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ORIGINAL ARTICLE / ÖZGÜN MAKALE

DEVELOPMENT AND VALIDATION OF GREEN RP-HPLC AND SPECTROPHOTOMETRIC METHODS FOR DETERMINATION OF ALPELISIB IN BULK AND PHARMACEUTICAL DOSAGE FORMS

FARMASÖTİK DOZAJ FORMLARINDA ALPELİSİB MİKTAR TAYİNİ İÇİN VALİDE EDİLMİŞ YÜKSEK PERFORMANSLI SIVI KROMATOGRAFİK VE SPEKTROFOTOMETRİK YÖNTEM GELİŞTİRİLMESİ

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ABSTRACT

Objective: *This study aims to develop and validate green analytical methods, specifically UV spectrophotometry, first-order derivative spectrophotometry, and reverse-phase HPLC (RP-HPLC), for determining Alpelisib (ALP) in bulk and pharmaceutical formulations. By comparing the methods using GAPI and AGREE metrics, the study evaluates their environmental friendliness, precision, and applicability.*
Material and Method: *The UV spectrophotometric and RP-HPLC analyses were conducted using Shimadzu UV 1800 and Agilent 1100 HPLC systems, respectively. The mobile phase for HPLC comprised 0.1% trifluoroacetic acid in water, acetonitrile, and methanol (50:25:25 v/v/v). ALP tablets were prepared and analyzed after dissolution in methanol/water (50:50 v/v) and filtration. Validation was conducted according to ICH guidelines.*
Result and Discussion: *The developed methods showed high precision, robustness, and sensitivity. UV and HPLC methods were effective in determining ALP in both bulk drug and tablet formulations, with detection limits of 0.078 µg/ml for direct UV spectrophotometry and 14 µg/ml for RP-HPLC. Greenness evaluation highlighted the methods' environmental compatibility, making them suitable for sustainable pharmaceutical analysis.*
Keywords: *Alpelisib, anticancer drug, greenness evaluation, RP-HPLC, spectrophotometric, validation*

ÖZ

Amaç: *Bu çalışma, Alpelisib'in (ALP) yığın ve farmasötik formlarda tayini için çevreci UV spektrofotometri, birinci derece türev spektrofotometri ve ters faz HPLC (RP-HPLC) yöntemlerini geliştirmeyi ve doğrulamayı amaçlanmıştır. Çalışma yöntemlerin çevre dostu olduğunu, doğruluklarını ve uygulanabilirliklerini değerlendirmek için GAPI ve AGREE metrikleri ile karşılaştırılmıştır.*

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Gereç ve Yöntem: *UV spektrofotometrik ve RP-HPLC analizleri sırasıyla Shimadzu UV 1800 ve Agilent 1100 HPLC sistemleri ile gerçekleştirilmiştir. HPLC için mobil faz, %0.1 triflorasetik asit suda, asetonitril ve metanolün (50:25:25 h/h/h) karışımından oluşmaktadır. ALP tabletleri, metanol/su (50:50 h/h) içinde çözülüp filtrelendikten sonra hazırlanmış ve analiz edilmiştir. Doğrulama, ICH kılavuzlarına göre yapılmıştır.*

Sonuç ve Tartışma: *Geliştirilen yöntemler yüksek hassasiyet, dayanıklılık ve duyarlılık göstermiştir. UV ve HPLC yöntemleri kullanılarak ilaç formülasyonlarında ALP'nin tayini yapılmış ve doğrudan UV spektrofotometri için 0.078 µg/ml, RP-HPLC için ise 14 µg/ml olarak tespit limitlerine ulaşılmıştır. Çevreci değerlendirme, yöntemlerin sürdürülebilir farmasötik analiz için çevre dostu uyumluluğunu vurgulamaktadır.*

Anahtar Kelimeler: *Alpelisib, antikanser ilaç, çevreci değerlendirme, doğrulama, RP-HPLC, spektrofotometrik*

INTRODUCTION

In 2019, a pivotal achievement marked the pharmaceutical landscape with the approval of Alpelisib (ALP), the maiden alpha-specific phosphoinositide 3-kinase (PI3K) inhibitor, by the United States Food and Drug Administration (FDA) [1]. Developed by Novartis, ALP garnered recognition for its application in treating hormone receptor (HR)c-positive, human epidermal growth factor receptor 2 (HER2)-negative, PIK3CA-mutated, advanced, or metastatic breast cancer, particularly in combination with Fulvestrant. Branded as "Piqray," this groundbreaking medication, administered in oral tablet form, signifies a profound leap in targeted cancer therapy [2].

The PI3K pathway, a critical signaling cascade, assumes a central role in various cancer types, influencing essential cellular processes such as growth, survival, and metabolism [3]. Particularly noteworthy is the involvement of the Class II PI3K alpha isoform (p110α) in insulin-mediated glucose uptake and cell growth [4]. Genetic aberrations in the PIK3CA gene, encoding p110α, and modifications in the phosphatase enzyme PTEN, integral to the dephosphorylation of PIP3 to PIP2, are prevalent in diverse cancers [5]. The perturbation of the PI3K pathway is deeply implicated in cancer development, progression, and resistance to antineoplastic therapies [6,7].

The approval of ALP by the FDA represents a significant change in cancer treatment by targeting PI3K. Current research is exploring its potential use beyond breast cancer, including treatments for colorectal and ovarian cancers [8,9]. This evolution in pharmaceutical progress underscores the profound impact of targeted PI3K inhibition in reshaping the landscape of cancer therapeutics.

Developing robust analytical methodologies for the determination of ALP with high sensitivity, precise, and reliable results remains a significant challenge. A few methods including high-performance liquid chromatography combined with a fluorescence detector (HPLC-FLD) [10], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [11], and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) [12], methods for the determination and analysis of ALP have been published in the literature. Also, electrochemical methods for the ALP determination in bulk and biological fluids samples have been reported [13,14].

To the best of our knowledge, no previously published papers have addressed the use of UV spectrophotometry for the determination of ALP. UV-spectrophotometry is a versatile technique widely used for the quantitative analysis of many pharmaceutical compounds [15]. Its simplicity and time efficiency, combined with low solvent consumption, make it more economical and environmentally friendly compared to other analytical methods. Although spectral overlaps can occur when analyzing mixtures with UV-spectrophotometry, this issue can be resolved using post-mathematical processing algorithms [16].

In addition, High-Performance Liquid Chromatography (HPLC) stands as a pivotal analytical technique with widespread applications across diverse fields including pharmaceuticals, food, cosmetics, biochemistry, and environmental analysis [17]. This technique is instrumental in delivering efficient, reliable, and precise analytical methods, playing a crucial role in the characterization and quantification of compounds in complex samples [18]. HPLC's versatility and sensitivity make it an indispensable tool for scientists and researchers seeking to unravel the composition of substances in different industries, contributing significantly to advancements in analytical chemistry and related

disciplines [19]. The incorporation of HPLC coupled with a Diode Array Detector (DAD) emerges as a cost-effective strategy for therapeutic drug monitoring [20]. This approach, despite the absence of stable isotope internal standards commonly utilized in mass spectrometry, offers reliable results. The utilization of HPLC with DAD presents a viable alternative, demonstrating efficacy in drug quantification while contributing to overall cost reduction in analytical methodologies. This pragmatic application underscores the versatility and economic advantages of HPLC-DAD for therapeutic drug monitoring in the absence of stable isotope internal standards [21].

Notably, to our knowledge, no published analytical method exists for the determination of ALP in bulk and tablet formulations using an RP-HPLC system equipped with a DAD and UV spectrophotometry. This underscores the uniqueness and importance of this research endeavor. The main aim of this study is to develop novel analytical methods that are sensitive, simple, economical, accurate, and reliable for the determination of ALP in bulk and tablet formulations. To evaluate their efficacy and practicality, all developed approaches were statistically compared using analysis of variability. Additionally, we assessed the environmental effect of the novel UV spectrophotometric and HPLC methods by conducting a greenness profile assessment using AGREE and GAPI metrics.

MATERIAL AND METHOD

Instrument and Software

Shimadzu UV 1800 double-beam UV-VIS spectrophotometer with Shimadzu UV Probe 2.7 system software was used for the spectrophotometric measurements. The samples' absorbance was recorded by placing them into 1 cm quartz cuvettes. The chromatographic analyses were conducted utilizing an HPLC system, specifically the Agilent 1100 Technologies model. This HPLC system was equipped with a quaternary pump featuring an automated membrane eluent degasser unit, an autosampler, a column oven, and a DAD to enable comprehensive detection and quantification of analytes. System operation and data acquisition were proficiently overseen by employing the Agilent Chemstation Plus Software, headquartered in Palo Alto, California, USA. To achieve optimal separation and analysis of target compounds, an Inertsil C8-3 column with dimensions of 4.6 x 150 mm and particle size of 3 µm, manufactured by Supelco, based in the United States, was utilized. To ensure the pH measurements throughout the experiments, a Mettler Toledo pH meter featuring a high-quality glass electrode was employed.

Chemicals and Solvents

ALP standard and pharmaceutical preparation was provided by FARMANOVA Health Services (Istanbul, Turkey). The chemicals and reagents utilized were of gradient-grade quality suitable for chromatography analyses. Methanol, acetonitrile, and analytical grade trifluoracetic acid were obtained from Merck (Darmstadt, Germany). Millipore Milli-Q system (Milford, MA, USA) was used to provide ultrapure water for the preparation of mobile phase solutions and sample dilutions. Before HPLC analysis, all mobile phases were meticulously passed using a 0.22 µm membrane filtration process, employing a vacuum pump for rapid and efficient filtration. Furthermore, to eliminate any potential sources of air bubbles or other inconsistencies, the filtered mobile phase was subjected to sonication.

Preparation of ALP Sample Solution for UV, and HPLC Method Development

The ALP tablets with Batch No. 1704007B are produced in various formulations, including 150, 200, and 250 mg of ALP. For the experiments conducted in this study, the pharmaceutical preparation containing 200 and 250 mg of ALP was utilized. The choice of this particular formulation allows for a focused investigation into the characteristics and stability of the ALP drug at the specified dosage. In that case, 10 tablets were further weighed and then dissolved in methanol/water (50:50 v/v) within a 500 ml volumetric flask after allowing it to rest in an ultrasonic bath for 20 minutes. The obtained solution was then filtered using a 0.45 µm syringe filter. Subsequently, the filtered solution was transferred to volumetric flasks and further diluted with the mobile phase for the determination of the pharmaceutical formulation.

Preparation of ALP Standard Solution for UV Spectrophotometric, and HPLC Method Development

To create the standard stock solution for developed methods, the ALP standard was dissolved in methanol/water (50:50 v/v) at a concentration of 1000 µg/ml. To achieve the working standard with different concentrations, further, dilute the previously prepared standard stock solution with the mobile phase made from the working standard. The mixture of methanol and water (50:50 v/v) was chosen as a solvent for preparing the standard stock solution for UV spectrophotometry. For the HPLC method, the mobile phase, composed of 0.1% trifluoroacetic acid in water, was meticulously prepared by dissolving 1 ml of trifluoroacetic acid in 1000.0 ml of ultrapure water. This carefully formulated mobile phase serves as a critical component in chromatographic analysis, providing an optimal environment for the separation and quantification of ALP in subsequent experiments.

Method I (Direct UV Spectrophotometric Method)

The principle of UV-spectroscopy is the most straightforward method for conducting various analyses. A blank solution for the mobile phase was maintained. The standard ALP solution was scanned within the 200-400 nm range. An absorption spectrum was identified at 313.6 nm, which was chosen as the analytical wavelength for subsequent analysis. The recorded spectrum is displayed in Figure 1.

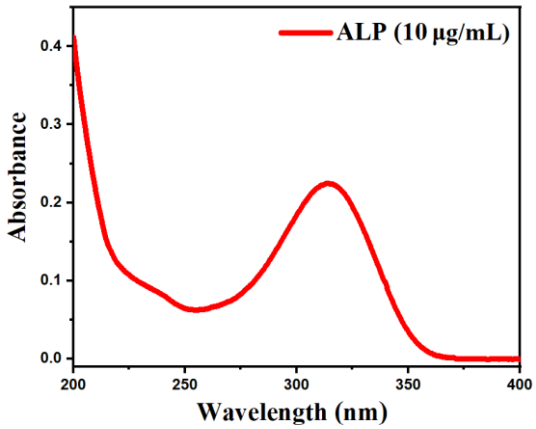

Figure 1. UV spectrum of ALP standard solution (λ_{max} = 313.6 nm)

Method II (First-Order Spectrophotometric Method)

The first-order spectrophotometric method can extract both qualitative and quantitative information from unresolved band spectra. A blank solution served as the mobile phase. Measurements were taken within the 200-400 nm range. Using the instrument's inbuilt software, zero-order spectra were converted into first-order derivative spectra (delta lambda 8.0, scaling factor 100). Analysis of the data for linearity revealed a λmax of 294.8 and 336.2 nm.

Method III (RP-HPLC Method)

The quantification of ALP was achieved through a rigorous developed and optimized process using an RP-HPLC system equipped with a DAD. To ensure accurate separation and analysis, an Inertsil C8-3 column (4.6 x 150 mm, 3 µm) was selected as the stationary phase. Various parameters such as flow rates, column temperature, and mobile phase composition were meticulously optimized to achieve optimal results. The mobile phase composition chosen for this analysis consisted of 0.1% trifluoroacetic acid, acetonitrile, and methanol in a volumetric ratio of 50:25:25 (v/v/v). The entire chromatographic run was 10 minutes, with a consistent flow rate of 1 ml/min. The injection volume was set at 10 µl, and the column temperature was maintained at a stable 20°C. In contrast, the samples were stored at a lower

temperature of 5°C to prevent any degradation or unwanted reactions before analysis. Peaks in the chromatogram were detected at a wavelength of 310 nm for the detection of ALP.

Optimization of Chromatographic Method

In the pursuit of developing and establishing a suitable RP-HPLC approach for the determination of ALP in both standard (A) and tablet forms (B), a series of meticulous preliminary tests were conducted. Various chromatographic conditions were systematically explored and scrutinized, ultimately leading to the development of optimized parameters detailed below. The conclusive analysis was executed utilizing a mobile phase consisting of 0.1% trifluoroacetic acid, acetonitrile, and methanol in a volumetric ratio of 50:25:25 (v/v/v) at a constant flow rate of 1 ml/min. The analytical procedure employed a detector wavelength of 310 nm, and an injection volume of 10 µl was introduced into the chromatographic system, facilitating a runtime of 10 minutes. The finalized method demonstrated its efficacy through the achievement of well-defined and distinct peaks, ensuring both sharpness and resolution. The optimized chromatogram, visually depicted in Figure 2, serves as a testament to the method's precision and suitability for the targeted analysis of both ALP standard and pharmaceutical formulations.

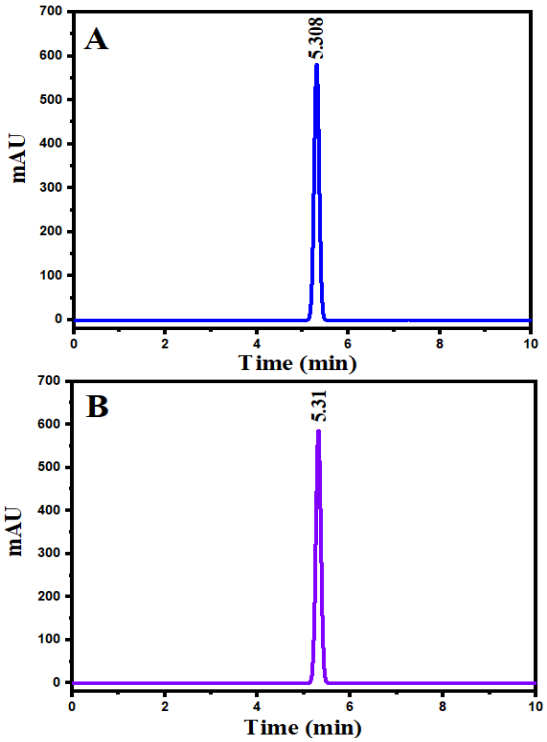

Figure 2. Chromatograms of ALP standard (A), and ALP tablet forms (B)

Method Validation

The constructed UV spectrophotometry and RP-HPLC analytical approaches of ALP have undergone validation in terms of robustness, accuracy, precision, linearity, sensitivity, and stability following ICH (Q2B, 1996) (Committee, 1996).

Robustness

The robustness of the analytical method was comprehensively assessed by subjecting it to various deliberate variations in critical parameters. In the case of the HPLC method, these parameters included the mobile phase composition, flow rate, column temperature, and detection wavelength. Two distinct mobile phase ratios were systematically investigated to ascertain the optimal conditions for

chromatographic analysis. The first composition, denoted as 50:25:25 (v/v/v), encompassed 0.1% trifluoroacetic acid, acetonitrile, and methanol. Simultaneously, an alternative ratio of 40:30:30 (v/v/v) with the same composition was explored to assess its chromatographic performance. The flow rate, another pivotal parameter, was intentionally varied over a range of 0.9, 1.0, and 1.1 ml/min, while the column temperature was modified at three distinct settings of 15, 20, and 25°C. Additionally, different detection wavelengths were explored, encompassing 308, 310, and 312 nm. For the spectrophotometric method, the absorption maxima shifted by ± 2 nm for the 25 µg/ml ALP solution. The relative standard deviation (RSD%) was calculated over five measurements.

Accuracy

The accuracy parameter was carried out using the recovery study method. The proposed method's accuracy was quantified using the standard addition method at 25 and 37.5 µg/ml for spectrophotometry. At the same time, the HPLC method, three different ALP standard concentrations, 200, 400, and 600 µg/ml, were tested to suit the low, medium, and high measurement points of the calibration chart. Then standard solutions were incorporated into the tablet solution at the mount levels of 50%, 100%, and 150% of the nominal standard concentration. The standards and prepared tablet solutions were injected into the RP-HPLC device three times and the mean, % relative standard deviation (RSD %), and recovery values were calculated from the results obtained. The recovery was evaluated by measuring the percentage recovery and quantifying the drug in three separate preparations at each concentration level. This approach provided a comprehensive assessment of the method's accuracy and its ability to produce results that are both precise and close to the true values across a wide concentration range.

Precision

The precision of Methods I and II was assessed by examining intraday precision (within one day) and interday precision (six consecutive days). For the HPLC method, precision intraday was calculated from the coefficient of variance for six replicates of injecting the standard. The ALP standard solution with the concentration of 400 µg/ml was injected in RP-HPLC six times and the area, theoretical number of plates, and tailing factor, for all six injections were compared. To evaluate the intermediate precision of the method, the standard solution at 400 µg/ml concentration and the pharmaceutical preparation with the same ALP concentration were injected for six days and the areas for all six injections were compared. The solutions were kept in amber-colored vials at 2-8°C after each injection. The change percentage between days for the ALP standard and pharmaceutical preparation was calculated. In the case of the spectrophotometric method, a concentration of 20 µg/ml of standard drug solution is evaluated for intraday and interday precision, and variations are investigated. The corresponding average absorbance (UV) and peak area (HPLC) were noted, and the results were reported as % RSD, with an acceptable limit set at below 2%.

Linearity and Sensitivity

In this study, ALP standard solutions were injected into Method I and Method II at concentrations ranging from 10 to 40 µg/ml, and into Method III at concentrations ranging from 100 to 600 µg/ml. The mean and RSD % were calculated from the results obtained. Subsequently, calibration curves and linear regression analysis were performed at the chosen wavelength for developed methods. The sensitivity assessment of the developed methods encompassed the determination of both the LOD and LOQ. The LOD represents the lowest concentration at which the analyte can be detected but not precisely quantified, while the LOQ signifies the lowest concentration within the linear range where precise and reliable quantification is achievable. Following ICH guidelines, these parameters were calculated using the following equations derived from the calibration curve: $LOD = 3.3\sigma/S$ and $LOQ = 10\sigma/S$. Here, σ represents the standard deviation of the y-intercept of the regression line, and S denotes the slope of the calibration curve. These calculations were integral to establishing the method's sensitivity and quantifying its capability to detect and quantify ALP at low concentrations.

Stability

The stability of ALP was studied by the HPLC method using 400 µg/ml ALP standard and

pharmaceutical preparation at several storage conditions for 24 hours. Both solutions were subjected to various storage conditions for 24 hours. The tested storage conditions included a dark environment at room temperature and refrigeration at 2-8°C. At hourly intervals within the 24-hour duration, the solutions were meticulously retrieved, and the areas of the chromatographic peaks were precisely determined. Subsequently, change percentages were then calculated by comparing the obtained areas before and after storage, providing a quantitative measure of any alterations in the stability of the solutions.

RESULT AND DISCUSSION

Validation of Developed Analytical Methods

Following the International Conference on Harmonization ICH guidelines, the developed I, II, and III methods were validated for robustness, accuracy, precision, linearity, sensitivity, and stability.

Robustness

The assessment of robustness, as an indicative measure of the method's resilience against small yet purposeful modifications in chromatographic parameters, was meticulously conducted. This involved a systematic exploration of the impact of small changes in mobile phase composition, flow rate, column temperature, and detection wavelength on the analytical performance. The pivotal parameters of retention time, theoretical plate count, and tailing factor were judiciously scrutinized and found to consistently adhere to predefined criteria. Consequently, the method demonstrated robustness in the face of variations across all tested conditions, substantiated by the empirical data presented in Table 1. Moreover, the robustness of the spectrophotometric method was evaluated by deliberately altering the maximum absorption wavelength. The relative standard deviation (RSD%) was found to be 1.2%, confirming the method's robustness and indicating no significant variations in absorbance values.

Table 1. Assessment of the robustness of ALP

		Retention time		Tailing factor		Theoretical plate count	
		Standard	**Sample**	**Standard**	**Sample**	**Standard**	**Sample**
Detection wavelength	**308**	5.283	5.274	1.1	1.1	10373	10088
	310	5.286	5.292	1.1	1.1	10409	10491
	312	5.282	5.301	1.1	1.1	10262	10413
Column temperature	**15**	5.309	5.305	1.1	1.1	10695	10251
	20	5.286	5.292	1.1	1.1	10409	10491
	25	5.282	5.287	1.1	1.1	9054	10654
Flow rate	**0.9**	5.862	5.857	1.1	1.1	11890	11059
	1.0	5.286	5.292	1.1	1.1	10409	10491
	1.1	4.838	4.839	1.1	1.1	9870	9960
Mobile phase composition	**40/30/30**	3.449	3.446	1.1	1.1	8875	8905
	50/25/25	5.286	5.292	1.1	1.1	10409	10491

Accuracy

The proposed method’s accuracy was quantified using the standard addition method at 25.0 and 37.5 µg/ml for direct (I) and First-order spectrophotometric (II) methods. At the same time, the accuracy of the RP-HPLC method was ascertained through a percent recovery study involving the addition of ALP standard solutions of known concentrations (50%, 100%, and 150%) to a tablet solution. Different replicate measurements using different methods I, II, and III, showed that the percent recovery was within the allowed ranges (Tables 2 and 3). It is noteworthy that all obtained RSD % values for methods I, II, and III are well within the ICH-approved limits, underscoring the robust accuracy of the developed analytical methodology. These results affirm the method's capability to yield accurate and reliable quantitative results across a range of concentrations.

Table 2. Recovery values obtained for the determination of ALP for the direct (I) and First-order spectrophotometric (II) methods

Set	ALP added (µg/ml)	ALP recovered (µg/ml)			Mean recovery %			RSD %		
		Method I	Method II (294.8 nm)	Method II (336.2 nm)	Method I	Method II (294.8 nm)	Method II (336.2 nm)	Method I	Method II (294.8 nm)	Method II (336.2 nm)
1	25.0	24.71	24.35	23.78						
2	25.0	24.89	24.35	23.91						
3	25.0	24.75	24.37	24.00	99.2	97.6	95.8	0.33	0.38	0.67
4	25.0	24.84	24.54	24.16						
1	37.50	37.47	37.23	36.66						
2	37.50	37.52	37.19	36.70	100.2	99.2	98.0	0.45	0.13	0.29
3	37.50	37.52	37.21	36.80						
4	37.50	37.84	37.16	36.90						

Table 3. Recovery values obtained for the determination of ALP for the RP-HPLC method

Recovery Levels %	Set	ALP added, mg/ml	ALP recovered		Mean recovery	RSD %
			mg/ml	%		
50	1	0.2	0.199983	99.9	99.9	0.05
	2	0.2	0.20034	99.9		
	3	0.2	1.99827	99.8		
100	1	0.4	0.406281	101.5	101.1	0.32
	2	0.4	0.403893	100.9		
	3	0.4	0.40212	101.0		
150	1	0.6	0.599941	100	99.9	0.1
	2	0.6	0.599763	99.9		
	3	0.6	0.598956	99.8		

Precision

In this study, six standard solutions of ALP were analyzed using RP-HPLC and spectrophotometric methods to evaluate intraday and interday precision. Under inter-day, the % RSD, calculated within the ICH limit of ± 2%, was found to be 0.09% for RP-HPLC and 1.06% for the spectrophotometric method, significantly assuring the precision of the proposed methods. The performance of intra-day precision and the percent RSD for the response of six replicate measurements in the spectrophotometric method were within the acceptable ranges (0.96%). In the intermediate precision analysis conducted after a six-day interval, notable differences in stability were observed between the ALP standard and the pharmaceutical preparation. The ALP standard exhibited a minimal 0.3 percent change over the six days, indicating a high degree of stability under the specified storage conditions. Conversely, the ALP in the pharmaceutical preparation demonstrated a more substantial 9.6 percent change, signifying a comparatively lower sensitivity to environmental factors when compared to the standard. These findings underscore the importance of considering formulation-specific stability, as variations in the drug solution may be influenced by additional excipients or interactions. The minimal change in the ALP standard reaffirms its robust stability, while the observed alteration in the drug solution warrants further investigation into factors affecting its stability under intermediate precision conditions.

Linearity and Sensitivity

Linearity samples were prepared through multiple dilutions of a standard stock solution. The measured responses of ALP at different concentrations using the developed methods were analyzed to create linear regression equations. Table 4 summarizes the key statistical parameters for each process. The results show an excellent correlation between the recorded responses and drug concentrations within the specified range, as indicated by high correlation coefficients. Moreover, the linearity calibration curves, visually represented in Figures 3-5, provide a clear illustration of the method's ability to generate

consistent and proportional responses across the specified concentration range. The sensitivity of the methods was successfully determined by assessing the limit of detection (LOD) and the limit of quantification (LOQ). The calculated LOD values were 0.078 µg/ml for Method I, 0.096 µg/ml for Method II, and 14 µg/ml for Method III. These results highlight the high sensitivity of the methods, demonstrating their capability to reliably detect ALP at low concentrations. This level of sensitivity is essential for the effectiveness of the methods in pharmaceutical and chemical analyses, particularly when dealing with samples containing low analyte concentrations.

Table 4. Linearity data for ALP by direct UV Spectrophotometry (I), first-order spectrophotometric (II), and RP-HPLC methods

Parameters	Method I	Method II		Method III
		(294.8 nm)	(336.2 nm)	
Linearity Range (µg/ml)	10-40	10-40	10-40	100-600
Calibration equation	y = 0.0221 x - 0.001	y = 0.047 x -0.004	y = -0.071 x + 0.023	y =11.73 x +5.122
Correlation coefficient (R^2)	0.9997	0.9996	0.9990	0.9996
LOD (µg/ml)	0.078	0.096	0.084	14.0
LOQ (µg/ml)	0.261	0.323	0.281	43.0

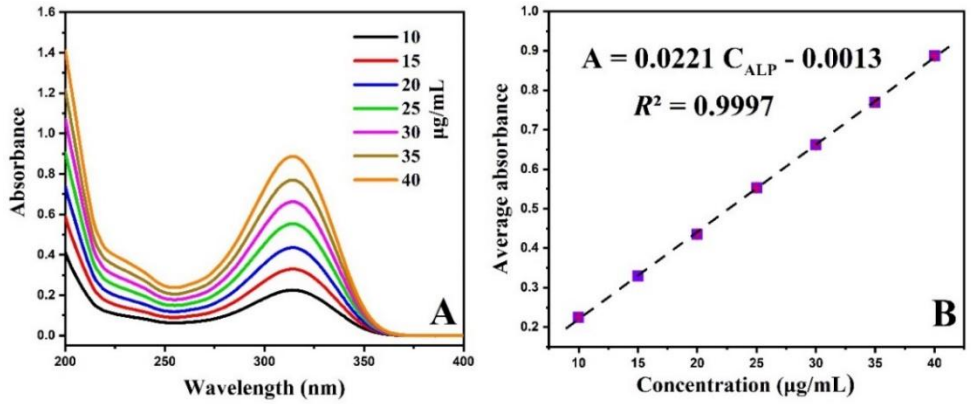

Figure 3. Spectrum of ALP for direct UV spectrophotometric method (10-40 µg/ml)

Stability

Following a meticulous examination of the stability profiles for both the standard and pharmaceutical preparation over 24 hours, including storage conditions of 2-8°C and room temperature, it was established that ALP demonstrated stability under these conditions. Throughout these trials, the highest recorded RSD% was 0.2%. This minimal variability indicates a high level of stability for ALP in both the standard and pharmaceutical preparation under the specified conditions over 24 hours. These findings affirm the robustness of the analytical method and its ability to maintain stability for ALP in various formulations and storage conditions over a short duration.

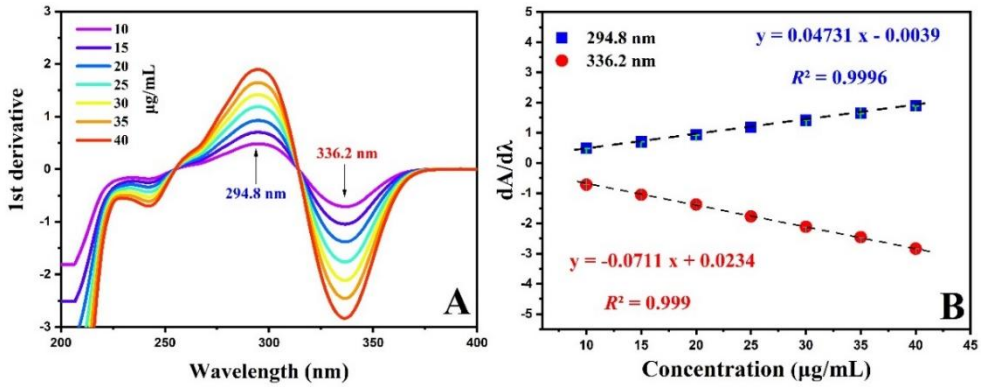

Figure 4. Overlay first-order derivative spectra with the linearity of ALP concentrations ranging from 10 to 40 µg/ml

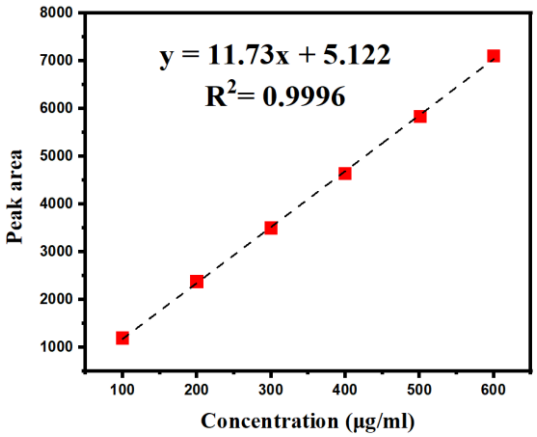

Figure 5. Calibration curve for ALP (100-600 µg/ml) for the RP-HPLC method

Greenness Assessment and Comparison of the Developed Methods

In this study, the environmental impact of both the UV spectrophotometric and HPLC approaches was evaluated in the context of green analytical chemistry principles using the Green Analytical Procedure Index (GAPI) and Analytical Greenness Measure (AGREE) tools.

Figure 6 illustrates the GAPI and AGREE evaluation of the proposed methods for determining ALP. The GAPI tool provides a comprehensive evaluation of the environmental impact of analytical methods through 5 pentagrams, each representing key steps in the process: sample collection, sample preparation, chemicals and solvents used, apparatus, and the goal of the method. GAPI employs a three-color system to reflect the environmental risk, with red indicating high risk, yellow indicating moderate risk, and green representing a lower risk and greater sustainability [22]. As can be seen in Figure 6, the green HPLC method demonstrates 4 green, 9 yellow, and 2 red pentagrams, with the red indicating the use of non-greener solvents such as methanol and trifluoroacetic acid (TFA) and the absence of waste treatment. The inclusion of TFA, along with the energy-intensive preparation steps, further reduces the method's overall greenness. Conversely, the UV spectrophotometric method shows 7 green, 7 yellow, and only 1 red pentagram, with the red similarly indicating no waste treatment. Overall, the UV spectrophotometric method is considered greener than the RP-HPLC method due to its lower energy consumption, reduced solvent use, and minimal waste generation.

Moreover, the AGREE tool was employed to assess the environmental effect of the constructed methods, providing a numerical representation of their greenness. AGREE is an accessible software that evaluates methods against the twelve key principles of green analytical chemistry. Each principle is rated on a scale from 0 to 1 and visualized using a red-yellow-green color gradient [23]. The AGREE

analytical scores of the developed spectrophotometric and HPLC methods were 0.77 and 0.69, respectively. According to the AGREE scale, scores below 0.50 indicate poor greenness, scores between 0.50 and 0.75 are acceptable, and scores above 0.75 represent excellent greenness. While both methods demonstrate environmentally friendly characteristics, the spectrophotometric method shows a higher level of greenness compared to the RP-HPLC method.

Based on green chemistry assessment tools including GAPI, and AGREE, the UV spectrophotometric method exhibited a higher green score due to its simpler, more eco-friendly sample preparation, making it a preferable choice from a sustainability perspective.

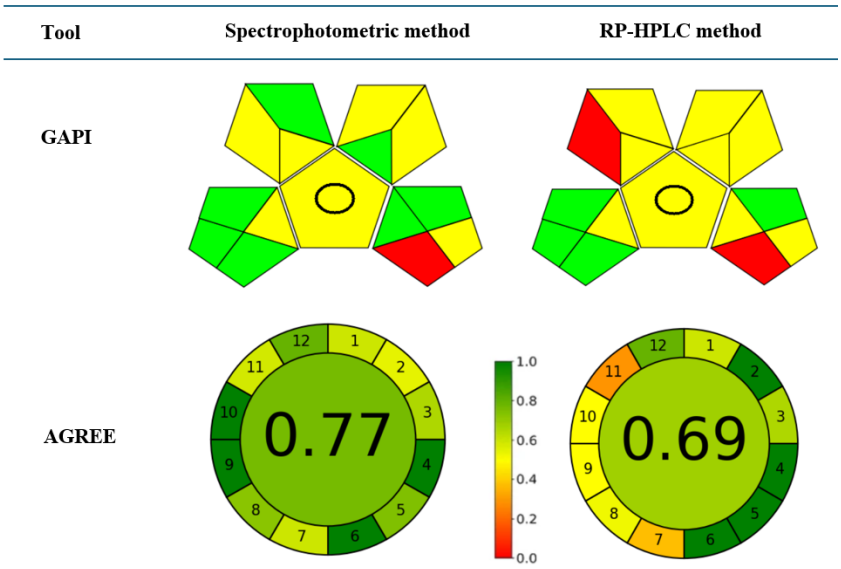

Figure 6. Comprehensive performance assessment of the developed spectrophotometric and RP-HPLC methods by GAPI and AGREE tools.

Conclusion

The creation and validation of green RP-HPLC and spectrophotometric methods for the determination of ALP in bulk drug and pharmaceutical dosage forms have been successfully accomplished. The study demonstrated the efficacy of three distinct analytical methods: direct UV spectrophotometry, first-order UV spectrophotometry, and RP-HPLC. Each approach was meticulously optimized and validated according to ICH guidelines, encompassing parameters including robustness, accuracy, precision, linearity, sensitivity, and stability. Direct UV spectrophotometry and first-order UV spectrophotometry provided simple, rapid, and environmentally friendly approaches for the quantification of ALP, with limits of detection as low as 0.078 µg/ml and 0.096 µg/ml, respectively. The RP-HPLC method, employing a Shimadzu Inertsil C8-3 column and a mobile phase composed of 0.1% trifluoroacetic acid, acetonitrile, and methanol, achieved reliable and precise results with a limit of detection of 14 µg/ml. The greenness assessment using AGREE and GAPI metrics underscored the environmental sustainability of the developed methods, highlighting their minimal solvent and chemical consumption and reduced waste production. Overall, the validated methods exhibit excellent potential for routine quality control analysis of ALP in pharmaceutical formulations, offering robust, precise, and environmentally benign alternatives to existing analytical techniques. This research not only advances the analytical methodologies for ALP determination but also reinforces the importance of green chemistry principles in pharmaceutical analysis. The successful implementation of these methods can significantly contribute to enhancing the efficiency, accuracy, and sustainability of drug quality assessment processes in the pharmaceutical industry.

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AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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