RESEARCH PAPER

Simultaneous estimation and method development of bioactive compounds

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ABSTRACT
A simple, precise, and accurate simultaneous equation method was successfully developed and validated for the quantitative estimation of withanolide A and aloin using UV spectrophotometry. Methanol was selected as the solvent for the analysis, with the absorbance of the two bioactive compounds measured at their respective wavelengths of 229 nm (withanolide A) and 297 nm (aloin).The method demonstrated good linearity within a concentration range of 10-60 µg/ml for aloin and 5-30 µg/ml for withanolide A. Excellent recovery rates were observed for both compounds (98.72% for aloin and 99.45% for withanolide A, confirming the method's accuracy. The validated procedure was accurate, precise, and highly applicable for the simultaneous analysis of these bioactive compounds, making it a suitable and robust method for formulation development in pharmaceutical research and industry.
Keywords: simultaneous, UV spectrophotometry, bioactive compounds, withanolide A, aloin and method's accuracy
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1.1 Introduction
In pharmaceutical and chemical analysis, UV-Visible Spectrophotometry serves as a vital tool for determining the quantity of various compounds within solutions.[1] The underlying operational principle is the Beer-Lambert Law [2], which dictates a direct correlation between the amount of monochromatic light a solution absorbs and both the analyte's concentration and the distance the light travels through the sample.[3,4]
Method development utilizing this technique involves systematically defining the optimum parameters for accurate measurement. Key steps typically include selecting an appropriate, transparent solvent (such as methanol) that dissolves the analytes without interfering with the absorbance readings, identifying the wavelength of maximum absorbance (λmax) and validating the method through established linearity ranges, accuracy assessments, and precision testing as outlined by guidelines (e.g., ICH).[5]
A powerful analytical technique for complex samples is the Simultaneous Equation Method, often referenced as Vierordt's method.[6] This approach enables the accurate quantification of multiple analytes within a mixture even when their absorption spectra significantly overlap, eliminating the need for time-consuming physical separation steps.[7] The method operates on the principle that total absorbance is additive. For a system containing two active components (A and B), measurements are acquired at two distinct wavelengths (λ1 and $\lambda 2$),

typically the peak absorbance wavelengths for each pure compound. This yields two equations: [8]

$$A1 = ax1Cx + ay1Cy$$
$$A2 = ax2Cx + ay2Cy$$

Where:
$A1$ and $A2$ are the absorbances of the mixture at λ1 lambda and $\lambda 2$, respectively.
C x and Cy are the unknown concentrations of components X and Y.
$ax1$and $ax2$ are the absorptivity values of component X at λ1 lambda and $\lambda 2$, respectively.
$ay1$and $ay2$ are the absorptivity values of component Y at λ1 lambda and $\lambda 2$, respectively
By first establishing the specific absorptivity coefficients ('a' values) for both components from known standard concentrations and subsequently measuring the mixture's total absorbance at both wavelengths ($A1$ and $A2$), these two simultaneous linear equations can be mathematically solved to determine the unknown concentrations (Cx and Cy) of each analyte present in the sample.[8]
Withanolides constitute a unique class of over 300 naturally derived polyoxygenated C_{28} steroidal lactone triterpenoids, characterized by their foundational ergostane skeleton. These secondary metabolites are primarily sourced from plants within the Solanaceae (nightshade) family.[9] Found predominantly in Withania somnifera

(Ashwagandha), withanolides like Withaferin A and Withanolide A are the main active phytochemicals. Their biosynthesis within the plant uses the mevalonic acid and methylerythritol phosphate pathways. This diverse class has been used for millennia in traditional medicine systems such as Ayurveda and continues to attract substantial pharmaceutical interest due to its potent effects.[10] Pharmacological studies highlight robust anti-inflammatory, immunomodulatory, neuroprotective, and antioxidant properties. Specific withanolides also show significant anticancer potential by inducing apoptosis in various cell lines, positioning them as strong lead candidates for future drug development given their ability to modulate multiple biological targets simultaneously.[11]

Aloin is a prominent, naturally occurring anthraquinone C-glycoside found primarily in the bitter yellow latex located just beneath the skin of Aloe vera and Aloe ferox leaves. Chemically, it exists as a mixture of two diastereomers, aloin A and aloin B (collectively known as barbaloin), characterized by an anthraquinone core linked to a glucose sugar moiety.[12] Historically and pharmacologically, aloin is highly valued for its potent stimulant laxative properties; once metabolized by gut bacteria into aloe-emodin anthrone, it stimulates intestinal motility.[13] While its traditional use is extensive, regulatory changes in some regions have restricted its use in oral over-the-counter products due to safety concerns regarding potential long-term carcinogenicity. Current scientific interest now largely focuses on aloin-free Aloe gel for topical applications or further research into aloin's potential antioxidant, antiviral, and anti-inflammatory activities.[14]

1.2 Material and Methods

1.2.1 Apparatus

UV spectrophotometer , Shimadzu 1700 (Japan) with a spectral width 2nm , wavelength accuracy of 0.5 nm and a pair of quartz cell with 10mm. A Digital weighing balance of Remi and ultrabath sonicator were used in this study.

1.2.2 Reagents and materials

Methanol used as a solvent was of Merck Pvt. Ltd, freshly prepared triple distilled water , withanolide A procured by Natural Remedies Pvt.Ltd. and aloin was procured by Sigma Aldrich.

1.2.3 UV estimation For Withanolide A and aloin

1.2.3.1 Preparation of standard stock solution:

According to Indian Pharmacopoeia standard stock solution for API were prepared for that 10 mg of Withanolide A was dissolve in 100 ml of methanol (100 µg/mL). Out of this stock 0.25-1.50 ml was pipetted and diluted up to 5 ml by solvent methanol (5-30 µg/mL) and examined between 200-800 nm and 10 mg of aloin was dissolve in 100 ml of methanol (100 µg/mL). Out of this stock 0.50-3.00 ml (10-60 µg/mL) was pipetted and diluted up to 5 ml by methanol and examined between 200-800 nm. The maximum absorbance was determined using UV-Vis Spectrophotometer (UV- 1700, Shimadzu, Japan) to confirm the λ max of the drugs.[15]

1.2.3.2 Simultaneous Equation Method

From the standard stock solutions both drug (100 µg/mL), of both the solutions were taken and made it to final concentration of 5-30 µg/ml and 10-60 µg/ml . Absorbance was measured at both the wavelengths by using solvent as blank. The reading was taken in triplicate. Absorbance maxima of both the drugs were recorded at both the wavelengths. The concentration was determined by using simultaneous equation method.

By using the below equations the concentrations in the samples were obtained

CX= A1ay2 – A2ay1 / ax1ay2 – ax2ay1 Eq. 1

CY = A1ax2 – A2ax1 / ay1ax2 – ay2ax1 Eq. 2

where A1 and A2 are absorbances of mixture at 229 nm and 297 nm respectively, ax1 and ax2 are absorptivities of Withanolide A at λ1 and λ2 respectively, ay1 and ay2 are absorptivities of aloin at λ1 and λ2 respectively, Cx and Cy are concentrations of Withanolide A and aloin respectively.[16]

A1 = ax1Cp + ay1Cs (At 229 nm)
A2 = ax2Cp + ay2Cs (At 297 nm)
A1 = ax1Cp + ay1Cs (At 229 nm)
A2 = ax2Cp + ay2Cs (At 297 nm)
A1 = absorbance value of the sample solution at 229 nm
A2 = absorbance value of the sample solution at 297 nm
ax1 = absorptivity of aloin at 229 nm
ax 2 = absorptivity of aloin at 297 nm
ay1 = absorptivity of withanolide A at 229 nm
ay2 = absorptivity of withanolide A at 297 nm
Cy = concentration of the withanoliode A in µg/ml
Cx = concentration of the aloin in µg/ml

1.2.3.3 Precision Study (validation for method during analysis)

Both Inter- day precision and Intra-day precision were carried out as per the statistical requirement to support reproducibility of the method.

a) Intra Day Assay (validation for method during analysis)

The assay procedure was carried out in the same day in the duration of 2 hours to 3 hours at fixed concentration and the results were compared.

b) Inter Day Assay (validation for method during analysis)

The assay procedure was carried out in the after day in the duration of 24 hours at fixed concentration and the results were compared.

1.2.3.4 Ruggedness study (validation for method during analysis)

The ruggedness of the method was determined by carrying out the analysis using two different analysts and the respective absorbance was noted. Ruggedness of the methods was assessed by

carrying out assay 6 reading with different analyst by using same equipment.

1.2.3.5 Robustness study (validation for method during analysis)

To determine the robustness, the same procedure was carried out by changing the temperature and the result was compared with the same previous procedure.

1.2.3.6 Accuracy

Accuracy expresses the closeness of procedure between the value which is accepted (i.e may be true value, reference value or value that will be found. It was performed by spiking (adding) the required amount of analyte into the sample and analyzing spike sample to report found value. It was calculated with the % recovery at 80% , 100% and 120% of the sample. The amounts of Withanolide A and Aloin were estimated by applying the obtained values to equation.[17]

1.2.3.7 Sensitivity

In the method validation the sensitivity of the system and method calculated with the help of Limit of detection (LOD) and Limit of Quantification. The limit of detection and the limit of quantification of the drug was derived by calculating the signal-to-noise ratio (S/N, i.e.,3.3 for LOD and 10 for LOQ) using the following equations designated by International Conference on Harmonization (ICH) guidelines. [17]

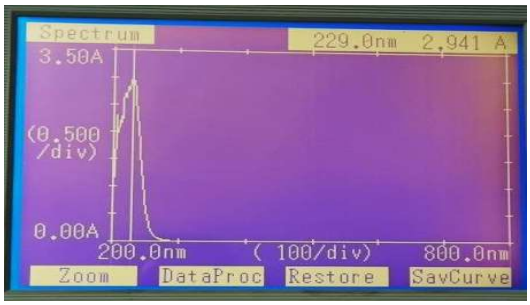
$LOD = 3.3 \times \sigma/S$

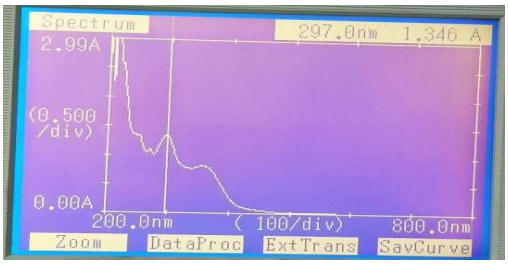
$LOQ = 10 \times \sigma/S$

Where, σ = the standard deviation of the response

S = slope of the calibration curve

1.3 Result





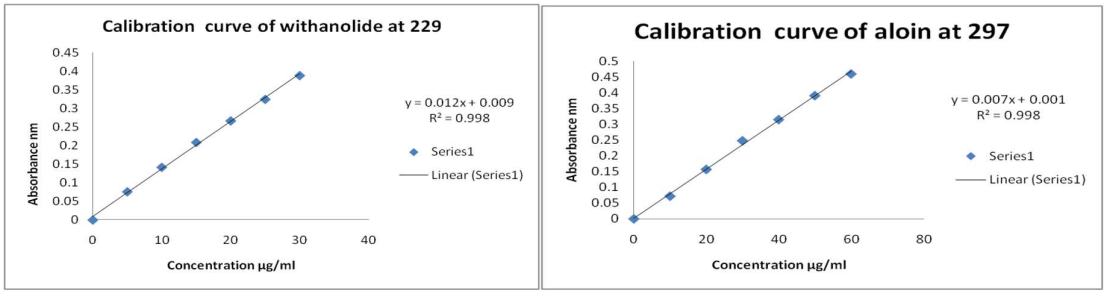
Graph 1: Lambda max of Withanolide A at 229 nm and aloin at 297 nm

Table no. 1: Calibration curve of Withanolide A and Aloin

Withanolide A	Aloin

The results of the validated UV spectrophotometric method for the simultaneous determination of withanolide A and aloin are summarized below. The method optimization utilized a Shimadzu 1700 UV-Visible spectrophotometer, establishing the maximum absorbance wavelengths (λmax) in methanol at 229 nm for withanolide A and 297 nm for aloin. A crucial aspect of the development was identifying a distinct isosbestic point at 245 nm within the overlay spectra (Graph 3). This finding confirms the suitability of the chosen wavelengths for the simultaneous equation approach, indicating an additive relationship in absorbance without chemical interference. Linearity was rigorously evaluated across specific concentration ranges: 5–30 µg/mL for withanolide A and 10–60 µg/mL for aloin (Graph 2; Table no. 1). Linear regression analysis provided high correlation coefficients R^2=0.998 for both compounds, confirming excellent adherence to the Beer-Lambert law within these ranges. The precision of the method was high, as demonstrated by low percentage relative standard deviation (%RSD) values obtained during both intra-day and inter-day precision studies (Table no. 2), which consistently remained below 3.0%. Accuracy was verified through a standard addition recovery study across four concentration levels. The resulting recovery percentages, ranging from 95.5% to 99.45% for withanolide A and 95.2% to 99.2% for aloin (Table no. 5), were well within acceptable analytical limits. The method's ruggedness was confirmed by comparing results obtained by two different analysts, while robustness was established by testing the impact of minor temperature variations (25°C vs. 30° C). In both tests, minimal impact on results and low %RSD values were observed, as detailed in Table and Table, respectively. Sensitivity parameters, the limits of detection (LOD) and quantification (LOQ), were calculated, yielding values for withanolide A and for aloin (Table no. 6).Overall, these results validate that the developed method is accurate, precise, robust, and rugged, confirming its suitability for routine quantitative analysis of withanolide A and aloin in combined formulation.

Concentration (µg/ml)	Absorbance (229nm)	Concentration (µg/ml)	Absorbance (297 nm)
5	0.076	10	0.072
10	0.142	20	0.157
15	0.209	30	0.248
20	0.267	40	0.315
25	0.325	50	0.391
30	0.389	60	0.460
Mean	0.10675	Mean	0.11925
SD	0.089515	SD	0.107168
%RSD	1.217228	%RSD	1.24127



Graph 2: Calibration curve of withanolide A and aloin

Table no. 2: Precision of Withanolide A and Aloin

Bio active compounds	Precision study	Results
Withanolide A	Intra day	0.61±0.001366
	Interday	0.63 ±0.002137
Aloin	Interday	2.6603 ±0.002345
	Intra day	2.1884±0.004131

Table no. 3: Result of ruggedness of Withanolide A and aloin

Bio active compounds	Ruggedness	Results
Withanolide A	Analyst-I	0.83± 0.001472
	Analyst -II	1.4±0.001941
Aloin	Analyst-I	1.8195±2.786874
	Analyst -II	2.5816±3.937004

Table no. 4 : Results showing robustness of Withanolide A and aloin

Bio active compounds	Robustness at	Results
Withanolide A	Temperature 25^0C	0.286 ± 0.000894
	Temperature 30^0C	0.285± 0.001304
Aloin	Temperature 25^0C	0.151167 ± 2.483277
	Temperature 30^0C	0.146333 ± 2.503331

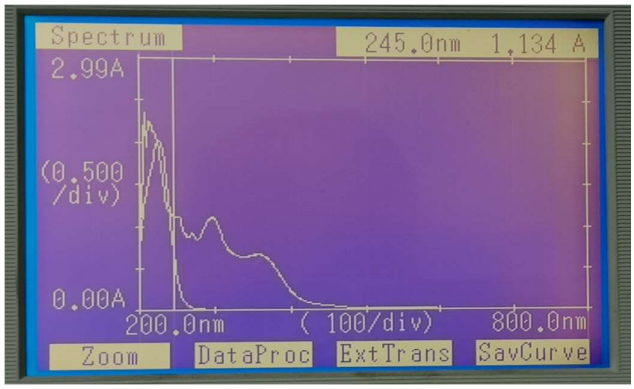
Table no. 5: Withanolide A Accuracy via % recovery

Accuracy level%	Actual amount (µg/ml)	Amount added (µg/ml)	Withanolide A		Aloin	
			Amount recovered (µg/ml)	% Recovery	Amount recovered (µg/ml)	% Recovery
0	20	0	19.10	95.5	19.11	95.5

80	20	16	35.27	97.9	34.28	95.2
100	20	20	39.59	98.9	39.70	99.2
120	20	24	43.76	99.45	43.28	98.72

Table no. 6 : LOD and LOQ

Sample	**LOD**	**LOQ**
Withanolide A	3.02	9.17
Aloin	3.04	9.21



Graph 3: Overlay spectra of Aloin and withanolide A with Iso absorptive point

Table no.7: Absorbance of the withanolide A and aloin at 229 and 297 wavelength

Name of the drug	**Concentration**	**Absorbance**	
		λ 1(229)	**λ 2(297)**
Withanolide A	5	0.076	0.085
	10	0.142	0.167
	15	0.209	0.224
	20	0.267	0.291
	25	0.325	0.346
	30	0.389	0.412
Aloin	10	0.061	0.072
	20	0.119	0.157
	30	0.231	0.248
	40	0.286	0.315
	50	0.373	0.391
	60	0.441	0.46

Discussion

Analytical Wavelengths and Linearity:

The method effectively utilized the intrinsic absorption maxima of each compound in methanol: 229 nm for withanolide A and 297 nm for aloin. A distinct iso-absorptive point (isosbestic point) was identified at 245 nm, confirming the suitability of these conditions for the simultaneous equation method. Excellent linearity was established using least squares regression analysis across specific concentration ranges: 5–30 µg/mL for withanolide A and 10–60 µg/mL for aloin. High correlation coefficients (R^2=0.998) for both indicate a strong linear relationship between absorbance and concentration, confirming adherence to the Beer-Lambert law within the tested ranges.

Accuracy and Precision:

The method demonstrated high accuracy, with recovery values falling within acceptable ranges (95.5–99.45% for withanolide A and 95.2–99.2% for aloin), minimizing systemic error. Precision was assessed through repeatability (intra-day) and intermediate precision (inter-day) studies, yielding low percentage relative standard deviation (%RSD) values (e.g., 0.61% intra-day for withanolide A; 2.1% intra-day for aloin). These low %RSD values underscore the high reliability and reproducibility of the measurements.

Robustness and Ruggedness:

The method's robustness was confirmed by analyzing samples under minor variations in analytical temperature (25°C and 30°C), which resulted in minimal shifts in results and low %RSD values (e.g., 0.31% and 0.45% for withanolide A). Ruggedness was also verified by comparing results obtained by two different analysts; the consistently low %RSD values indicate the method is insensitive to minor variations in laboratory conditions or operators.

Conclusion:

In summary, the developed simultaneous UV spectrophotometric method is validated according to ICH Q2(R1) guidelines. It is a precise, accurate, rugged, and robust analytical tool suitable for the routine quantitative analysis of withanolide A and aloin in combined dosage forms within pharmaceutical research and industry.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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