45 µm membrane filter. The mobile phase used was buffer (pH adjusted to 3.0 and acetonitrile in the ratio 15:85 at a flow rate of 1.0 mL/min, column temperature maintained at 35°C, and a detection wavelength of 294 nm using a UV-visible detector. The injection volume was 20 µL for every injection.[8]

Selection of Wavelength

By taking UV spectrum readings between 200 and 400 nm for different drug solutions of AZA and CAM, then overlapping them, the best wavelength for the HPLC analysis was found. These one marker's UV overlain spectra revealed that the medications absorb noticeably at 294 nm. Hence 294 nm was chosen as the detection wavelength for HPLC analysis.[9]

Preparation of Standard Solution

Weighed 0.1 mg of AZA and 10 mg of CAM were transferred into the 10 mL volumetric flask, dissolved in the mobile phase, and diluted up to the mark. And Shake for 15 minutes and makeup volume with the mobile phase. Stock solutions had concentrations 10 and 1000 µg/mL, respectively.

Working Sample Preparation

Appropriate aliquots were taken out. AZA (10 µg/mL) and CAM (1000 µg/mL) stock solutions were diluted up to 10 mL with mobile phase to obtain standard solutions of AZA 0.1, 0.3, 0.5, 0.7, and 0.9 µg/mL and 20,60,100,140, and 180 µg/mL of camphor.

Analytical Method Validation

The following parameters—specificity, linearity, sensitivity, precision, and accuracy of analytical solutions—were used to validate the developed RP-HPLC technique. The validation was done in accordance with the standards for validating analytical processes established by the ICH.[7]

Specificity

The specificity of the HPLC method was evaluated to ensure that there was no interference from the excipients present in the formulations. The specificity was studied by injecting the excipients.

To make sure that the excipients used in the formulations didn't interfere the specificity of the HPLC method was assessed.

System Suitability

By preparing standard solutions, the system suitability parameters were calculated. The solutions were injected six times, and the parameters of %RSD, peak tailing, resolution, and the oreticalplate number and retention time were measured.

Linearity and Range

Five standard working solutions of AZA (0.1–0.9 µg/mL) and CAM (20–180 µg/mL) were injected into an HPLC system in three separate duplicates to examine the linearity of the analytical process. A calibration curve was created by graphing the concentration of AZA and CAM on the X-axis and the

Table 1: Optimized condition for simultaneous estimation of Azadirachtin and Camphor

Mode of separation	*Isocratic elution*
Mobile phase	buffer (pH 3.0): Acetonitrile (15:85v/v)
Column	C18 column (250 X 4.6 mm, 5 µm).
Flow rate	1.0 mL/min
Detector wavelength	294 nm
Injection volume	20 µL

Table 2: Parameters evaluated to optimize chromatographic separation

Drug	*Retention time(min)(RT)*	*Area*	*Tailing factor*	*Theoretical plate*
Azadirachtin	3.5	17920	1.187	5460
Camphor	4.8	243851	1.144	8374

average peak area on the Y-axis to show that the instrument response was proportional to the analyte concentration. The linear regression analysis was used to calculate the regression equation and the value of the correlation coefficient.

Accuracy

Recovery tests were conducted at three concentration levels (0, 50, 100, and 150%), which were used to evaluate the assay method's accuracy. And three samples were put into each concentration. For each of the replicate samples, the %RSD and the % recovery of the additional sample were calculated.

Precision

By analyzing standard working solutions at three distinct concentration levels, precision was evaluated. Three times on the same day, standard solutions of AZA and CAM were analyzed at concentrations of 0.3–0.7 g/mL and 60–140 g/mL, respectively. The same solution was analyzed 3 times ovver the course of a week to determine the inter-day precision (%RSD). The relative standard deviation (RSD) values for intra-day and inter-day analyses with acceptance criteria of not more than 2% were calculated to determine the precision of the suggested method.

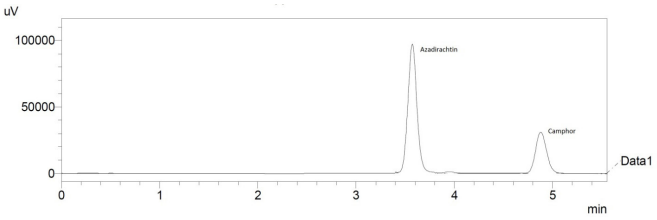

Figure 3: Chromatogram for simultaneous estimation of Azadirachtin and Camphor

LoD and LoQ Determination

The detection limit (LoD) and quantification limit (LoQ) were used to determine the method's sensitivity. LoD is the lowest analyte concentration that reliably produces a reaction but cannot always be precisely quantified. LoQ is the lowest analyte concentration at which an accurate result can be obtained. The standard deviation of response (SD) and the slope was used to calculate the LoD and LoQ (S). Method using the formula listed here. At least three separate analyses were done for each study.

LoD = 3.3 x SD/S

LoQ = 10 S x D/D

where SD is the response's standard deviation

S is the calibration curve's slope.

Robustness

The method's resilience was investigated by making small but deliberate adjustments to variables, including the mobile phase's composition, pH, buffer, flow rate, detecting wavelength, etc.

Assay of Formulation

A 10 mg of the formulation was accurately weighed and dissolved in 10 mL of Mobile phase in a volumetric flask. The solution was diluted with the mobile phase to achieve a final concentration of 1000 µg/mL, and then the appropriate volume of the aliquot was transferred to a 10 mL volumetric flask, and the volume was made up to the mark with the mobile phase to obtain a solution containing 10 µg/mL then analyzed by HPLC. The content of AZA and CAM was calculated based on the calibration curve.

Table 3: System suitability parameters for the proposed method (n = 6).

No. of runs	*Retention time (min)*		*Theoretical plates*		*Tailing factor*	
	Azadirachtin	*Camphor*	*Azadirachtin*	*Camphor*	*Azadirachtin*	*Camphor*
1	3.545	4.855	5460.20	8374.90	1.187	1.144
2	3.552	4.875	5462.27	8339.82	1.188	1.152
3	3.552	4.857	5410.27	8215.74	1.187	1.141
4	3.568	4.852	5415.26	8380.20	1.176	1.154
5	3.568	4.847	5378.18	8369.25	1.185	1.142
6	3.567	4.880	5471.40	8305.19	1.186	1.141
Avg	3.558	4.861	5432.93	8330.85	1.184	1.145
SD	0.0101	0.00134	37.16	62.99	0.0044	0.0054
%RSD	0.29	0.27	0.60	0.75	0.37	0.47
Limit	<2		>20000		<2	

RESULTS AND DISCUSSIONS

Optimization

The pure drug solution of AZA and CAM were injected individually into HPLC system and allow to run in different mobile phases like methanol, water, acetonitrile and phosphate buffer in different proposition to find the optimum conditions for the separation of azadirachtin and camphor. It was found that mobile phase containing buffer (pH 3.0):Acetonitrile (15:85 v/v) at a flow rate of 1.0 mL/min with detection wavelength 294 nm gave satisfactory results with sharp, well defined and resolved peaks with minimum tailing as compared to other mobile phase. The optimized chromatographic conditions shown in Table 1. Under this conditions the retention time were typically 3.5 minutes for AZA and 4.8 minutes for CAM, respectively as shown in Table 2 Figure 3 shows the chromatogram of the optimized condition.

Validation of Method

System Suitability

As seen in Table 3, all of these parameters' RSD values for AZA and CAM were less than 2%, indicating that the proposed HPLC method's parameters all meet ICH standards. As a result, it is determined that the devised HPLC method is appropriate and efficient for the analysis.

Table 4: Data for linearity study

Marker	*Concentration range (ppm)*	*Regression equation*	R^2
Azadirachtin	0.1-0.9	y = 12211x + 4831.1	0.999
Camphor	20-180	y = 2395.7x + 1787.5	0.999

Linearity

The results show an excellent correlation between peak areas and concentrations level within the tested concentration range of 0.1–0.9 ppm for AZA and 20–180 ppm for CAM (Table 4). The correlation coefficients were greater than 0.999 for each marker, which meets the method validation acceptance criteria; hence, the method is said to be linear (Figure 4,5).

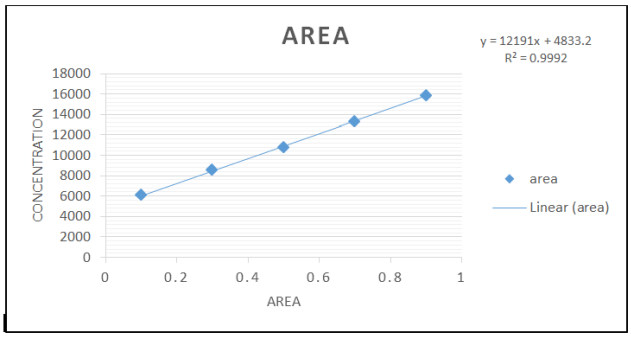

Figure 4: Calibration curve of azadirachtin

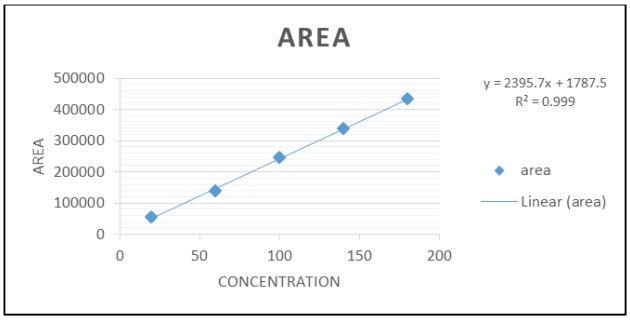

Figure 5: Calibration curve of camphor

Table 5: Intraday precision results for the developed RP-HPLC method.

Drug	*Conc.(µg/mL)*	*Peakarea*			*Meanpeakarea*	*SD*	*%RSD*
		I	*II*	*III*			
	0.3	8540	8525	8440	8501.66	53.92	0.63
AZA	0.5	10755	10599	10690	10681.33	78.36	0.73
	0.7	13337	13237	13330	13301.33	55.82	0.42
	60	138343	139233	138580	138718.66	460.91	0.33
CAM	100	243851	246270	245684	245268.33	1261.93	0.51
	140	338102	335266	337222	336863.3	451.62	0.43

Table 6: Inter-day precision results for the developed RP-HPLC method

Drug	*Conc. (µg/mL)*	*Peak area*			*Mean peak area*	*SD*	*%RSD*
		Day 1	*Day 2*	*Day 3*			
	0.3	8540	8555	8528	8541	13.52	0.158
AZA	0.5	10755	10599	10725	10693	82.77	0.774
	0.7	13337	13420	13251	13336	84.50	0.633
	60	138343	139020	137552	138305.0	9737.72	0.53
CAM	100	243851	242889	246995	244578.3	2147.45	0.88
	140	338102	336296	338081	337493.0	1036.68	0.31

Table 7: LoD and LoQ value for AZA and CAM

Parameter	*AZA*	*CAM*
SD of the Y-Intercepts of 5 Calibration curve	78.019	22.63
Mean slope of 5 calibration curve	12068.66	2382.93
LoD(µg/mL)	0.0213	0.031
LoQ(µg/mL)	0.0646	0.094

Precision

Two levels of precision were examined: repeatability (daily) and intermediate precision (inter-day). The findings are shown in %RSD (as shown in Table 5,6), which stayed below 2% and demonstrated the exceptional precision of this technique.

LoD and LoQ

The results of the LOD and LOQ have been mentioned in Table 7.

Table 8: The result obtained for different wavelength data for AZA and CAM

Drug	*Conc. (µg/mL)*	*Peak area*			*Mean peak area*	*SD*	*%RSD*
		293	*294*	*295*			
	0.3	8455	8540	8515	8503.33	43.68	0.51
AZA	0.5	10755	10499	10835	10696.33	175.51	1.64
	0.7	12990	13337	13102	13143.0	177.096	1.35
	60	139952	138343	139956	139417.0	930.11	0.67
CAM	100	239168	243851	245165	242728.0	3152.27	1.30
	140	329851	338102	336211	334721.33	4322.50	1.29

Table 9: The result obtained for different wavelength data for AZA and CAM

Drug	*Conc. (µg/mL)*	*Peak area*			*Mean peak area*	*SD*	*%RSD*
		0.9 mL/min	*1.0 mL/min*	*1.1 mL/min*			
	0.3	8720	8540	8595	8618.33	92.240	1.070
AZA	0.5	10623	10755	10833	10737	106.15	0.988
	0.7	12899	13337	13252	13129.3	219.8	1.674
	60	141105	138343	138992	139480.0	1444.2	1.040
CAM	100	247210	243851	246511	245857.33	1772.3	0.72
	140	342582	338102	339600	34009.6	2280.59	0.68

Table 10: The result obtained for different wavelength data for AZA and CAM

Drug	*% Level*	*Amt. of a sample taken (µg/mL)*	*Amt. of Standard spiking (µg/mL)*	*Total Amt. (µg/mL)*	*Conc.Found (µg/mL)*	*%Recovery*
	I (50 %)	0.1	0.05	0.15	0.160	100.056
AZA		0.1	0.05	0.15	0.098	98.25
		0.1	0.05	0.15	0.152	101.812
	II (100 %)	0.1	0.1	0.2	0.25	101
AZA		0.1	0.1	0.2	0.19	99.50
		0.1	0.1	0.2	0.25	100.06
	III (150%)	0.1	0.15	0.25	0.28	100.40
AZA		0.1	0.15	0.25	0.20	98.521
		0.1	0.15	0.25	0.24	99.98
	I (50%)	20	10	30	30.321	100.52
CAM		20	10	30	30.405	100.00
		20	10	30	29.454	98.57
	II (100%)	20	20	40	39.445	98.86
CAM		20	20	40	39.458	99.50
		20	20	40	40.201	100.12
	III (150%)	20	30	50	50.256	100.56
CAM		20	30	50	50.260	100.9
		20	30	50	49.501	98.49

Table 11: Accuracy data

Drug	*Retention time*	*Area*	*Tailing factor*	*Found %*
Azadirachtin	3.5	37857	1.187	2.7
Camphor	4.8	87755	1.144	35.8

Robustness

Different Wavelength

Changes in wavelength, flow rate, and pH were used to evaluate robustness. For AZA and CAM, the %RSD was determined. The result obtained for different wavelength data for AZA and CAM presented in Table 8 and results obtained for different flow rate presented in Table 9.

Different Flow Rate

The Result obtained for Different wavelength data for AZA and CAM.

Accuracy

The method's accuracy was verified by a recovery analysis using formulations at three levels of standard addition% recovery of AZA, and CAM was found between 98 to 102%. The results data presented in Table 10.

Analysis of Formulation

The result of the formulation analysis has been presented in Table 11.

CONCLUSION

A rapid, accurate, convenient and precise RP-HPLC method has been developed for the simultaneous estimation of AZA and CAM The proposed method followed the ICH guidelines. The proposed method can be used for the routine analysis of AZA and CAM without interference of excipients.

REFERENCE

1. Morgan ED. Azadirachtin, a scientific gold mine. Bioorganic & Medicinal Chemistry. 2009 Jun;17(12):4096–105 Available from: doi.org/10.1016/j.bmc.2008.11.081
2. Koul O, Isman MB, Ketkar CM. Properties and uses of neem, Azadirachta indica. Canadian Journal of Botany. 1990 Jan 1;68(1):1–11. Available from: doi.org/10.1139/b90-001
3. Butterworth Jh, Morgan Ed, Percy Gr. Chem In form Abstract: Struktur Von Azadirachtin, Die Funktionellen Gruppen. Chemischer Informationsdienst. 1972 Dec 26;3(52) Available from: doi.org/10.1002/chin.197252456.
4. Biological activities of different neem leaf crude extracts used locally in Ayurvedic medicine. Pacific Science Review A: Natural Science and Engineering [Internet]. 2016 Jul 1 [cited 2020 Oct 28];18(2):128–31. Available from: https://www.sciencedirect.com/science/article/pii/ S2405882316300667
5. Hamidpour R, Hamidpour S, Hamidpour M, Shahlari M. Camphor (Cinnamomum camphora), a traditional remedy with the history of treating several diseases. International Journal of Case Reports and Images. 2013;4(2):86. . Available from: doi.org/10.5348/ijcri-2013-02-267-ra-1
6. Chaursiya AC, L. Dumpala R. A Review on Revision of ICH Q2 (R1) and New ICH Q14 Guidance. Global Journal for Pharma and Allied Sciences. 2020 Nov 15;1(6):1–6. Available from: doi.org/10.47583/gjfpas.2020.v01i06.001
7. Khristi A, Prajapati N. Development and validation of RP-HPLC method for estimation of fluticasone propionate and mupirocin in a combined topical dosage form. International Journal of Pharmaceutical Sciences and Research, 2020 11(9):4411. Available from: doi.org/10.13040/IJPSR.0975 - 8232.11(9).4411-19
8. Joshi H, Khristi A. RP-HPLC method development and validation for the simultaneous estimation of Teneligliptine Hydrobromide Hydrate (TEN) and Metformin Hydrochloride (MET) in tablet dosage form. Indian drugs, 2019 Aug 28;56(08):49–56. Available from: doi.org/10.53879/id.56.08.11253
9. Khristi A, Baraka J, Dasvani B. Analytical Method Development and Validation of First Order Derivative Spectrophotometric Method for Simultaneous Estimation of Telmisartan and Metformin Hydrochloride in their Combined Pharmaceutical Dosage Form. International Journal of Pharmaceutical Quality Assurance, 2020 Mar 25;11(01):60–8. 10.25258/ijpqa.11.1.9