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A Highly Sensitive RP HPLC-PDA Analytical Method for Detection and Quantification of a Newly Synthesized (E-2-((E)-4-(5-Ethoxy-3-Methyl-1-Phenyl-1H-Pyrazole-4-yl)but-3-en-2-Ylidene)) Hydrazine-1-Carbothioamide in Nanosuspension

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ABSTRACT

Analytical methods development and validation are vital for the precise detection, quantification, and characterization of novel therapeutic compounds, especially those with poor aqueous solubility, such as pyrazolone derivatives. This study aimed to develop and validate a sensitive, accurate, and efficient RP HPLC-PDA method for the detection and quantification of novel (E-2-((E)-4-(5-ethoxy-3-methyl-1-phenyl-1H-pyrazole-4-yl)but-3-en-2-ylidene) hydrazine-1-carbothioamide in nanosuspension. The method was optimized for high sensitivity and specificity using a Shim-pack GIST C18 (5 µm, 150 × 4.6 mm) column, with an isocratic mobile phase of ACN and 0.1% TFA in water (75:25 v/v). It employed a 0.5 mL/min flow rate, a 20 µL injection volume, and detected the compound at 333 nm. The method showed excellent linearity ($R^2 = 0.9994$) over a concentration range of 2.5–50 µg/mL, with high precision, accuracy, and reproducibility, in compliance with ICH Q2 (R1) guidelines. The LOD and LOQ were 2.43 and 7.38 µg/mL, respectively. Recovery rates ranged from 110% to 112%, with RSD below 2%. The validated RP HPLC-PDA method was effectively applied to detect, characterize, and quantify the novel compound in its nanosuspension form. This method offers a reliable analytical tool for the quality control of this novel compound, both in raw material and finished product forms, as well as for impurity profiling, drug release, and stability testing, which will, in turn, facilitate new drug development.

1 | Introduction

The pyrazolone core is one of the most explored precursors among diverse fused heterocycles, capable of multiple roles in different pathophysiological conditions [1]. Pyrazolone derivatives are a class of heterocyclic compounds found in many drugs and synthetic products and have been valuable pharmaceutical intermediates. The development of pyrazolone derivatives is regarded as the epitome of medicinal and pharmaceutical chemistry [2]. They are five-membered heterocyclic compounds containing one ketonic group and two adjacent nitrogen atoms and are viewed as derivatives of pyrazole possessing an additional carbonyl (C═O) group. Several analogs have been explored from antipyrine, and the precursor designing methods have evolved from structural

modification to fragment-based drug design and high-throughput screening simultaneously [2, 3]. Pyrazolones can potentially substitute quite a significant percentage of chemical frameworks with an analogous chemical structure and similar biological profiles, offering substantial functionality for drug design and development [4]. As a result, they have received significant attention in chemical, medicinal, and pharmaceutical research as a structural framework in various drugs [5]. Considering the many advantages associated with pyrazolone-based compounds, poor aqueous solubility remains a major drawback to their efficient clinical translation [6, 7].

Currently, researchers are focusing on the encapsulation of poorly soluble compounds in appropriate nanoparticle (NP) systems as an ideal solution to overcome the limitations of poor aqueous solubility and further improve pharmacokinetics and bioavailability [8–10]. Nanosuspensions are colloidal dispersions of drug particles (generally in the size range of 100–600 nm) in solvents [11]. As an efficient hydrophobic drug delivery system, the NPs of the drug with increased specific surface area to volume ratio interact better with the surrounding medium. This results in improved solubility, increased drug loading, reproducibility of oral absorption, a low incidence of side effects of excipients, optimized in vivo distribution, low cost, non-toxicity, and reduced patient burden, a follow-on to generally improved performance of such drugs [12–15]. Hence, this study explores the formulation of a poorly water-soluble novel bioactive compound as a nanosuspension. This then necessitated the development and validation of an accurate and precise analytical method suitable to identify, characterize, and quantify the compound.

Analytical methods, particularly the HPLC method, play an essential role in discovering, developing, and manufacturing pharmaceuticals. Several analytical methods, such as UV–vis and HPLC, are well described in the literature for the detection and quantification of drug substances in nanoformulations [16–19]. Several methods involving liquid chromatography-mass spectrometry, high-performance thin-layer chromatography (HPTLC), and liquid chromatography with fluorescence scanning detection have also been reported to determine bioactive compounds [20–22]. UPLC- and HPLC-developed methods have been used to quantify curcumin and its derivatives in commercial samples [23].

Typically, RP HPLC-PDA was used by Yaneva et al. to determine a newly synthesized N-Isonicotynoyl-N′-(3-fluoro benzal) hydrazone in aqueous phase [24]. At the same time, Sivagam et al. reported a highly sensitive, validated reversed-phase HPLC method for a novel pyrazoline derivative with an LOD of 4 µg/mL and an LOQ of 15 µg/mL [25]. Again, Alfei et al. recently described an HPLC method for a novel bioactive pyrazolone, 2-(4-bromo-3,5-diphenyl-pyrazol-1-yl)-ethanol, using an HPLC JASCO system equipped with an ODS C18 column (250 × 4.6 mm, 5 µm) and a JASCO PU-980 pump with a mobile phase of ACN and 10 mM K_2HPO_4 aqueous buffer solution (15/85, v/v). The total run time was 20 min, and the compound was detected with the UV-970-975 UV/Vis detector at 253 nm [26]. The long run time and variations in this instrument's parameters made the described method unsuitable for this analysis. Moreover, the development of analytical methods for newly synthesized compounds is crucial for several reasons. These methods facilitate the evaluation of purity as part of quality control testing, determination of concentration in biological samples during pharmacokinetic studies, assessment of chemical stability, and content assay during formulation development, including entrapment efficiency and drug loading capacity in nanocarriers [27–29].

Herein, we described the development and validation of a reversed-phase HPLC-PDA method for the detection and quantification of a novel pyrazolone compound (E-2-((E)-4-(5-ethoxy-3-methyl-1-phenyl-1H-pyrazole-4-yl)but-3-en-2-ylidene) hydrazine-1-carbothioamide (PBC)) with a molecular weight of 344 g/mol and a melting point of 151.1°C. This compound was designed and synthesized in our laboratory, and the methods of synthesis, characterization, and properties have previously been reported by Obakachi et al. [6]. Our preliminary in silico studies indicated that the selected compound possesses potential biological activity but has poor aqueous solubility. Therefore, to address this shortfall, we proposed formulating it as a nanosuspension to enhance its water solubility and biological activity. However, to characterize the developed nanosystem in terms of entrapment efficiency, drug loading, drug release, and solubility, a highly sensitive and robust HPLC-PDA method is essential for determining the compound's concentration. This analytical approach forms the basis of the current study.

The compound in nanosuspension was obtained by encapsulating the drug in PLGA/Poloxamer-188 nanosuspension. The developed method was validated according to the recommendations of the ICH guidelines and updated international convention in which the linearity of response, precision, accuracy, suitability, specificity, and robustness were assessed and validated. The validated method was then used to efficiently determine the encapsulation efficiency and drug loading capacity of the nanosuspension, further highlighting the accuracy and efficiency of the method in the detection and quantitation of the novel pyrazolone compound in the formulation.

2 | Materials and Methods

2.1 | Reagents and Chemicals

PBC was previously synthesized in our lab, chemically characterized, and reported by Obakachi et al. [6]. The chemical structure is depicted in Figure 1. Poly (lactic-co-glycolic acid) (PLGA) 50:50, Poloxamer-188, TFA, HPLC-grade solvents (methanol, ACN), and phosphate-buffered saline (PBS) tablets were purchased from Sigma–Aldrich. All other solvents are of analytical grade. The compound-containing nanosuspensions and the solutions remained stable throughout the experiments for a month.

2.2 | Instrumentation and Apparatus

The Shimadzu HPLC system (Kyoto, Japan) outfitted with binary high/low-pressure gradient pumps, a degasser, a PDA detector, and an autosampler. Shim-pack GIST C18 (5 µm, 150 × 4.6 mm) column and the LC Solution 5.106 SPI system software for obtaining the results. An analytical balance was used to weigh standard compounds. The mobile phase was filtered with a 0.45-micron membrane filter (Millipore filter) using a membrane-

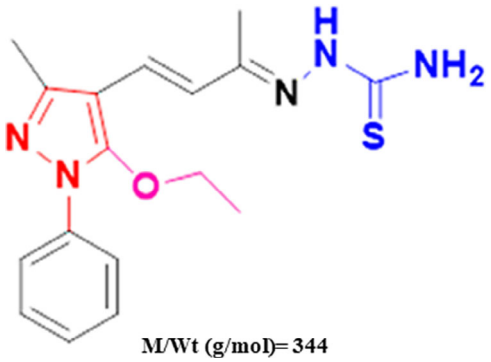

FIGURE 1 | Novel (E-2-((E)-4-(5-ethoxy-3-methyl-1-phenyl-1H-pyrazole-4-yl)but-3-en-2-ylidene) hydrazine-1-carbothioamide (PBC) [6].

holder vacuum filtration system and degassed with an ultrasonic bath sonicator. A digital pH meter was used to adjust the pH of the buffer, and an Elix 10 water purification system (Millipore Corp., USA) was used for ultra-pure water.

2.3 | Method Development and Optimization

The experimental conditions were optimized to achieve good separation and proper chromatographic conditions for analysis. The assessed separation conditions included solvent type, sample volume, injection volume, mobile phase composition (ratios, gradient, flow rate), medium pH, column type, and column temperature. A planned/systematic study, as well as optimization of the mentioned parameters, achieved the experimental conditions for analysis.

2.3.1 | Chromatographic Conditions

After the extensive screening of the parameters mentioned above, the best separation of the compounds was obtained by isocratic elution, and the detection was carried out at a wavelength of 333 nm. The mobile phase used for separation was ACN and water (0.1% TFA) (75:25 v/v). The flow rate was kept fixed at 0.5 mL/min, the column temperature was ambient (25°C), and the injection volume was 20 µL.

2.4 | Preparation of Mobile Phase

One milliliter of TFA was diluted to 1000 mL with ultrapure water to prepare the inorganic phase of the mobile phase, then ultrasonicated. The acidified water and ACN were filtered separately through a 0.45 µm membrane filter and degassed. A freshly prepared mobile phase was used for every experiment.

2.5 | Preparation of Standard Stock Solution

The standard stock solution was prepared by weighing 10 mg of the sample accurately, dissolved in 5 mL of methanol in a 10 mL volumetric flask, and made up to volume with methanol to give a stock concentration of 1 mg/mL. The calibration curve was constructed in the range of 0.25–50 µg/mL. The samples were prepared by withdrawing varying amounts from the stock solution and diluted to 10 mL volume in a volumetric flask using methanol. All samples were filtered before injection into the HPLC instrument.

2.6 | Analytical Method Validation

The optimized method was validated according to the ICH Q2 (R1) guidelines [30] to check the method's reliability. The following validation parameters were evaluated.

2.6.1 | System Suitability

The system suitability of the optimized method was evaluated by injecting six replicates of 50 µg/mL of the test sample to assess whether or not the method was suitable for its intended use. The number of theoretical plates (NTP), percentage tailing factor ($Tf_{10\%}$), and peak asymmetry were calculated and compared with the recommended limits.

2.6.2 | Specificity and Selectivity

These parameters confirm whether the developed method can specifically resolve the test compound, interference from excipients (used in the nanosuspension preparation), and blank at the retention time. These were assessed by injecting three replicates of each diluent and placebo sample into the HPLC system.

2.6.3 | Linearity

Refers to the procedure's ability to deliver test findings proportionate to the test compound's concentration over a given range. The linearity of measurement was evaluated by analyzing standard solutions of the pyrazolone compound in the field of 2.5 to 50 µg/mL and constructing a calibration curve.

2.6.4 | Limit of Quantitation (LOQ) and Limit of Detection (LOD)

LOD and LOQ of the novel compound were determined using the calibration curve. Solutions of the compound were prepared in the range of 2.5–50 µg/mL and injected in triplicate. According to the ICH guidelines, the LOQ and LOD are determined by the standard deviation (SD) from the regression line, y-intercepts, and calibration curve slope. The LOD is calculated using the following equation:

$$\mathrm{LOD} = \frac{3.3\varphi}{S} \tag{1}$$

Then, the LOQ is calculated from the following equation:

$$\mathrm{LOQ} = \frac{10\varphi}{S}, \tag{2}$$

where φ is the SD of response, and S is the slope of the regression line.

2.6.5 | Accuracy

The method's accuracy was calculated by recovery studies at three levels, 10, 30, and 50 μg/mL, and reported as a percentage of nominal. The analyzed solutions were prepared using a blank and stock solution of the test sample and were done in triplicate.

2.6.6 | Precision

Six independent sample solutions of 10 μg/mL from the analyte were used for the precision of the method and done in triplicate. The repeatability and intermediate precision were studied by comparing assays on the same day and on different days. The SD and %RSD were calculated and reported.

2.6.7 | Robustness

The influence of slight changes in the chromatographic conditions, such as a change in wavelength of detection ± 2 nm, flow rate ± 0.2 mL/min, and the percentage TFA used in the analysis, were studied to establish the robustness of the optimized method, and their %RSD was determined. One-factor analysis of variance (ANOVA) was also carried out using the Excel Analysis tool kit to establish further variability in the data obtained using the p-value.

2.7 | Application of the Method for Quantifying Novel Pyrazolone-Based Derivative in Nanosuspension

The nanoprecipitation technique prepared the nanosuspension with slight modification [31]. Briefly, 60 mg of PLGA, 1% poloxamer 188, and 6 mg of the compound were weighed. The 60 mg PLGA was dissolved in 5 mL of acetone and stirred for 2 h. The compound was dissolved in 500 μL of suitable organic solvent and mixed with the PLGA solution under stirring and then added in drops to 10 mL poloxamer in water under sonication for 5 min. Then, it was kept stirring at 600 rpm overnight. A blank was also formulated in this manner. The nanosuspensions were characterized in terms of particle size distribution, zeta potential, and polydispersity index using Zeta Sizer (Nano ZS, Malvern Instruments, UK).

The total drug content and the entrapment efficiency of the nanosuspension were calculated using the indirect method. Briefly, a 2 mL sample was centrifuged at 12 000 rpm at 4°C for 30 min, and the supernatant was filtered and analyzed to obtain the unentrapped compound in the formulation using the developed method. For the drug content, 1 mL of methanol/acetone was added to 500 μL of nanosuspension and kept under sonication for 15 min, then was made up to a volume of 10 mL in a volumetric flask, filtered, and analyzed using the developed method. The experiment was performed in triplicates. The drug content and entrapment efficiency were calculated using Equations (3) and (4) as given below:

$$\text{Total drug content recovered from nanosuspension } \left(\frac{\text{mg}}{\text{mL}}\right) = \frac{\text{Amount of the drug in a nanosuspension} - \text{unentrapped}}{\text{The total weight of the nanoparticles}} \quad (3)$$

$$\text{Entrapment efficiency } (\%) = \frac{\text{Amount of drug in nanosuspension} - \text{unentrapped}}{\text{Total amount of the drug in nanosuspension}} \times 100 \quad (4)$$

TABLE 1 | Summary of optimum system conditions of the developed method.

Parameter	Observed
Mobile phase	ACN:water (0.1% TFA)
Flow rate	0.5 mL/min
Injection volume	20 μL
Column type	Shim-pack GIST C18 (5 μm, 150 × 4.6 mm)
Column temperature	25°C
Retention time	4.82 min
NTP	2265 (> 2000)
% Tailing factor	1.4 (< 2%)

3 | Results and Discussion

3.1 | Development and Optimization of the Chromatographic System

Chromatographic separation was accomplished using the Shimadzu HPLC system (Kyoto, Japan) with the Shim-pack GIST C18 (5 μm, 150 × 4.6 mm) column at 25°C. A binary mixture of 70:25 ACN and 0.1% TFA was optimal for the isocratic elution of the compound at a 0.5 mL/min flow rate. PDA detection was monitored at a wavelength of 333 nm after scanning. The HPLC elution was completed in below six minutes with a total run time of 10 min, and 20 μL was used as the sample injection volume. These conditions successfully eluted the pyrazolone-based compound with high specificity, as shown in Figure 3. The conditions of the developed method are summarized in Table 1. Figure 2 depicts a concise summary of the workflow of this study, highlighting the different validation assessments carried out following the recent ICH guideline, 2022 [32], as well as the steps followed in the preparation of the nanosuspension.

3.2 | System Suitability

The system suitability test is commonly used to prove that the system perfectly separates the compound of interest with

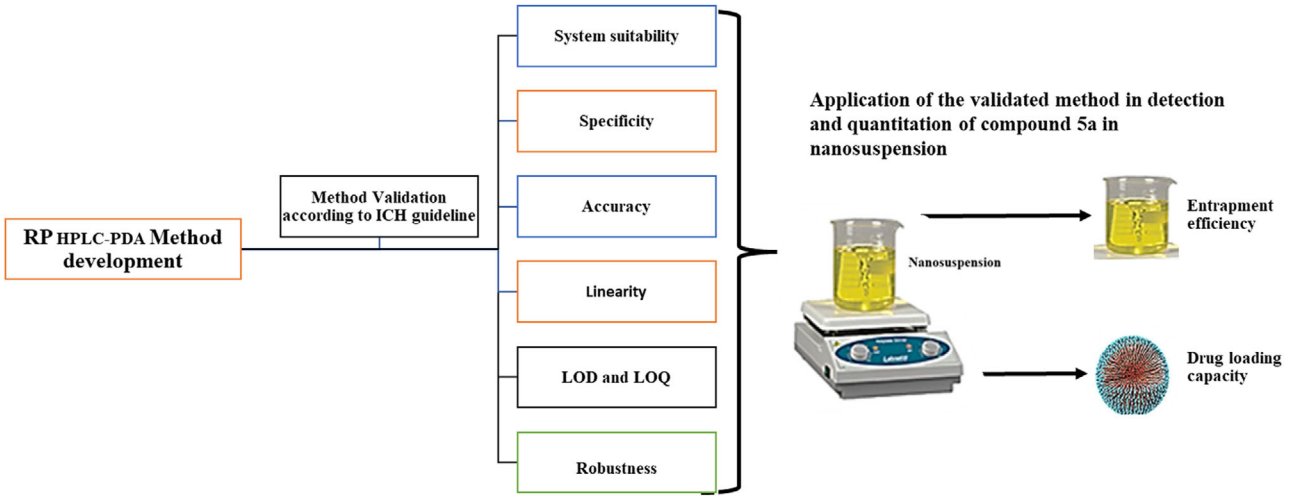

FIGURE 2 | Concise summary of the workflow of this study.

high efficiency. Various parameters, including resolution, column efficiency, retention time, theoretical plate number, tailing factors, and repeatability of a chromatographic system, must be checked to ensure the adequacy of a particular analysis method. As illustrated in Table 1, the system suitability test revealed acceptable performance since all checked parameters are within the accepted limits, indicating the system's suitability to analyze the compound for its intended use. RSD of peak areas obtained from injecting the six samples is 1.192 ($\leq$ 2%) at an average retention time R_t of 4.82 min. The average NTP and $Tf_{10\%}$ were 2265 (> 2000) and 1.4 (< 2%), respectively. All other observed response parameters were within the recommended criteria, indicating that the system for analysis is suitable for its intended use.

3.3 | Specificity and Selectivity

Specificity and selectivity describe the ability of the analytical method to detect the analyte in the presence of other excipients, degradation products, matrix components, and impurities [30]. These parameters were assessed by comparing the chromatograms of the pure compound, compound-loaded nanosuspension, and blank nanosuspension, represented in Figure 3A,C,D). From the stated figure, the peak purity analysis noted no co-elution and significant interfering peaks. Again, comparing chromatograms in Figure 3, it can be inferred that the developed method is specific and selective for the test compound.

3.4 | Linearity

The calibration curve method was used in assessing the linearity of the method over a concentration range of 2.5–50 µg/mL, and the results are summarized in Table 2. The calibration curve showed a coefficient of determination (R^2) of 0.9994, demonstrating a strong linear relationship between the peak area (as seen in chromatograms Figure 3C,D) and the analyte concentration. This ultimately confirms the suitability of this method for the efficient separation and detection of the compound.

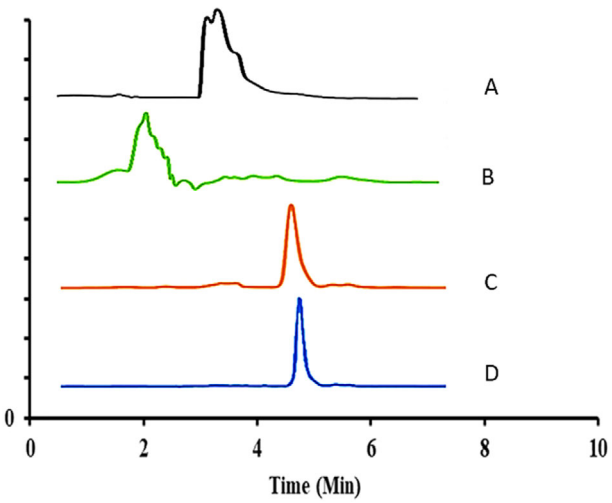

FIGURE 3 | Chromatograms of the blank formulation (A), methanol: solvent for dilution (B), the compound-loaded nanosuspension (C), and pure compound (D), which further established the specificity of the method and its suitable application in detecting the compound in nanosuspensions.

TABLE 2 | Linearity results obtained over a concentration range of 2.5–50 µg/mL.

Parameter	Result
Coefficient of determination (R^2)	0.9994
Curve equation	$Y = 274470x - 94958$

3.5 | Accuracy

Accuracy is established across the reportable range of an analytical procedure, typically demonstrated by comparing the measured results with an expected value. The method's accuracy was established using three different concentrations and calculating the percentage recovery and percentage relative SD

TABLE 3 | Assessment of the accuracy of the method developed in this study.

Injected concentration (μg/mL)	Observed concentration (μg/mL)	% Recovery	SD	RSD (%)
10	11.02	110.20	0.066	0.60
30	32.53	108.43	0.098	0.30
50	56.32	112.64	0.48	0.86

TABLE 4 | Observed data for repeatability and intermediate precision assessment.

Theoretical/standard concentration (μg/mL)	Observed concentrations (μg/mL)		
	Intraday	Interday 1	Interday 2
10	10.79	10.28	11.44
10	10.82	10.75	11.52
10	10.80	10.31	11.54
10	10.56	10.74	11.55
10	10.76	10.57	11.64
10	10.69	10.51	11.29
Mean	10.74	10.53	11.50
SD	0.09	0.19	0.11
RSD (%)	0.82	1.76	0.94

of the observed concentrations, as shown in Table 3. The mean percentage recoveries for the three concentrations are between 110 and 112, with a percentage relative SD ranging from 0.30 to 0.86 (≤ 2%). The result of accuracy testing showed that the method is accurate within the acceptable limits.

3.6 | Precision

Precision is the variability of the results in repeated sample analysis under the same experimental conditions. Intraday precision of the developed method was evaluated by assaying freshly prepared solutions in triplicate at a known concentration on the same day. Interday precision was evaluated using freshly prepared solutions in triplicate on different days. The experiment was performed as described in the methods section, and the result is presented in Table 4. The %RSD obtained for the study is 0.82 and 0.94 (≤ 2%) for repeatability and intermediate precision, respectively, representing excellent precision for the method.

3.7 | LOD and LOQ

The calibration curve method was used to determine the detection and quantification limits. Regression analysis was performed using the linearity results to determine the standard error of the intercept. This value was then used to calculate the LOD and LOQ, resulting in values of 2.43 and 7.38 μg/mL, respectively, using Equations (1) and (2). The LOD is the lowest concentration from which it is probable to assume the presence of the test compound. At the same time, the LOQ is calculated as the lowest concentration of the detected compound in a sample that may be quantified. These parameters are fundamental in analytical method validation as they correspond to the method's sensitivity to the analyte, especially in cases where detecting minimal impurities is essential. The values obtained are low enough to permit a good assessment of the test compound level in nanosuspension compared to that reported for the similar compound N-Isonicotynoyl-N′-(3-fluorobenzal) hydrazone by Sivagam et al. [25].

TABLE 5 | Results from the method's robustness under different conditions.

Parameter	Setting	R_t (min)	RSD (%)	*p*-value
Wavelength (nm)	331.0	4.82	0.40	0.13
	333.0	4.82	0.86	
	335.0	4.82	0.41	
Flow rate (mL/min)	0.3	8.08	9.65	0.01
	0.5	4.89	0.86	
	0.7	3.46	6.60	
%TFA	0.1	4.83	0.87	0.22
	0.2	4.83	0.25	
	1.0	4.83	0.73	

3.8 | Robustness

Robustness is a parameter that establishes the analytical method's suitability within the intended operational environment. This

TABLE 6 | Summary of the results from physicochemical characterization of the nanosuspension, the %EE and %DLC.

Sample	Size (nm)	PDI	Zeta potential (mV)	Entrapment efficiency (%)	Drug loading capacity (%)
F1	193.1	0.164	−13.2	79.33	0.10
F2	193.67	0.15	−13.67	80.42	0.10
F3	190.8	0.094	−9.98	84.81	0.13
SD	1.07	0.03	1.43	2.05	0.0016

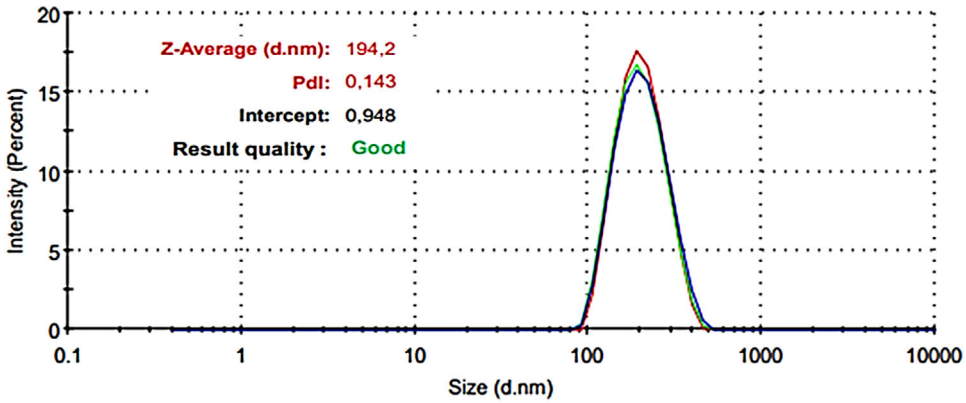

FIGURE 4 | Size analysis graph for the nanosuspension.

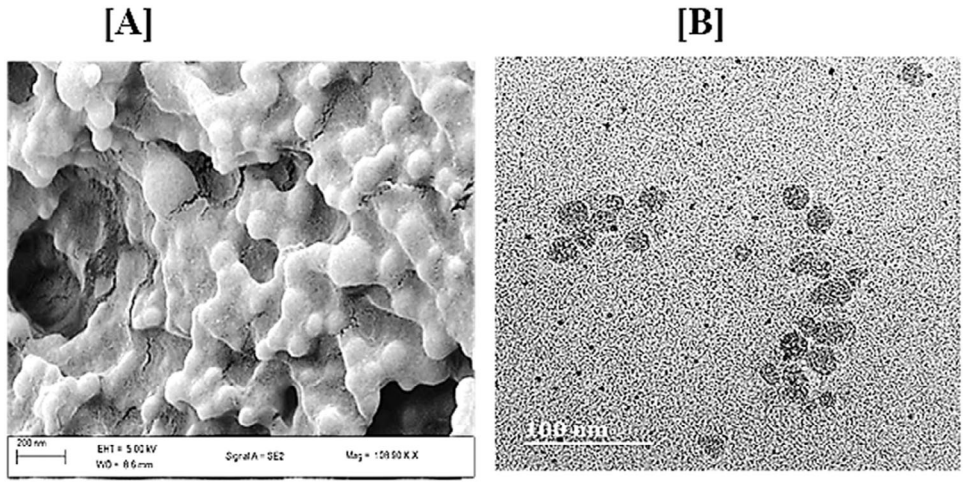

FIGURE 5 | Surface morphology of the nanoparticles obtained from scanning electron microscopy (A) and transmission electron microscopy (B).

parameter was studied by deliberately varying the flow rate and wavelength and increasing the percentage of TFA in the mobile phase used in the analysis. The calculated %RSD and *p*-values of peak areas obtained from the ANOVA are represented in Table 5. All %RSD obtained was within the recommended limit (≤ 2%) except for that obtained in the flow rate analysis, where the %RSD was 9.65 and 6.60 at flow rates of 0.3 and 0.7 mL/min, respectively. Therefore, to ensure precision and accuracy in detecting the compound, it is recommended to maintain a flow rate of 0.5 mL/min. However, further studies are needed to thoroughly investigate the impact of significant variations in retention time when the flow rate is altered, particularly as it applies to the method's broader applications. The *p*-values further established a significant change in the flow rate (< 0.05); however, no significant change was observed in the wavelength and percentage TFA in the mobile phase. This indicates that the method is very suitable within its intended operational environment.

3.9 | Application of the Validated Analytical Method

The nanosuspension was prepared and characterized for surface morphology using a scanning electron microscope, particle size distribution, zeta potential, and polydispersity

index using Zeta Sizer (Nano ZS, Malvern Instruments, UK) to ensure the particles are within the nanorange as described in the literature. The method was used to detect the novel pyrazolone moiety in the nanosuspension by calculating the entrapment efficiency and drug loading capacity of the nanosuspension. A typical chromatogram of the compound in the nanosuspension is depicted in Figure 3C. Table 6 summarizes the results from the physicochemical characterization of the nanosuspension. The encapsulation efficiency and drug loading capacity were obtained by applying the validated method to quantify the compound in the prepared nanosuspension. Also, Figures 4 and 5 show the size distribution curve and the surface morphology image of the nanosuspension. From these results, monodispersed, spherical-shaped anionic NPs with greater than 70% encapsulation efficiency were observed, highlighting the successful preparation of the nanosuspension formulation.

4 | Conclusion

An RP HPLC-PDA method for detecting and quantifying the novel pyrazolone-based compound was successfully developed and found to be accurate and specific with an unprecedented resolution. The validation results show that the developed RP HPLC-PDA method is suitable for detecting newly synthesized PBC in nanosuspension, owing to its specificity, precision, accuracy, and linearity within the study range. The method can be applied in further compound analysis, in vitro dissolution testing in dosage formulations, stability testing, impurities profiling, and raw material analysis.

Author Contributions

Nkeiruka N. Igbokwe: conceptualization, data curation, formal analysis, investigation, methodology, project administration, validation, visualization, writing–original draft, writing–review and editing. **Eman A. Ismail:** conceptualization, formal analysis, methodology, validation, writing–review and editing. **Vincent A. Obakachi:** investigation, validation, writing–review and editing. **Mlindeli Gamede:** formal analysis, visualization, writing–review and editing. **Rajshekhar Karpoormath:** supervision, validation, visualization. **Mbuso Faya:** project administration, supervision, validation, visualization.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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