Nanoencapsulation of novel Pyrazolone-based compounds to enhance solubility and biological activity.

by

Nkeiruka Nkeonyere Igbokwe

(***B. Pharm (Hon.)- University of Maiduguri, Nigeria***)

Student number: 221120500

Dissertation submitted in fulfilment of the requirements for the degree of Master of Pharmacy in the discipline of Pharmaceutical Sciences, School of Health Sciences at the University of KwaZulu-Natal

Supervisor: Dr Mbuso Faya
(PhD, University of Kwazulu-Natal, Durban, South Africa)

Co-supervisor: Prof. Rajshekhar Karpoormath

(PhD, University of Kwazulu-Natal, Durban, South Africa)

Date submitted: DECEMBER 2022

"The future belongs to those who believe in the beauty of their dreams."

-Eleanor Roosevelt

"This Thesis is dedicated to every young girl who has ever dared to dream……. Your dreams are valid!

DECLARATION–PLAGIARISM

I, Ms **Nkeiruka Nkeonyere Igbokwe,** declare that:

1. Except when otherwise noted, the research data contained in this dissertation represents original work.

2. This work has not been submitted for any degree or examination at any other University or Institution.

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Dr Andile Mbuso Faya, the supervisor of the M. Pharm study, now consents to submit this M. Pharm dissertation.

Signed…… [redacted] ……. Date…….. 14/02/2023

Prof. Rajshekhar Karpoormath, a co-supervisor of the M. Pharm study, now consents to submit this M. Pharm dissertation.

Signed: …… [redacted] ……...Date…. 14/Feb./2023

Declaration 2 – Publications

Contributions to publications that are based on or involve research reported in this thesis:

The following papers were submitted to renowned high-impact factor peer-reviewed journals.

Development and validation of Simple RP HPLC-PDA method for detection and quantification of a novel Pyrazolone-based compound in nanosuspensions

Nkeiruka N. Igbokwe; Eman A. Ismail; Vincent A. Obakachi; Rajshekhar Karpoormath; Mbuso Faya*, Journal of Pharmaceutical and Biomedical analysis (Manuscript ID: JPBA-D-22-02114).

Nanoencapsulation of novel Pyrazolone-based compounds to enhance solubility and biological activity.

Nkeiruka N. Igbokwe; Eman A. Ismail; Sthabile Mokoena; Blessing Ike; Vincent A. Obakachi; Aviwe Ntsethe; Rajshekhar Karpoormath; Mbuso Faya*

The first-author manuscripts may be seen in Chapters three and four of this dissertation.

Statement of ethics

An ethics exemption **(00019373)** was obtained from the UKZN Research Ethics office, as the study does not involve animals or humans.

Ms Nkeiruka N. Igbokwe helped to conceptualise and create the papers. She was also in charge of data collection/generation and analysis, drafting articles, and overseeing all changes. She helped to design and validate the analytical technique for the new compounds, as well as optimise and characterise the PBC-PLGA nanoformulations in terms of particle size, polydispersity index, zeta potential, surface morphology, entrapment efficiency, and in vitro biological activities (antibacterial, cell permeability, and antioxidant assay). She was also in charge of the analysis and interpretation of all results. Ms Eman A. Ismail assisted with concept creation, technique development, and article proofreading and editing. Sthabile Mokoena and Blessing Ike assisted with the antimicrobial component. Vincent A. Obakachi created the pyrazolone-based chemicals, and Mr Aviwe Ntsethe assisted with the Flow cytometry assay. The project was co-supervised by Rajshekhar Karpoormath and Mbuso Faya.

Research output from the dissertation

First authored Publications

The following research papers were submitted as results generated from the specific objectives of this study and they include:

- **Development and validation of simple RP HPLC-PDA method for detection and quantification of a novel Pyrazolone-based compound in nanosuspensions**

  Nkeiruka N. Igbokwe; Eman A. Ismail; Vincent A. Obakachi; Rajshekhar Karpoormath; Mbuso Faya*, Journal of Pharmaceutical and Biomedical analysis (Manuscript ID: JPBA-D-22-02114).

- **Nanoencapsulation of novel Pyrazolone-based compounds to enhance solubility and biological activity**

Nkeiruka N. Igbokwe[1]*; Eman A. Ismail[1,2]; Sthabile Mokoena[1]; Blessing Ike[1]**;** Mohammed Mshelia[1]; Vincent A. Obakachi[1]**;** Aviwe Ntsethe[1]; Rajshekhar Karpoormath[1]; Mbuso Faya[1]*

Acknowledgements

I am eternally grateful to God for his wisdom and kindness in guiding me through this career phase. I express my gratitude to my mother and my sisters, Success, and ND, for their encouragement, contributions, and prayers supporting this endeavour. Then, I express my gratitude to my special friend Onyebuchi for his support, care, and guidance.

I am especially grateful to Dr Mbuso Faya, who welcomed me as a student with open arms and without reservation. You have been a source of inspiration, encouragement, direction, and compassion. Your perseverance in pursuing perfection and always pointing me in the correct route will be remembered.

Professor Rajshekhar Karpoormath, my co-supervisor, I appreciate you for creating an enabling environment for me to work. I am grateful for your encouragement, drive, and assistance during this process.

Thank you, Ms Eman Ismail, for your assistance in the design of this project, as well as your continued inspiration and guidance. I will be eternally grateful to you for being there for me every step of the way and assisting me in resolving any issues that arose, imparting vital skills that will be critical in my future job.

Words will also fail me to express my gratitude to members of the SMCRG group for their continuous support and encouragement during this effort. I thank Sthabile, Marvela, Blessing, Mshelia, Sachin, Baba, Darko, Mr Ken, and John for the times you came through for me.

I sincerely thank Mr Almahi Mohamed Idris, Mr Aviwe Ntsethe of the Physiology department for his assistance with the flow cytometry, and the Electron microscopy unit at the UKZN technical support group.

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LIST OF ABBREVIATIONS

sodium dodecyl sulphate (SDS) ---------- *36*
2,2-diphenyl-1-picrylhydrazyl (DPPH) ---------- *141*

A

active pharmaceutical ingredients (APIs) ---------- *2*
Administration, Distribution, metabolism, and Excretion

(ADME) ---------- *90*

American Society for Testing and Materials

(ASTMF) ---------- *156*

Analysis of Variance

(ANOVA) ---------- *96*

B

biopharmaceutics classification system

(BCS) ---------- *9*

blood-brain barrier

(BBB) ---------- *48*

British Pharmacopeia

(BP) ---------- *15*

C

Center for Drug Evaluation and Research

(CDER) ---------- *16*

Central nervous system

(CNS) ---------- *55*

critical micelle concentration

(CMC) ---------- *49*

critical pressure

(Tp) ---------- *32*

critical temperature

(Tc) ---------- *32*

cyclodextrin-glycosyltransferase

(CGT) ---------- *31*

D

deoxyribonucleic acid

(DNA) ---------- *88*

di-methyl-aceto-amide

(DMA) ---------- *34*

dimethyl-sulfoxide

(DMSO) ---------- *34*

E

European Medicines Agency

(EMEA) ---------- *16*

F

ferric reducing ability

(FRAP) ---------- *141*

fluorescence-activated cell sorting

(FACS) ---------- *140*

forward scatter

(FSC) ---------- *140*

G

gastrointestinal

(GI) ---------- *44*

H

high-performance liquid chromatographic

(HPLC) ---------- *91*

high-throughput screening

(HTS) ---------- *2*

hydroxypropyl-methylcellulose

(HPMC) ---------- *30*

hydroxypropyl-β-cyclodextrin

(HP-β-CD) ---------- *49*

I

International conference of harmonisation guidelines

(ICH) ---------- *95*

L

light scattering

(DLS) ---------- *137*

Limit of Detection

(LOD) ---------- 95

Limit of Quantitation

(LOQ) ---------- 95

Loading capacity

(LC) ---------- 149

M

maximum orally absorbable dose

(MAD)---------- 22

minimal inhibitory concentrations

(MICs) ---------- 139

N

nanoparticles

(NP) ---------- 90

nanostructured lipid carriers

(NLCs) ---------- 42

new chemical entities

(NCEs) ---------- 54

New drug entity

(NDE) ---------- 1

nitric oxide

(NO) ---------- 142

nonsteroidal anti-inflammatory drugs

(NSAIDs) ---------- 55

Number of Theoretical Plates

(NTP)---------- 94

O

Oil in water

(O/W)---------- 44

P

phosphate buffered saline

(PBS)---------- 93

photodiode array

(PDA)---------- 91

Poly (amidoamine)

(PAMAM) ---------- 46

poly (ethylene-imine)

(PEI)---------- 49

poly (Ɛ-caprolactone)

(PCL) ---------- 48

poly (n-butyl cyanoacrylate)

(PBCA) ---------- 50

poly (N-isopropyl acrylamide

(PNIPAM)---------- 47

polyethene glycol

(PEG)---------- 30

povidone or polyvinylpyrrolidone

(PVP) ---------- 30

propidium iodide

(PI)---------- 140

R

red blood cells

(RBC)---------- 140

relative standard deviation

(RSD) ---------- 99

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ribonucleic acid

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(SNEDDSs) ---------- *44*

side scatter

(SSC) ---------- *140*

sodium dodecyl sulphate

(SDS) ---------- *34*

sodium dodecylbenzene sulfonate

(SDDBS) ---------- *34*

Sodium Lauryl Sulphate

(SLS) ---------- *138*

Solid Lipid nanoparticles

(SLNs) ---------- *42*

standard deviation

(SD) ---------- *96*

structure-activity relationships

(SARs) ---------- *22*

structure-property relationships

(SPRs) ---------- *22*

supercritical fluid

(SCF) ---------- *26*

U

United Nations Sustainable Development Goals

(UN SDGs) ---------- *88*

United States Pharmacopeia

(USP) ---------- *15*

W

World Health Organisation

(WHO) ---------- *16*

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Abstract

The biological activity of pyrazolone-based derivatives has been thoroughly documented; nonetheless, low stability and water solubility are their main drawbacks, preventing effective translation to clinical application. Based on this, two previously reported weakly soluble pyrazolone-based compounds, PBC-301 and PBC-302, were encapsulated using PLGA: poloxamer complex to improve their solubility and further examine the influence of solubility augmentation on their biological activities. We first developed and validated a simple, accurate RP HPLC-PDA method for detecting, measuring, and standardising the compounds in nanoformulations to achieve this wide goal. Efficient separation and quantification were carried out using Shim-pack GIST C18 (5 μm 150 × 4.6 mm) column, maintained at 25 ℃ with isocratic elution using acetonitrile and acidified water (0.1% Trifluoracetic acid) (75:25 v/v) at 0.5 mL/min flow rate. The injection volume was 20 μL, and eluents were detected at 333 nm at a retention time of 4.82 mins. Method validation was done following ICH guidelines. Results demonstrated that the method is specific, precise, and accurate within the recommended limits. The method showed good linearity with a 0.9994 correlation coefficient over a concentration range of 2.5-50 μg/ml. The method efficiently detected and quantified the novel pyrazolone compound in the nanosuspension. The obtained nanoformulations PBC-PLGA 301 and PBC-PLGA 302 were characterised using various *in vitro* techniques. Size, PDI and ZP of the optimised nanoformulations were 166.6 ± 7.12 nm, 0.129 ± 0.042, -14.14 ± 2.90 mV for PBC-PLGA 301 and 192.5 ± 1.08 nm, 0.132 ± 0.025, -10.77 ± 1.515 mV for PBC-PLGA 302 with the encapsulation efficiency being 84.20 ± 0.930 and 81.5 ± 2.051, respectively. The compound release from the nanovesicles followed a sustained release pattern, with PBC-PLGA 301 and PBC-PLGA 302 attaining a cumulative release of approximately 37% and 53% in 48 hours. The biological activity assays showed a better-enhanced activity with the nanoformulations compared to the non-encapsulated PBC 301 and PBC-302. In vitro antibacterial activity revealed

that the compound-loaded nanovesicles have better activity against the two gram-positive bacteria *S. aureus* and *Methicillin-resistant S. aureus* compared to the standard drug vancomycin and the non-encapsulated compound. On the other, the cell penetration assay further revealed that the compound-loaded nanovesicles achieved greater than 90% propidium iodide penetration (translating to cell death) at the reported MIC well for *S. aureus* while showing 86% and 89% cell penetration for *Methicillin-resistant S. aureus.* Also, the nanoformulations showed improved radical scavenging activity in a concentration-dependent manner, with PBC-PLGA 301 exhibiting the best antioxidant activity against DPPH, FRAP and nitric oxide compared to the standard antioxidant-gallic acid and the non-encapsulated compounds.

In conclusion, the aqueous solubility of the two pyrazolone compounds, PBC-301 and PBC-302, was greatly enhanced by their encapsulation into a nanosystem, resulting in improved biological activities. Therefore, the nanoformulations of the pyrazolone-based derivatives can be exploited as potential pharmaceutical agents to fight bacterial infections and other diseases triggered by oxidative stress, cancer, and hepatic and vascular diseases. The data from this study has resulted in two first-authored research publications.

CHAPTER 1. INTRODUCTION

1.1 Introduction

This chapter provides a background for the study, highlighting the problems associated with innovative drug candidates' poor solubility during the development, industrialisation, and clinical stages. In addition, it introduces nanoformulation techniques as a practical and efficient way to make bioactive compounds more soluble, which leads to the study's suggested goals and objectives, emphasises the study's originality and significance once more and concludes with a discussion of the thesis' organisation and content.

1.2 Background

Finding new compounds with desirable qualities has received much attention during the past 20 years, particularly in the pharmaceutical sector. Drug discovery is essentially a technique for discovering chemical entities that have the potential to become new curative agents, with the primary goal of recognising novel molecular entities that may be useful in treating diseases with "unmet medical demands". These illnesses are really or potentially fatal and lack treatments that are unquestionably effective [1]. As shown in Figure 1, the discovery and development of a novel drug entity (NDE) into a clinically and commercially viable medicine is difficult, expensive, and time-consuming. According to estimates, numerous potential drug candidates would have been screened for each new medicine that enters clinical trials before narrowing down the search for the new therapeutic molecule based on the leads discovered during screening. It was originally projected that this search for NDE would take at least five years and cost at least $200 million [2, 3]. Even at this point, having a promising lead compound in hand does not ensure that it will be used successfully in clinical settings. These compounds may fail in the subsequent development phase due to unfavourable toxicity, a lack of *in vivo* efficacy in desired disease models, high first-pass metabolism by cytochrome enzymes, susceptibility to efflux mechanisms, business unattractiveness, and poor biopharmaceutical properties such as solubility and permeability, all of which represent substantial barriers to the successful

development of therapeutic products [4]. This puts the cost of drug development, from the first discovery to approval, at approximately US $800 million for a single drug, with one of the reasons being that only one in ten developed drugs makes it to final approval [5]. Considering that just one in ten produced treatments receives final clearance, the cost of drug development, from the initial discovery to approval, is around US $800 million for a single drug [5]. However, the success rate in pharmaceutical research utilised for drug discovery has recently grown thanks to computer-based techniques such as combinatorial chemistry, high-throughput screening (HTS), computer-aided and structure-based drug design, and fragment-based lead identification [6-8]. Although, these techniques usually result in many lipophilic active pharmaceutical ingredients (APIs), forcing formulation scientists to deal with their poor water solubility and dissolution. As a result, they have done more harm than good. According to estimates, 75 and 90 per cent of medications are now under research, and 40 per cent of pharmaceuticals on the market have low water solubility [9–12]. Poor water solubility is thus still a problem for many treatment candidates in the pipeline of pharmaceutical development today [13-15].

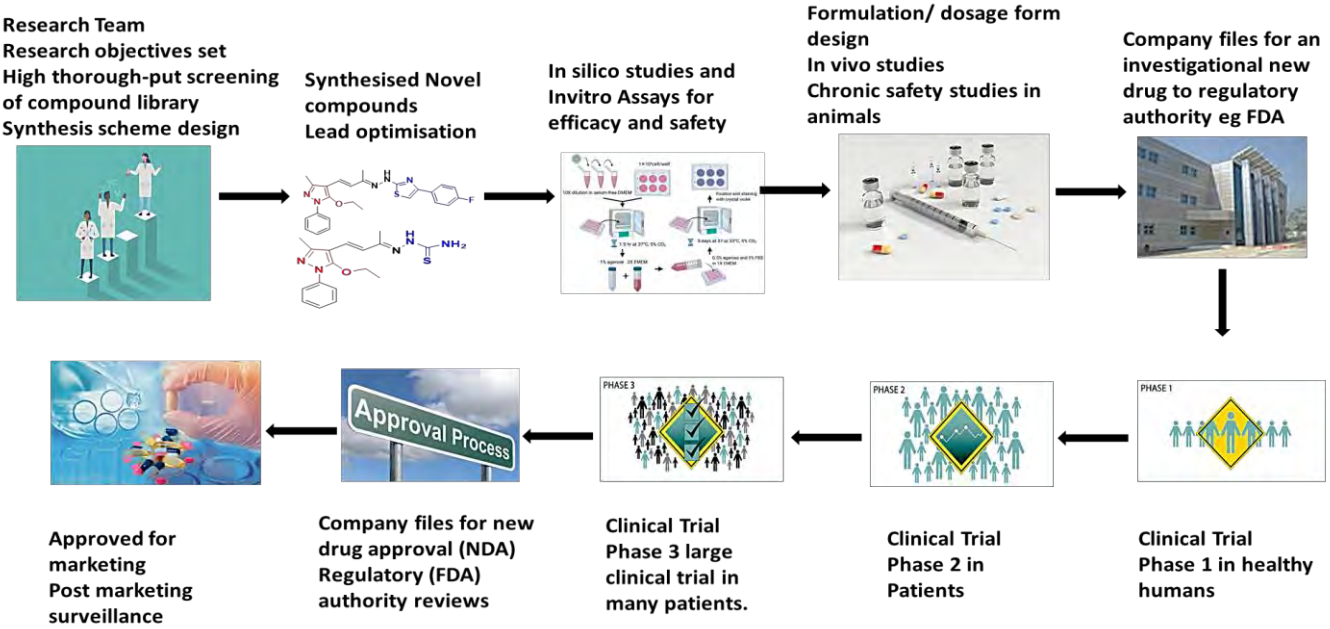

Figure 1. A schematic diagram describing the drug development pipeline

Poor solubility of new pharmaceuticals impacts biological assay performance in the drug discovery stage, poses a problem for generating effective drug formulations, and leads to poor bioavailability in the clinical stage by interfering with the ease of manipulation during production and testing in the laboratory, the potential for drug absorption after oral administration, and the ability to give the drug intravenously. Since toxicological investigations typically need larger dosage exposure than pharmacologic or pharmacokinetic studies to verify their safety, limitations in solubility might affect the quality of data on both *in vitro* tests and *in vivo* toxicity evaluations. Furthermore, poor solubility in the test medium provides inaccurate information on the drug's capabilities in vitro, particularly in cell culture experiments (efficacy, membrane permeability properties, and genotoxicity) required to demonstrate the drug's tolerability. Therefore, a therapeutic molecule must be adequately dissolved in solution to quantify relevant actions *in vitro* and achieve sufficient absorption *in vivo* for safety and effectiveness evaluation in preclinical models and clinical trials. Once more, one of the main factors affecting a drug's dissolution rate is its solubility. Therefore, low solubility from restricted dissolution results in low bioavailability from orally taken medications [16, 17], which culminates in the drug's limited therapeutic potential, resulting in therapeutic failure and suboptimal clinical outcomes. Additionally, the solubility of the active pharmaceutical ingredient (API) is a crucial consideration during the drug development process, particularly when it comes to dosage form development and clinical trial selection, designing experiments to find potential salt forms, cocrystal forms, polymorphic forms, solvates, and hydrates, developing analytical methods, and developing drug manufacturing strategies. According to these features, it is important to incorporate solubility evaluation into the discovery and development process, and it has to be regularly checked and improved as compounds evolve [18].

The literature reports several methods/techniques to improve the water solubility, rate of dissolution, and, eventually, the bioavailability of medicinal compounds with low solubility. Poor solubility has been solved using co-solvent addition, micelle solubilisation inclusion complexation, hydrotropy,

particle size reduction, and crystal engineering. However, drawbacks, including a broader distribution of particle size (PSD), potential product heat and chemical deterioration, and high operational costs, are their main restrictions and limit their uses [19]. Therefore, a different and promising strategy, such as nanoformulations, is required to create a homogenous particle size distribution with superior solubility improvement prospects, medication stability and efficacy, and pharmacoeconomics.

In the last three decades, researchers have paid increasing attention to nanoparticulate systems to produce formulations for insufficiently water-soluble medications (BCS class II and IV). They enhance their innate characteristics by making medication molecules more aqueously soluble [20]. For improving the solubility of poorly soluble substances, nanosystems such as nanocrystals/nanoparticles, nanoemulsions, lipid-based nanoparticles, polymeric nanoparticles/micelles, and nanogels/nanomatrices are widely used. According to a survey conducted by Babadi and his colleagues, polymer-based systems (polymeric nanoparticles and micelles combined) have been the most often employed nanosystems for improving water solubility over the past ten years (Figure 2). With Poly D, L lactide-co-glycolide (PLGA) emerging as the most frequently used polymer in their preparation, a feat well attained due to their ease of preparation, long-term stability, less toxic properties, and ability to efficiently encapsulate hydrophobic drugs to improve solubility and bioavailability [20, 21]. One of the well-known degradable and biocompatible polymers used as carriers for transporting proteins, vaccines, genes, and peptides is polylactic-co-glycolic acid (PLGA) [22]. Hydrophobic drugs/bioactive compounds, including organic phytochemicals like curcumin, crocetin coumarin, synthetic bioactive compounds such as novel pyrano [2,3-c] pyrazole heterocyclic [23], and many others have been successfully encapsulated in PLGA nanoparticles to improve solubility and biological applications.

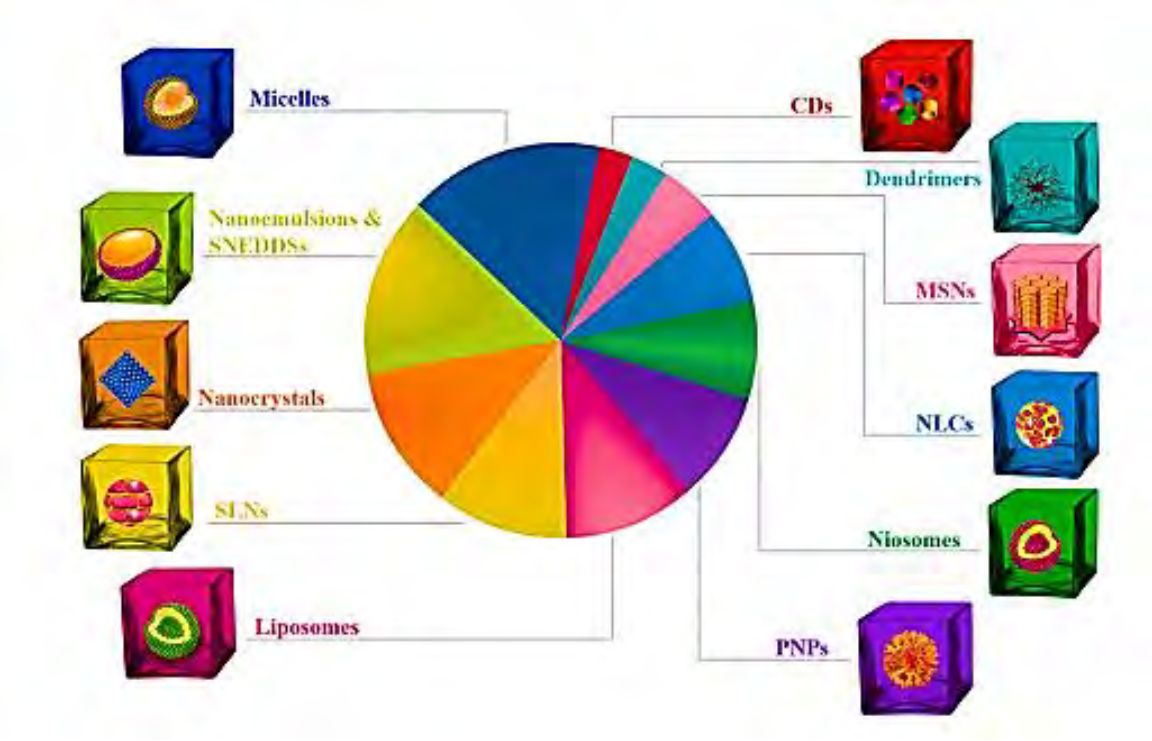

Figure 2. Frequency of recent ten-year studies on the use of nanoformulations for bioavailability enhancement of poorly soluble compounds [20].

1.3 Problem statement

As the number of drug candidates classified as poorly soluble has increased significantly, low aqueous solubility continues to be a unique issue for novel drug candidates in today's pharmaceutical development pipeline [13–15]. Poor solubility makes it difficult to create effective drug formulations and impairs the results of biological assays throughout the drug discovery process. At the clinical stage, it also has a low bioavailability effect. Obakachi et al. created and synthesised a novel series of pyrazolone-based compounds with potential biological activities that were anticipated using computational methods [24, 25]. However, the compounds' limited solubility in an aqueous media prevented further research into their *in vitro* biological activities.

Furthermore, even if these novel pyrazolone-based derivatives were shown to be biologically active *in vitro*, their clinical uses would still be constrained without water-soluble formulations of the substances. This study, therefore, aimed to establish an HPLC-PDA analytical method for the

detection, quantification, and standardisation of the compound in nanoformulations, develop a nanoformulation to improve the solubility of two novel pyrazolone-based compounds from the previously synthesised series, and further evaluate the impact of solubility enhancement on their *in vitro* antioxidant and antimicrobial activities.

1.4 Hypothesis or research question

We postulated that encapsulating the two promising novel pyrazolone-based compounds into a nanosystem would enhance their aqueous solubility and further improve their biological activities and, ultimately, their pharmaceutical/biological applicability.

1.5 Aims and objectives of this study.

This research seeks to enhance the solubility of Two (2) novel pyrazolone-based compounds by nanoencapsulation and to assess solubility enhancement's effect on their biological activities *in vitro.*

To actualise this broad aim, the objectives of the study were:

1. To develop and validate an HPLC-PDA analysis method using the ICH guideline to detect and quantify the compounds in nanoformulations.
2. To optimise the formulation parameters for preparing stable pyrazolone-based compounds encapsulated nanoparticles (PBC/PLGA NPs) and to characterise them based on particle size, polydispersity index, zeta potential, surface morphology (SEM and TEM), differential scanning calorimetry (DSC), release studies and stability.
3. Owing to previous reports in the literature on the antibacterial and antioxidant activity of the pyrazolone scaffold, *in vitro* assay of the compounds and their nanoformulations will be tested for antibacterial and antioxidant activity. Also, haemolysis assay will be carried out to establish the safety of the formulations in blood circulation.

1.6 The novelty of the study

The investigation results presented in this study, as depicted in chapters 3 and 4, are novel because the compounds are novel. Therefore, no related studies have been carried out on them.

1.7 Significance of the study

Insolubility issues continue as a massive setback in drug discovery and development at all levels, with a significant hindrance to the progression of active bioactive compounds into applicable pharmaceutical entities. Therefore, the potential benefits of the study include the following:

1. New pharmaceutical products: The improvement in the solubility of these novel pyrazolone-based compounds will enable their possible biological and pharmaceutical applications. The PBC-PLGA nanoformulation will be a new pharmaceutical product that has never been reported. Therefore, they can serve as new drug entities for effective therapeutic outputs, contributing to producing low-cost drug entities in the pharmaceutical industry.
2. Improved patient therapy and disease treatment: the search for new and improved therapy is imminent. This study foresees a possible transition of the nanoformulations of the novel bioactive compounds to clinical usage, especially in infectious disease and cancer therapy.
3. Addition of new knowledge to the scientific community: it is envisaged that the proposed study can lead to the addition and creation of new knowledge in drug discovery, delivery, and development, which may include:
   i. Solubility enhancement of bioactive compounds using nanoformulation strategy
   ii. Analytical method development and validation using the HPLC.
   iii. Biological activities of pyrazolone-based derivatives
4. Design of ideal novel compounds for lead optimisation in drug candidate development.
5. Simulation of new research:

i. The proposed nanoformulation of the two promising pyrazolone-based compounds will stimulate research towards developing nanoformulations of other poorly soluble pyrazolone-based derivatives, consequently accelerating the discovery of new drugs for therapy.

ii. Enhancing the solubility of the compounds will further enable other biological and *in vivo* assays to be performed with them. Further biological activity testing against cancer, HIV, diabetes, and inflammation can be done on the compounds

iii. The analytical method development and validation in this study could be a useful tool for the standardisation and quality control of the compounds.

1.8 Overview of the Dissertation

The research work is presented in this dissertation in the publication format, according to the University of KwaZulu-Natal College of Health Sciences guidelines. It stipulates the inclusion of a brief introductory chapter, published papers and a final chapter on the conclusions.

CHAPTER 1. Introduction

This chapter outlines the background of the study, highlighting the problems associated with poor solubility of new drug candidates at the development stage, industry, and clinical stage. It further introduces nanoformulation strategies as a capable and convenient method for solubility enhancement of poorly soluble bioactive compounds resulting in the study's proposed aims and objectives. Then highlight the novelty and significance of the study and concludes with the structure and content of the thesis.

CHAPTER 2. LITERATURE REVIEW

This chapter outlines an overview of solubility and the biopharmaceutics classification system (BCS), factors affecting solubility and its importance at drug discovery, pre-formulation/formulation, and clinical stages of drug development. It further outlines the different strategies deployed to enhance the solubility of poorly soluble compounds. Then describes different nanoformulation systems, features

and preparation methods used in enhancing solubility and concludes with an overview of pyrazolone-based derivatives and their biological importance.

CHAPTER 3. EXPERIMENTAL PAPER 1

This chapter addressed objective one (1) of the study and is a first-authored research paper submitted to an international journal and presented here in the required journal format; it describes the development and validation of a simple RP HPLC-PDA method for the detection and quantification of a novel Pyrazolone-based compound in nanosuspensions.

CHAPTER 4. EXPERIMENTAL PAPER 2

Addresses the aim and objectives 2-4 of the study and is a first-authored research article to be submitted to an internationally recognised journal. This paper highlights the encapsulation of two novel pyrazolone-based compounds using PLGA and poloxamer to simultaneously enhance their solubility and biological activities.

CHAPTER 5. CONCLUSION

This chapter includes the general conclusions from the research findings, provides more information on the significance of this study and makes recommendations for future research work on pyrazolone-based derivatives.

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CHAPTER 2. LITERATURE REVIEW

2.1 Introduction

This chapter outlines the concept of solubility and the biopharmaceutics classification system (BCS), factors affecting solubility and its importance at drug discovery, pre-formulation/formulation, and clinical stages of drug development. It further outlines the different strategies deployed to enhance aqueous solubility. Then describes the different nanoformulation systems, features and preparation methods used in enhancing solubility and concludes with an overview of pyrazolone-based derivatives and their biological importance.

2.2 The concept of Solubility

Solubility is the percentage of a constituent that dissolves at a certain temperature and pressure when there is an equilibrium between the dissolved and undissolved [1], that is a solute's capacity to evenly mix with a solvent (solid, liquid, or gas) to create a solution. As seen with the precipitation of solids, solubility is the outcome of the dissolution and phase joining phenomena, which coincide and oppose each other under equilibrium. When dissolution and phase joining occur at a consistent pace, an equilibrium is reached, and if certain criteria are satisfied, the equilibrium solubility results in a metastable supersaturated solution [2]. The solubility of a material in a known solvent is approximated as the saturation concentration when the addition of more solute does not increase its concentration in the solution [2-4]; this ranges widely from extremely soluble to weakly soluble, as shown with silver chloride in water. Notably, a substance's solubility should not be confused with its capacity to dissolve or liquefy since both processes result from dissolution and a potential chemical reaction. For instance, whereas zinc chloride is soluble in hydrochloric acid, zinc is not [2]; instead, it dissolves, thereby chemically interacting with the acid to form zinc chloride and hydrogen. The usage of the phrase "Solubility" from multiple perspectives has led to its representation in numerous ways, such as mass (gramme per kilogramme, millilitre, or litre of solvent), concentration (C), and mole-fraction, molarity, or molality as the case may be. These solubility expressions have the benefit of being simple, but they

have a downside in that they can heavily rely on the existence of different ion groups in the solvent (known as the common ion effect). Because this is an equilibrium process, solubility constants can occasionally characterise saturated solutions of ionic substances with limited solubility. Despite being strongly influenced by temperature, these constants reflect the equilibrium between dissolved ions from salt and undissolved salt and are independent of other species in the solvent.

A substance's solubility can be generally influenced by temperature, pressure, and the solvent or solvent type. However, additional elements, particularly concerning pharmacological compounds, have been implicated, including molecular structure, particle size, the impact of complex formation, the presence of solubilizing agents, the impact of pH, the presence of additives, electrolytes, and non-electrolytes. Several parameters, such as enthalpy of fusion and entropy, have been used to forecast solubility. Additionally, the Hansen solubility parameters, Lipinski's rule of five, the Hildebrand solubility parameters, and The Flory-Huggins solution theory (a theoretical model to characterise polymer solubility) are empirical approaches for predicting solubility [2, 5]. The USP, BP, and Biopharmaceutics classification systems have also characterised solubility based on particular criteria (Table 1). The USP and BP solubility classifications are unaffected by the solvent (only in quantification).

Table 1: USP and BP Criteria for Solubility

Description	**Part of the solvent required per part of solute**
Very soluble	Less than 1
Freely soluble	from 1 to 10
Soluble	from 10 to 30
Sparingly soluble	from 30 to 100
Slightly soluble	From 100 to 1000
Very slightly soluble	from 1000 to 10,000
Practically insoluble	10,000 and over

2.3 The Biopharmaceutics Classification System (BCS)

The Biopharmaceutics Classification System (BCS) is a scientific classification system for medicinal compounds based on water solubility and intestinal permeability. Since its creation, it has been the most critical instrument for global medicinal product regulation [6, 7]. This categorisation is also a drug-development tool since it evaluates three key parameters affecting oral drug absorption from immediate-release solid oral dosage forms: dissolution, solubility, and intestinal permeability [8]. BCS, which was first introduced into the regulatory decision-making process in the guidance document on immediate-release solid oral dosage forms: Scale-up and post-approval changes [9], allows drug designers to manipulate the structure or physiochemical properties of the lead drug candidates while also minimising drug exposure to a large panel of human subjects and, in some cases, shortening drug product development time in addition to significant cost reduction [4]. Again, regulatory authorities have used the BCS to enable the use of *in vitro* dissolution data to demonstrate drug product *in vivo* bioequivalence. The US Center for Medicinal Evaluation and Research (CDER), World Health Organisation (WHO), and European Medicines Agency (EMEA) have approved a BCS-based biowaiver for drug products comprising BCS class I medicines with fast dissolution [10-12]. It is worth noting that the WHO and EMEA have expanded the BCS-based biowaiver for medications in BCS class II with mild acidic characteristics and BCS class III. From the preclinical to the clinical stages, BCS has been employed for biowaiver and formulation design [13]. BCS classified drugs and APIs according to their solubility and permeability [9, 12, 14].

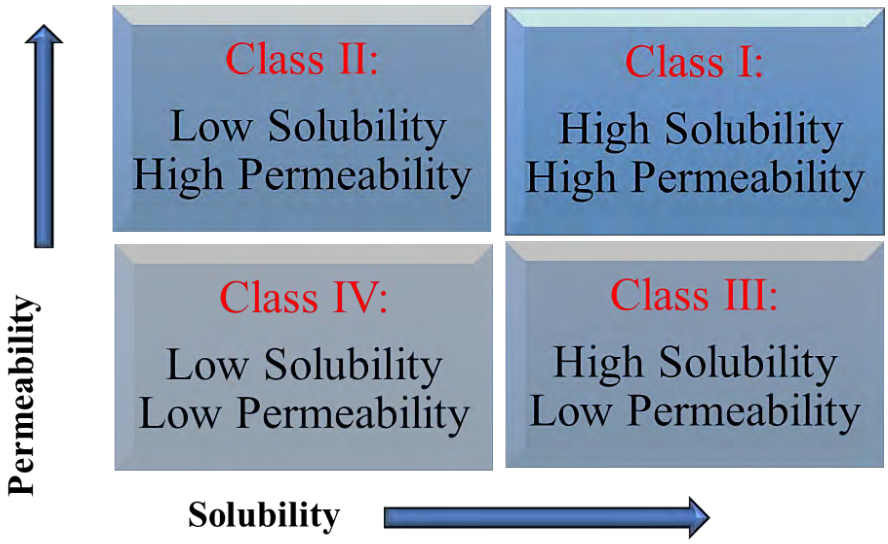

Figure 2.1. Biopharmaceutics classification of drugs based on solubility and permeability

Compounds are described as highly soluble when the amount of medicine contained in the greatest strength of an immediate-release dosage form is soluble in 250 ml of water over a pH range of 1.2-7.5 in the gastrointestinal tract. When tested *in vitro* or *in vivo*, highly permeable substances are 90% absorbed or have a permeability equal to, or more than, a 90% absorbed reference drug [15-17].

2.3 1. Class I: High Solubility-High permeability

Drugs in this category do not have issues with intestinal absorption and bioavailability; they are highly soluble and permeable. Metoprolol, propranolol, and theophylline are in this class [18]. Here, there is no rate-limiting step for oral absorption. Therefore, Immediate release solid oral dosage forms such as conventional tablets or capsules are designed to ensure rapid dissolution in the gastrointestinal tract.

2.3 2. Class II: Low Solubility-High permeability

Class II drugs have low dissolution but high absorption [19]. However, their absorption is still lower than Class I. Examples are listed in table 2 [18]. Their bioavailability is rate-limited by dissolution such that even a tiny increase in dissolution rate can result in a significant increase in bioavailability [11]. Thus, the enhancement of the dissolution rate of the drug is a key feature for bioavailability enhancement and can be rightly achieved by manipulating several factors related to the dissolution

rate, such as the effective surface area, the diffusion coefficient, the diffusion layer thickness, the saturation solubility, the amount of dissolved drug, and the volume of dissolution media [20, 21]. These can be achieved through pre-formulation studies and formulation design involving particle size reduction, crystal modification and amorphisation, self-emulsification, and pH modification.

2.3 3. Class III: High Solubility-Low permeability

Drugs in Class III display a high disparity in the rate and extent of absorption, as the rate-limiting step for drug absorption is permeability. The dissolution here is rapid; hence this variation is attributed to the modification of membrane permeability and physiological factors rather than the dosage form factors. Their absorption is by membrane permeation. Examples of such drugs are listed in table 2 [22]. Permeation enhancers, such as fatty acid, bile salts, surfactants, and polysaccharides, play a vital role in enhancing the permeability of these drugs via the paracellular pathway; however, some have damaging membrane effects [23, 24]. There is little information on the effective and safe dosage options for BCS class III drugs; hence, immediate-release solid dosage forms are designed for clinical use [25].

2.3 4. Class IV: Low Solubility-Low permeability

Class IV pharmaceuticals generally provide difficulties with successful oral administration due to challenging chemical features such as limited solubility and permeability. Solubility and permeability are rate-limiting phases in absorption; physiological variables such as stomach emptying and gastrointestinal transit time affects BCS class IV drug absorption. Absorption of drugs in this class varies greatly across and within subjects [26]. This diversity poses a significant obstacle to their formulation and development approaches. Viable formulation alternatives widely used in BCS class II medicines are used to enhance dissolving behaviour. However, most ways to improve their permeability are still in the early stages of research, and their safety is not well-proven. This class includes Acetazolamide, amphotericin B, chlorthalidone, cyclosporin, and ritonavir [27].

Table 2: Examples of drugs with their BCS classes [4, 18, 27, 28].

Class I	Class II	Class III	Class IV
Propranolol	Cyclosporin	Atenolol	Aprepitant
Metoprolol	Griseofulvin	Cimetidine	Albendazole
Theophylline	Itraconazole	Metformin	Curcumin
Chloroquine	Ketoconazole	Acyclovir	Co-Enzyme Q10
Paracetamol	Phenytoin	Captopril	Ellagic Acid
Diltiazem	Carbamazepine	Ranitidine	Furosemide
Verapamil	Donazole	Neomycin B	Taxol
Nifedipine	Glibenclamide	Amoxicillin	Amphotericin B
Quinidine	Troglitazone	Pravastatin	Chlorthalidone
	Ritonavir	Fexofenadine	Digoxin
	Saquinavir		Talinolol

2.4 FACTORS AFFECTING SOLUBILITY

Factors such as temperature, pH, particle size, crystal characteristics/molecular structure of solute, polarity/nature of the solvent, and dielectric constant can affect the solubility of a substance.

1. **Temperature:** The absorption of heat translating to an increase in temperature influences the solubility of drugs rightly described below:

i. Dissolution involving a positive hit of a solution results in a rise in temperature leading to an increase in the solubility of the solid. Potassium nitrate in water is an example.

ii. On the other hand, if the liberation of heat is involved in the dissolution of a solid, then an increase in temperature decreases solubility-for example, calcium acetate in water.

In other words, the temperature is directly proportional to solubility.

2. **pH:** most drug substances are either weak acids or bases that undergo ionisation in solution. The ionisation of a drug in solution highlights its solubility, which depends on its dissociation constant pKa and pH of the medium [29]. The dissociation constant of a drug helps predict the extent of

ionisation depending on the pH of the environment. The ionised species generally have greater aqueous solubility than the unionised species. For example, phenobarbitone is more soluble in alkaline pH and begins to precipitate as the pH decreases, whereas the reverse is the case with alkaloidal salts. Equations 1 and 2 below explain the relationship between solubility, the pKa Value and pH of the medium.

For a weakly acidic drug: $pH = pKa + LogS - \frac{So}{So}$ **Equation 1**

For a weakly Basic drug: $pH = pKa + \frac{LogSo}{S} - So$ **Equation 2**

So is the solubility of the unionised form expressed in moles/litre, S is the overall solubility also expressed in Moles/litre, and pKa, is the dissociation constant of the drug.

3. **Particle size:** solubility of a drug is inherently related to the particle size-the surface area to volume ratio increases as particle size decreases. Larger surface areas allow more interaction with the solvent, which causes an increase in solubility and vice versa.
4. **Physical forms of solute-crystal characteristics and molecular structure of solute:** Many drugs exhibit different crystalline forms of the same substance (polymorphism). The polymorphic forms possess different lattice energies, evident in the changes observed with their solubility and melting points. With forms of solute/drug, the following have

been well established: substances in amorphous forms are considered more soluble than their crystalline counterparts; The metastable forms of solutes have greater aqueous solubility than the stable forms; anhydrous forms possess greater aqueous solubility than hydrates forms; organic solvates have better aqueous solubility than non-solvated forms; salt forms of drugs have more excellent aqueous solubility than non-salt forms (provided there is no common ion effect). The structural orientation of the solute is also considered here, as a slight change in structure can affect the compound's solubility.

5. **Polarity and nature of solvent:** Polarity is associated chiefly with hydrogen bonding. Like dissolves, solubility is highest in compounds and solvents with similar polarity.

6. **Dielectric Constant:** solubility depends on the dielectric constant of the polar and nonpolar medium; thus, increasing the dielectric constant with hydrophobic drugs leads to decreased solubility.
7. **Pressure:** this factor does not appreciably affect Liquids and solids. However, as stated by Henry's Gas Law, it is a significant factor in the solubility of gases. Henry's gas law states that the amount of gas dissolved is directly proportionate to the partial pressure of that gas, provided the temperature is kept constant.

$$Cg = KPg \qquad \textbf{Equation 3}$$

Where Cg is the dissolved concentration, K is the Henry Law constant, and Pg is the partial pressure of the gas.

2.5 IMPORTANCE OF SOLUBILITY ENHANCEMENT

A substance's aqueous solution is crucial as it affects the prospect of its absorption and bioavailability after oral administration, parenteral administration and the ease of manipulation during manufacture and laboratory testing.

The drug discovery stage: The drug discovery process begins with the identification of a pharmacophore by screening a library or recombinant chemical series using a biological test, such as blocking an enzyme or binding to a receptor. Following high-throughput pharmaceutical profiling of typical hit compounds, compounds with requisite biological and pharmacological properties are tested *in vivo* for activity. Following that, more lead optimisation via chemical synthesis is carried out. A therapeutic chemical must be properly in solution to measure significant *in vitro* activities and obtain sufficient absorption for safety and efficacy evaluation in preclinical models and clinical trials. As a result, solubility testing is now an integral element of the research and development process and is constantly monitored and improved [30].

Additionally, during compound design and optimisation, solubility information is obtained using high-throughput approaches to influence learning cycles, allowing teams to construct structure-property relationships (SPRs) and structure-activity relationships (SARs) for enormous amounts of data [31]. Furthermore, a compound's solubility provides information on the maximum orally absorbable dosage (MAD) and human dose prediction for initial developability evaluation [32], allowing researchers to make informed judgments about chemical series and compound prioritising. As a result, this is the ideal stage for detecting solubility difficulties and exploring structural modification techniques for improvement.

Preformulation and formulation development stage: Preformulation entails recognising the physicochemical, mechanical, and biological features of the novel pharmacological entity using a structured programme, evaluating potential give-and-take interactions with multiple excipients utilised in the formulation, and evaluating stability [33]. Dissociation constant and solubility are two basic qualities required to develop a novel synthesised pharmacological entity or introduce new therapeutic applications for existing medications. These qualities indicate the need to increase bioavailability, which is occasionally compromised due to the drug molecules' low solubility. Preformulation studies are important in drug development because they give logical solutions to formulation challenges for various liquid, semisolid, and solid medicinal agents with systemic or local action [34]. Solubility, pH effect, lipid/water partition coefficient, salt formation, chemical structure/pharmacological activity link, and stability of active therapeutic ingredient in solution and solid state are essential features investigated during the pre-formulation phase [12, 35].

Also, disintegration and dissolution are essential for medication absorption and the optimal therapeutic response. As a result, one of the most important components of creating dosage forms, particularly those intended for oral or transdermal administration, is enhancing solubility and dissolving rate. The physicochemical features of the drug, the excipients, and the pharmaceutical dosage form all influence the successful delivery of a drug entity in a pharmaceutical form (pharmaceutical availability) [36]. As

a result, there is a greater need to accurately determine drug availability in formulations using a variety of *in vitro* tests that are more easily determinable and reproducible than *in vivo* tests, allowing the study of factors that can influence physicochemical processes without introducing physiological variables. These determinations are required because bioavailability is affected by physical properties such as particle size, crystalline form, method of manufacture of pharmaceutical forms, excipients used, and solvent pH, which affects *in vitro* dissolution- a factor that influences drug formulation and future therapeutic efficacy.

Furthermore, the formulation design of a medicinal product with a high drug load is often complicated—a high drug load results in poor powder flow and sticking during granulation and tabletting, as well as an overall increase in cost. Poor solubility is recognised as a critical issue that affects the effectiveness of oral medication administration [2, 37] as a novel medicinal entity with strong solubility seldom causes formulation issues and has good bioavailability for practically every route of administration. Therefore, it is crucial to guarantee that drug entities are sufficiently soluble in water.

Clinical Stage: The solubility and dissolution rate of a pharmacological substance are factors that have a direct impact on the absorption and bioavailability of the new medicinal entity. Solubility is critical for attaining the systemic concentration and establishing an effective therapeutic response, and hydrophobic medicines require high doses to achieve the desired therapeutic plasma concentrations after delivery. Limited drug dissolution frequently results in low bioavailability of orally administered drugs due to their dissolution-limited absorption, which in turn triggers dose escalation to achieve therapeutic concentration range further, predisposing the patient to potential toxicity in the gastrointestinal tract and hindering patient compliance when such drugs are administered. Other disadvantages of poorly soluble medications include their unsuitability for IV dosage, development issues that increase development cost and time, and ultimately shifting risk to the patient (frequent high-dose administration).

2.6 Solubility and dissolution rate

The aqueous solubility of a medication is a crucial quality that influences not only the convenience of handling and testing in the laboratory and during manufacturing but also the likelihood of drug absorption after oral administration and the capacity to inject the drug parenterally. Solubility is a measure of equilibrium, whereas dissolution is the rate at which a bare solid medication or drug in a formulation dissolves [38]. Because intestinal transit time is restricted after oral administration, the drug dissolving rate must greatly surpass the transit rate to maximise absorption. In most cases, if the dissolution rate is modest, the solubility limit may never be achieved within the intestinal transit period, resulting in inadequate absorption of a decently soluble medicine. Similarly, even though the rate of dissolution is relatively fast, if the equilibrium solubility is low, the quantity of medication accessible in solution for absorption may not be adequate to maintain rates of flow across the gut sufficient to absorb the complete drug dosage in the time available. Solubility or dissolution rate may be a limiting factor for absorption for different drug entities and under different situations.

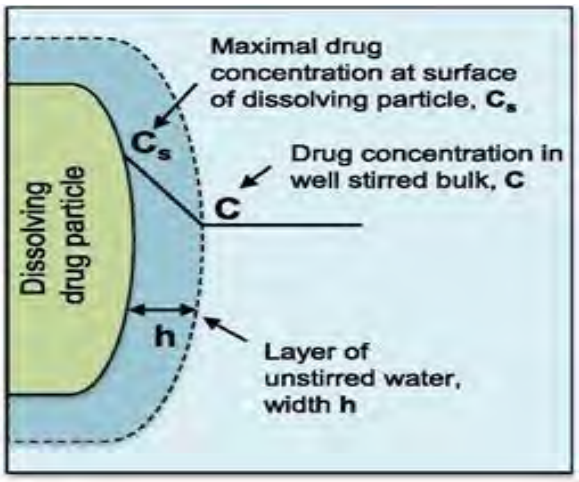

Figure 2.2. depicts the dissolution process of a drug. Reproduced from [39]

The diagram above **(Figure 2.2)** depicts an unstirred water layer of width (h) on the dissolving solid's surface and an established concentration gradient that drives dissolution across the unstirred water layer. The concentration gradient can be attributed to the disparity in drug concentration between the

surface of the dissolving solid (Cs) and the concentration in bulk (C). The Noyes-Whitney equation rightly established the relationship between the above parameters that the rate of transfer across the unstirred layer is a function of the concentration gradient across the same layer, the width of the diffusion layer (h), the surface area of contact of the solid with the dissolution fluid (A), and the diffusion rate of the drug in water (D). However, it also showed that the concentration gradient depends on the maximum drug concentration at the surface of the dissolving solid (drug solubility Cs) and the concentration in bulk (C) [40].

$$\frac{dcx}{dt} = \frac{DA}{h}(cs - c) \qquad \textbf{Equation 4}$$

However, when sink conditions are established, where the concentration of the drug in bulk solution (C) is low relative to the concentration on the surface of the dissolving solid (Cs), the above equation becomes;

$$\frac{dcx}{dt} = \frac{DA}{h}(cs) \qquad \textbf{Equation 5}$$

However, it is crucial to note that the diffusion rate constant in water (D) for most small molecules is rather large, and changing drug structure has no significant effect on D to influence the dissolution rate. At the same time, whereas stirring or agitation can change the breadth of the diffusion layer *in vitro*, it cannot be easily adjusted *in vivo*. Surface area and solubility are the primary drivers of *in vivo* medication dissolving rate. Given that solubility is determined by the strength of the crystal lattice and the affinity of the solute (drug) for the aqueous environment, three (3) major strategies have been implemented to efficiently increase solubility or dissolution rate while keeping in mind that increases in solubility inherently increase dissolution rate [39]:

• Dropping the intermolecular forces in the solid state (increases solubility and dissolution rate).

• Boosting the strength of solute-solvent interactions in solution (increases solubility and dissolution rate).

• Increasing the surface area available for dissolution (increases dissolution rate and the potential to moderately increase solubility at small particle sizes < 1 mm).

Based on the strategies mentioned above, several approaches/techniques, as depicted in **Figure 2.3**, are well reported in the literature to enhance aqueous solubility, dissolution rate and ultimately, the bioavailability of poorly soluble drug candidates. These may include and are not limited to particle size/micronisation, supercritical fluid (SCF) process, nanocrystals [41], cyclodextrins complexes [42, 43], nanoparticles and nanosuspensions [44], salt formation [45], amorphous solid dispersions [46].

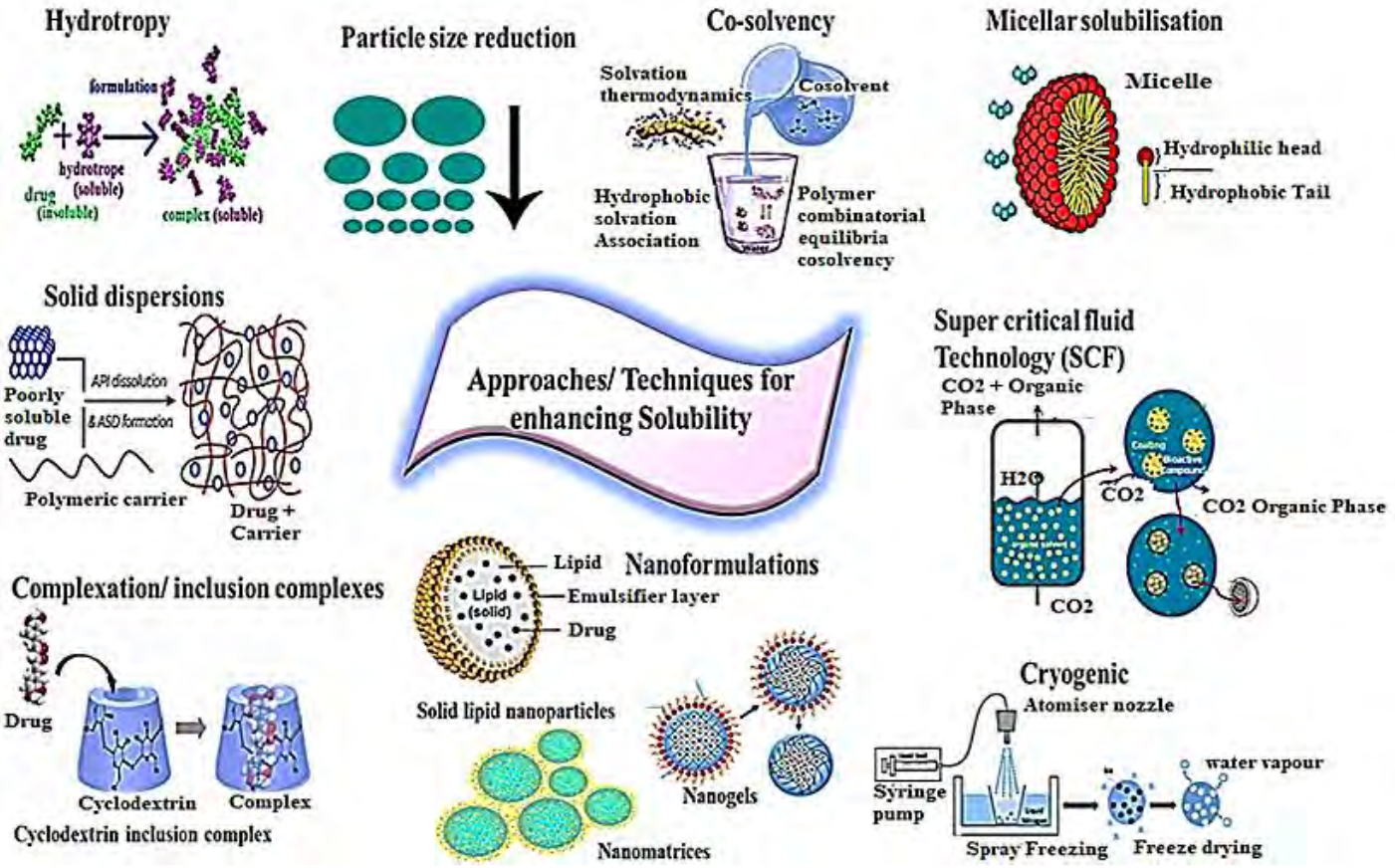

Figure 2.3. Summary of the different approaches for solubility enhancement of poorly soluble drugs

2.7 Approaches/ techniques for solubility enhancement of poorly soluble drug candidates

As discussed earlier, solubility is required to achieve the desired drug concentration in the systemic circulation. It directly affects *in vivo* bioavailability and clinical effects and poses a substantial setback in drug discovery and development. Therefore, enhancing the solubility of poorly soluble drug candidates is very crucial.

Techniques to enhance solubility can be broadly categorised into:

i. **Techniques involving physical modifications:** Particle size reduction: Micronisation/nanonisation, alteration of the crystal habits of the drug, drug dispersal in carriers such as solid solutions, solid dispersions, eutectic mixtures and cryogenic techniques.
ii. **Techniques involving chemical modifications:** The use of a buffer, derivatisation, change of pH, complexation and salt formation.
iii. **Others are** supercritical fluid processes, using adjuvants such as surfactants, solubilizers, co-solvency, hydrotropy, and novel excipients.

2.7.1 Particle size reduction approach for solubility enhancement

Particle size reduction is directly related to solubility; it is well known that solubility is enhanced when particle size is reduced, which can also be expected to enhance *in vivo* drug bioavailability [47]. Solubility is intrinsically related to drug particle size, the surface area to volume ratio increases as the particle becomes reduced. The larger surface area allows more interaction with the solvent, thus causing an increase in solubility, as postulated by the Ostwald Freundlich equation [48, 49]. Particle size reduction of the drug can be done by milling and grinding techniques using a jet mill, rotor-stator, and colloid mills; their reduction mechanism is well illustrated in **Figure 2.4**. Conventional particle size reduction methods, such as spray drying and comminution, which rely on mechanical stress to disaggregate the active compound, are well reported in the literature. These methods have been reported to permit an efficient, reproducible, and economic solubility enhancement. However, their

limitations, such as possible thermal and physical stress on drug products leading to degradation, restricted opportunity to control the shape, size, morphology, surface properties, and electrostatic charges of the final product and their ability to cause recrystallisation of amorphous regions in a hot or humid environment, since they are known to be thermodynamically unstable. Therefore, the particle size reduction technique is unbefitting for thermodynamically unstable drugs and high dose numbers because it does not change saturation solubility (Blagden et al., 2007), and the milling process may alter surface properties in an uncontrolled manner [50]. This technique was applied to enhance the solubility of silymarin, griseofulvin, itraconazole, progesterone, spironolactone, zaleplon, diosmin, fenofibrate, domperidone, and curcumin [51-55]; with well-improved bioavailability and clinical efficacy.

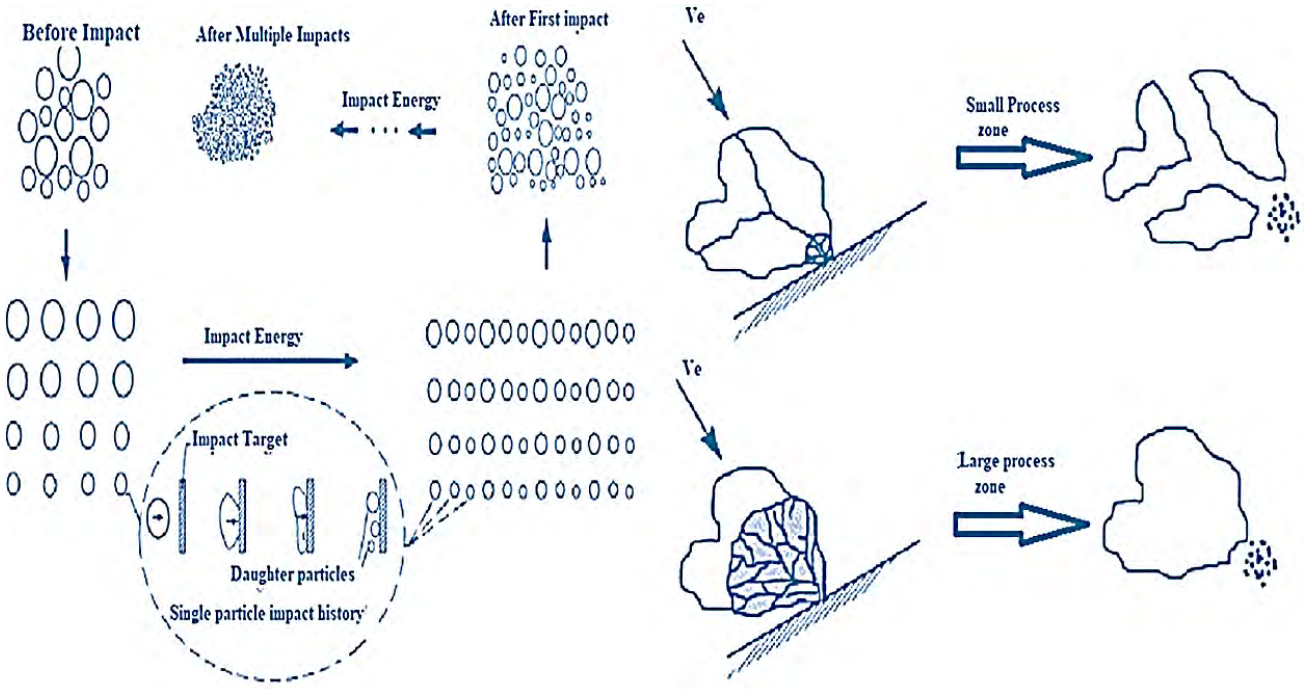

Figure 2.4. Mechanism of particle size reduction technique [56]

2.7.2 Solid dispersions approach for solubility enhancement

Solid dispersion implies a group of solid products constituting at least two components: a hydrophobic drug and a hydrophilic matrix [57]. The concept of solid dispersions was first suggested by Sekiguchi

and Obi, who researched the creation and dissolution performance of eutectic melt products of a sulphonamide drug and a water-soluble carrier in the early 1960s [58]. Solid dispersion is a useful pharmaceutical technique to improve dissolution and bioavailability. The first-generation solid dispersions were prepared using crystalline carriers such as urea and sugars, forming crystalline solid dispersions, posing a major drawback. Although crystalline solid dispersions were thermodynamically stable, they failed to release the drug quickly as the amorphous counterparts, which led to the development of second-generation solid dispersions, investigated using water-soluble amorphous carriers instead of crystalline [59]. Synthetic polymers such as polyethene glycol (PEG) povidone or polyvinylpyrrolidone (PVP), polymethacrylates, and natural polymers such as ethyl-cellulose, hydroxypropyl-methylcellulose (HPMC), hydroxypropyl-cellulose, and starch derivatives cyclodextrin are commonly used in the preparation of amorphous solid dispersions [60]. Third-generation solid dispersions with surface or self-emulsifying capabilities, such as inutec SP1, compritol 888 ATO, gelucire 44/14, poloxamer 407, Tween 80, and docusate sodium, have recently demonstrated improved dissolution and bioavailability profiles [61]. Solid dispersion enhances the solubility of weakly water-soluble pharmaceuticals when prepared using hot-melt extrusion, media milling, hot-melt (Fusion), a solvent evaporation method, and high-pressure homogenisation [62]. Solid dispersion enhances dispersibility and wettability by converting crystalline medications to amorphous form, regulating drug particle aggregation and agglomeration, and lowering particle size. Fenretinide and griseofulvin have enhanced solubility thanks to solid dispersion methods [63, 64].

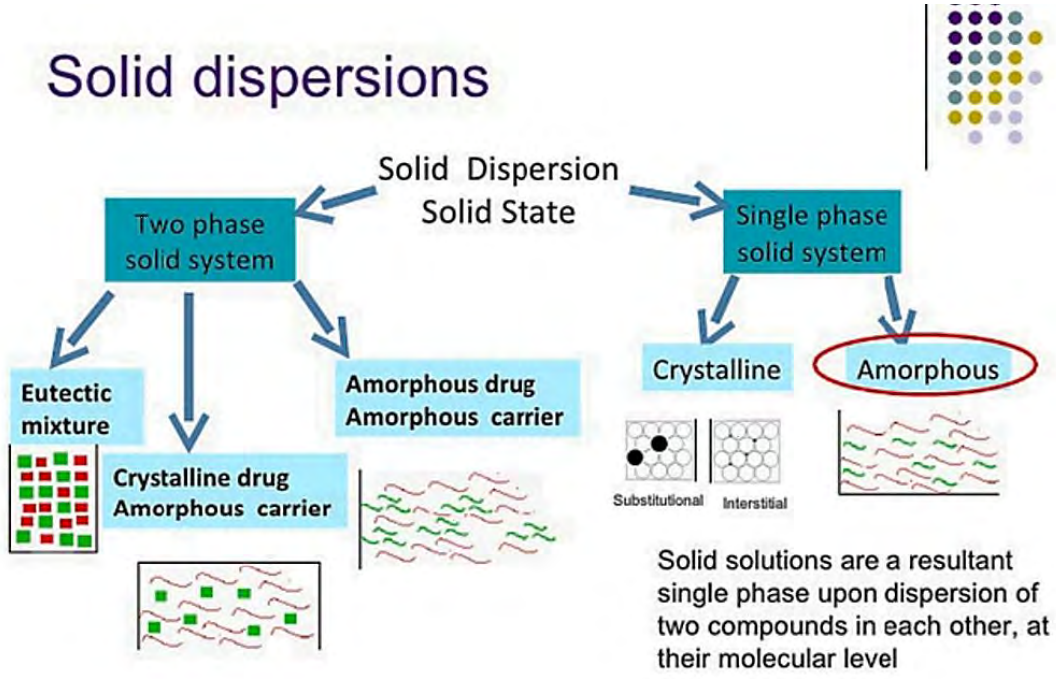

Figure 2.5. Mechanism of solubility enhancement using solid dispersion technique

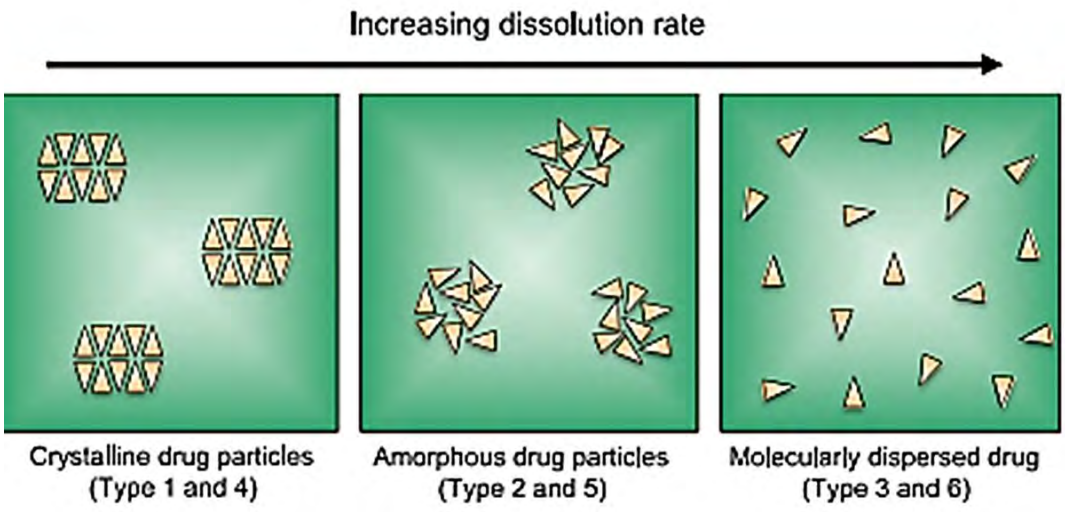

Figure 2.6. A diagram showing three different molecular arrangements of drug molecules within a solid dispersion [39]

2.7.3 Complexation/inclusion complexes

The complexation/inclusion complex formation-based technique, as depicted in **Figure 2.7**, has been commonly used in pharmaceutical industries for dissolution, solubility, and bioavailability

enhancement of poorly soluble drugs. These are formed by the addition of nonpolar compounds (hydrophobic), or their nonpolar part called guest molecules, into the cavity of the second molecule called the host molecule. For complexation/inclusion complex-based techniques, cyclodextrins are the commonly used host molecule [65]. Cyclodextrins are non-reducing, water-soluble, crystalline, and cyclic oligosaccharides made up of glucose monomers arranged in a doughnut-shaped ring with a hydrophobic hollow and a hydrophilic outer surface, produced by enzymatic degradation of starch by cyclodextrin-glycosyltransferase (CGT). The exterior portion of the cyclodextrin molecule is hydrophilic, while the interior portion is hydrophobic (lipophilic), enabling cyclodextrin to form complexes with hydrophobic compounds. Cyclodextrins are used in various drug delivery systems, food, chemical, agricultural, and pharmaceutical industries [66]. Various technologies involving lyophilisation/freeze-drying, kneading, and microwave irradiation have prepared the inclusion complexes of poorly water-soluble drugs using cyclodextrins. Yan, dos Santos Lima, Khalid and Kim, with their co-workers, used this technique to enhance the dissolution rate, solubility and bioavailability of poorly soluble methocarbamol, dexibuprofen and albendazole [43, 67-69].

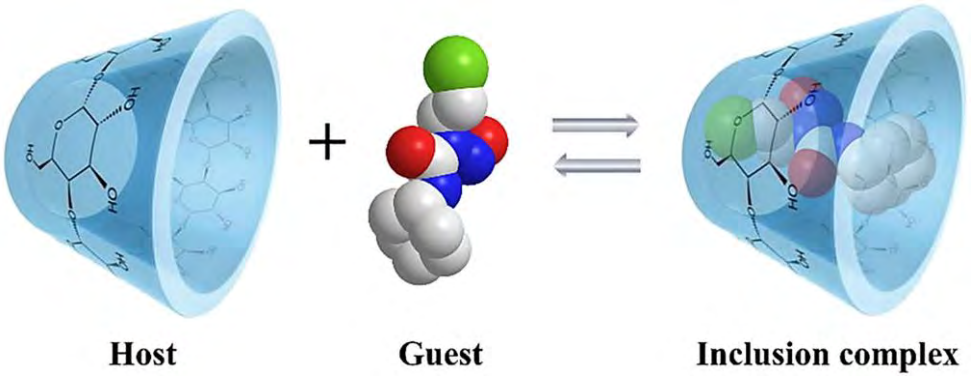

Figure 2.7. Illustration of a cyclodextrin inclusion complex for solubility enhancement [70]

2.7.4 Supercritical fluid Technology

The supercritical fluid process is a novel nanonising and solubilisation technology whose application in the pharmaceutical industry has intensified in recent years. It entails particle size reduction via supercritical fluids **(Figure 2.8)**. Supercritical fluids are fluids with temperatures and pressures greater

than their critical temperature (Tc) and critical pressure (Tp), allowing them to adopt the properties of both gas and liquid [71]. These fluids are environment-friendly (non-toxic, non-reactive, non-inflammable, non-polluting) and economical. Examples are carbon dioxide (CO_2), ethylene, diethyl ether, nitrous oxide, propylene, propane, ethanol, acetone, and water (H_2O) [72, 73]. The supercritical fluid (SCF) technology has been used to obtain active compounds with improved performance indices on the subject of stability, solubility, and bioavailability [74].

Recently, researchers have used supercritical fluid (SCF) technology for poorly soluble drugs' solubility and bioavailability enhancement. Xie et al. enhanced the solubility of curcumin using this technique [75]. Han et al. also applied the SCF technique to prepare solid dispersions of ibuprofen with improved oral bioavailability [76].

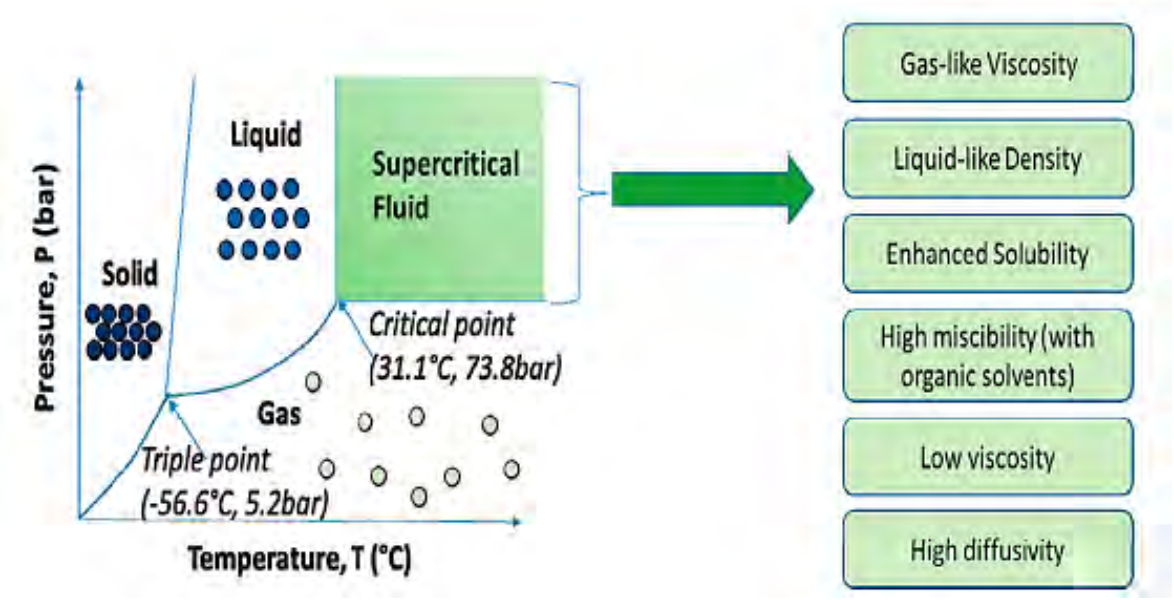

Figure 2.8. Supercritical fluid process for solubility enhancement [77]

2.7.5 Micellar solubilisation

Micellar solubilisation is a technique that entails using surfactants to improve the solubility of poorly soluble drugs. Surfactants are possibly the primary and oldest method used to enhance solubility [2]. They reduce the interfacial tension in aqueous media, improve solids' wetting and increase the solid

disintegration rate into finer particles. As the concentration of surfactants increases from their critical micelle concentration (0.05–0.10%), the formation of micelle occurs, resulting in the entrapment of the drug within the core of micelles. The process of drug entrapment within the core of the micelle is known as micellisation and generally results in an enhancement in the aqueous solubility of poorly aqueous soluble drugs [78]. In addition to enhancing solubility, surfactants as drug carriers can enhance permeability, reduce toxicity, and longer residence time in the system [79]. Commonly used surfactants are tween-80, polysorbates, poly-oxy ethylated castor oil, poly-oxy ethylated glycerides, sodium dodecyl sulphate (SDS, sodium dodecylbenzene sulfonate (SDDBS), lauryl macro glycerides, and mono, di-fatty acid esters of low molecular weight polyethene glycols [80, 81]. The solubility of fenofibrate, danazol, and rosuvastatin has been enhanced using this technique [82, 83]. The lack of *in vitro* model for the assessment of the formulations and the high chances of drug instabilities due to high surfactant concentrations constitute the major shortcomings of the technique [84]. **Figure 2.9** illustrates the process of micelles solubilisation.

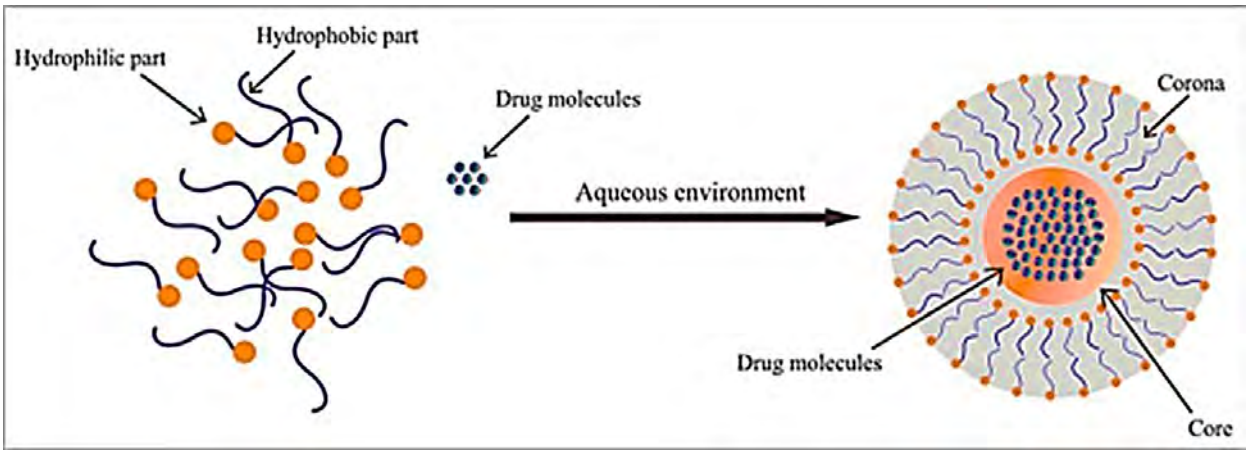

Figure 2.9. Mechanism of Micelle solubilisation [85]

2.7.6 Co-solvency

The aqueous solubility of pharmaceuticals can also be increased by mixing them with water-miscible solvents in which the drug is freely soluble; a technique known as co-solvency, and the solvents employed are known as cosolvents. A co-solvent is a solution composed of water and one or more

water-miscible solvents that reduces the interfacial tension between a hydrophobic solute and an aqueous solution [86]. Methanol, glycerine, ethanol, di-methyl-aceto-amide (DMA), dimethyl-sulfoxide (DMSO), propylene glycol, acetonitrile, isopropanol, and polyethene glycol 400 (PEG-400) are the solvents employed in this procedure [87, 88]. Most cosolvents are composed of hydrogen bond acceptor or donor groups and small hydrocarbon regions. Water-loving hydrogen bonding groups promote water miscibility, whereas hydrophobic hydrocarbon regions obstruct water's hydrogen bonding network, lowering water's intermolecular attraction. Co-solvents improve aqueous solubility by reducing the propensity of water to squeeze out nonpolar, hydrophobic molecules by modifying their self-association [86]. This approach has certain documented shortcomings, such as solvent-related toxicities and uncontrolled precipitation when diluted with an aqueous medium, which have been addressed partly by combining this technique with other solubility augmentation techniques to provide superior results. Kfoury et al. investigated the combined impact of complexation and co-solvency approach on caffeic acid solubility improvement, whereas Zadaliasghar et al. observed enhanced solubility of ketoconazole at different temperatures utilising binary mixes of water and 2-propanol [89, 90]. Maheri and Barzegar-Jalali et al. employed the co-solvency approach to improve the solubility of dexamethasone and ketoconazole, respectively [91, 92].

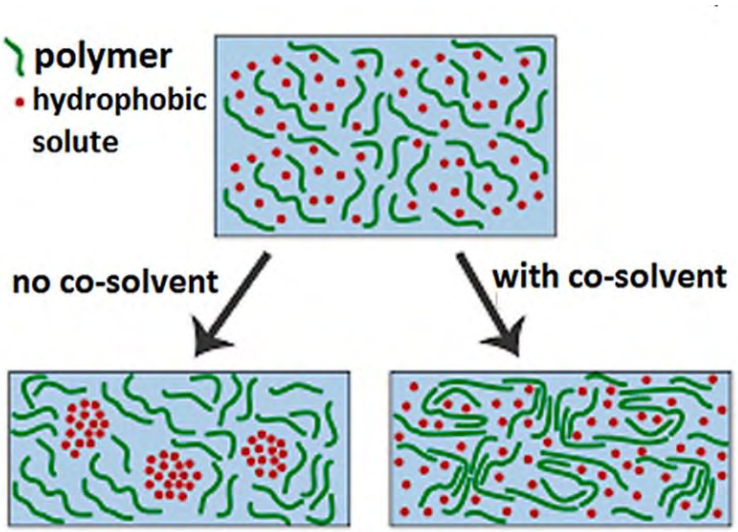

Figure 2.10. Cosolvent technique for solubility enhancement [93]

2.7.7 Hydrotropy

Hydrotropy is a solubilisation approach in which a substantial amount of a second solute, known as a hydrotropic agent, is added to improve the water solubility of the first solute [94]. Neuberg originally documented Hydrotropy in 1916. Later, Everson and Booth verified that adding various organic salts, such as sodium citrate, sodium benzoate, sodium salicylate, urea, benzene sulfonate, nicotinamide, and cumene sulfonate, to a solution might enhance the solubility of poorly soluble substances [95]. Hydrotropic agents are ionic organic salts made up of alkali metal salts of various organic acids. Additives or salts that improve solubility in a certain solvent are called "salt in", whereas those that decrease solubility are "salt out". Salts containing large anions or cations that are very soluble in water cause "salting in" of non-electrolytes known as "hydrotropic salts," a phenomenon known as hydrotropism. As a result of a substantial number of additives, hydrotropy leads to a rise in water solubility. Hydrotrope classification based on the molecular structure is problematic because many substances have been shown to display hydrotropic behaviour. Aromatic alcohols (pyrogallol, resorcinol, catechol, naphthols and salicylates), ethanol, dodecylated oxidibenzene, alkaloids (caffeine and nicotine), and ionic surfactants (diacids, SDS (sodium dodecyl sulphate) are specific examples [96]. The most researched compounds are aromatic hydrotropes with anionic head groups. They are numerous due to isomerism, and their efficient hydrotropic activity can be linked to the availability of interacting pi (π) orbitals [97]. The promising processes responsible for hydrotropic solubilisation may be classified based on their ability to self-assemble, the production of micelles-like structures, and the structure breakers/makers [98, 99]. Hydrotrope assemblies are distinguishable from other solubilisers by their distinct structural properties and connection patterns [100]. Structure breakers and structure builders are further subdivided into "Kosmotropes" and "Chao-tropes" [101]. Using hydrotropic approaches, Soni and Khan et al. enhanced the solubility of the weakly soluble medicines norfloxacin and piroxicam [102, 103].

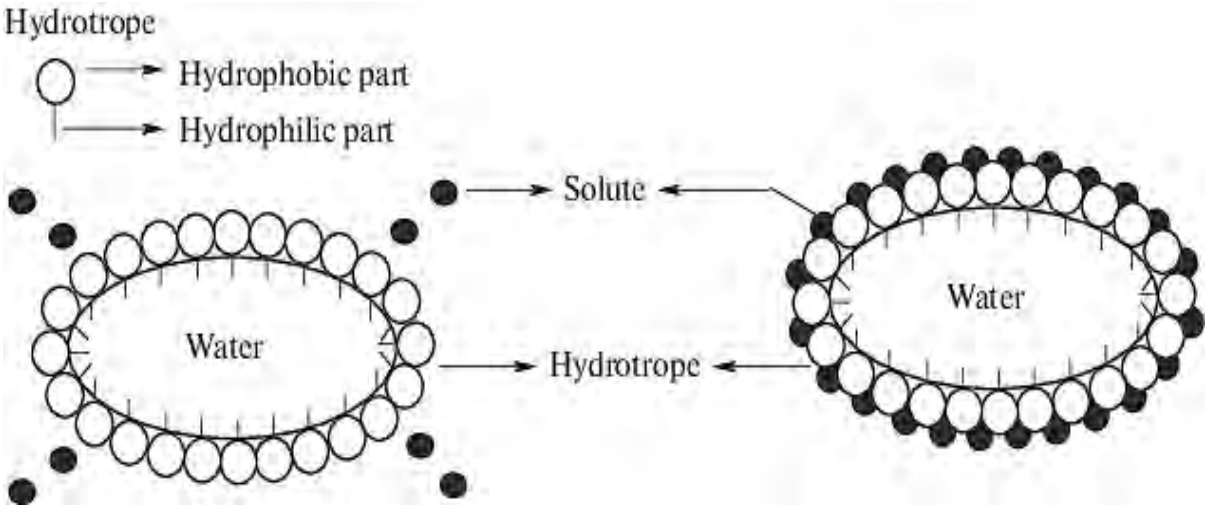

Figure 2.11. The solubilisation mechanism of Hydrotropes [104]

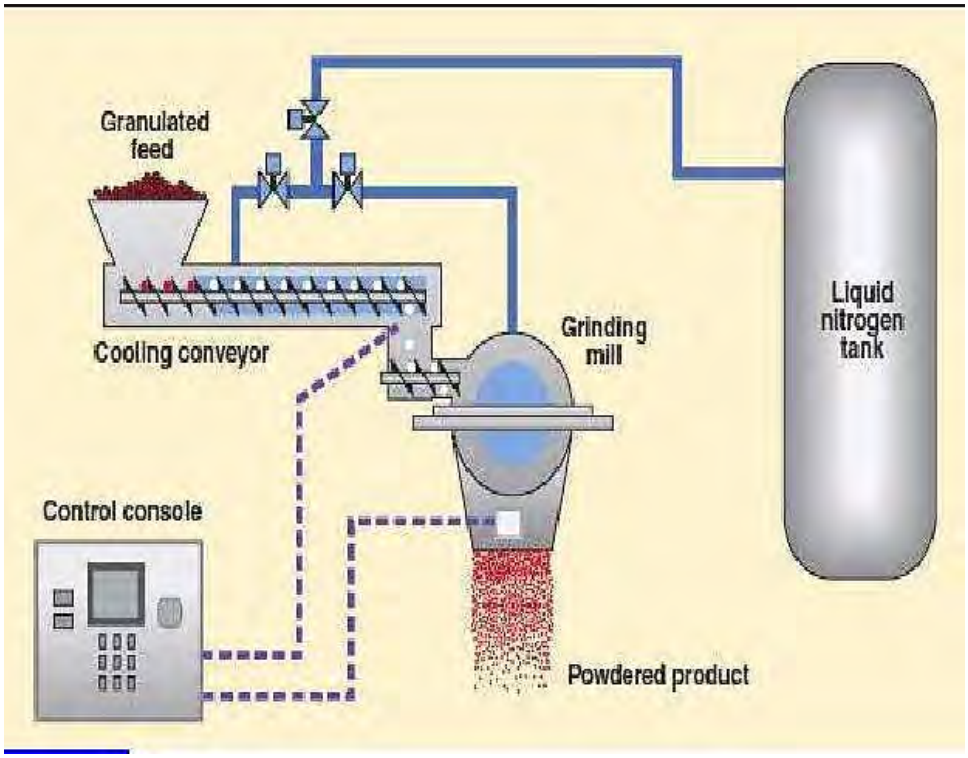

Figure 2.12. Grinding mechanism in cryogenic technique for solubility enhancement [105].

2.7.8 Cryogenic technique

Cryogenic techniques improve the dissolution rate and bioavailability of poorly soluble drugs by creating nanostructured amorphous particles with larger porosity at low temperatures [106]. It is a

novel size reduction technique and can be best explained by the nozzle location (above or under the liquid level), the type of injection device (rotary, capillary, pneumatic, or ultrasonic), and the nature/composition of the cryogenic liquids. The cryogenic mechanism is illustrated in **Figure 2.12**. Dry powders are usually obtained from this technique by spray freeze drying, vacuum freeze drying, atmospheric freeze drying, and lyophilisation. Spray freeze drying cryogenic technique includes spray freezing onto cryogenic fluids, spray freezing into cryogenic liquids, and spray freezing into vapour over liquids to produce reduced-size drug particles with enhanced wettability and solubility. Moinuddin et al. developed a co-amorphous system of atenolol and hydrochlorothiazide utilising cryogenic milling [107].

2.7.9 Crystal engineering technique

As stated earlier, the surface area of a drug available for solubility depends on the size of the particle and the degree of wetting by luminal fluids. The particle size, on the other hand, depends on the conditions of crystallisation and on comminution methods such as impact milling and fluid energy milling, which produce highly heterogeneous, charged cohesive particles, having the potential to cause problems downstream processing and overall product performance. The need to solve the pitfalls of conventional particle size reduction techniques led to the development of crystal engineering techniques which are useful for the controlled crystallisation of drugs to produce high-purity powders with a well-defined particle size distribution, crystal form (amorphous or crystalline), crystal habit, surface nature, and energy [108]. This is achieved by manipulating the crystallisation conditions, such as using different solvents, changing the stirring, or adding other components to the crystallising drug solution, thus producing crystals with different packing arrangements, known as polymorphs. Polymorphs for the same drug are sometimes observed to differ in their physicochemical properties, such as melting point, solubility, dissolution rate, and stability, making it pertinent to develop the most thermodynamically stable polymorph of a drug to assure reproducible bioavailability over its shelf-life

at different real-world storage conditions. Chloramphenicol palmitate suspensions, oxytetracycline, carbamazepine, mifepristone and dexamethasone are typical examples of polymorphs [109, 110].

Crystal engineering technique also involves the preparation of Hydrates and Solvates. This is achieved during the crystallisation process, where it is possible to trap solvents within the particle lattice. When the trapped solvent is water, the resultant crystals are Hydrate; when other solvents are trapped, the crystals are called solvates. Typical examples are glibenclamide (as pentanol and toluene solvates), theophylline (hydrate), and erythromycin (dihydrate) [2]. Recently this approach has been used to enhance the solubility of clinically useful drugs such as azilsartan [111], norfloxacin and sulphathiazole [112]. It is unsuitable to use solvates for pharmaceutical drugs as organic solvent residues may remain in the formulated preparation predisposing the end users to toxicity-related issues.

Other approaches by which crystal engineering technique is used to enhance solubility are pharmaceutical co-crystal formation [113], spherical crystallisation [114], and chemical modification of the drug to form salts [115]. Traditional crystallisation methods include sublimation, controlled crystallisation/precipitation from solutions, desolvation, evaporation, thermal treatment, or grinding/milling [116]. However, these are being replaced with novel crystallisation methods, such as SCF technologies and melt sonocrystallisation, to produce pharmaceuticals with desired dissolution rate and stability [117]. Based on these reports on crystal engineering, there is a need further to explore the use of crystal engineering in solubility enhancement.

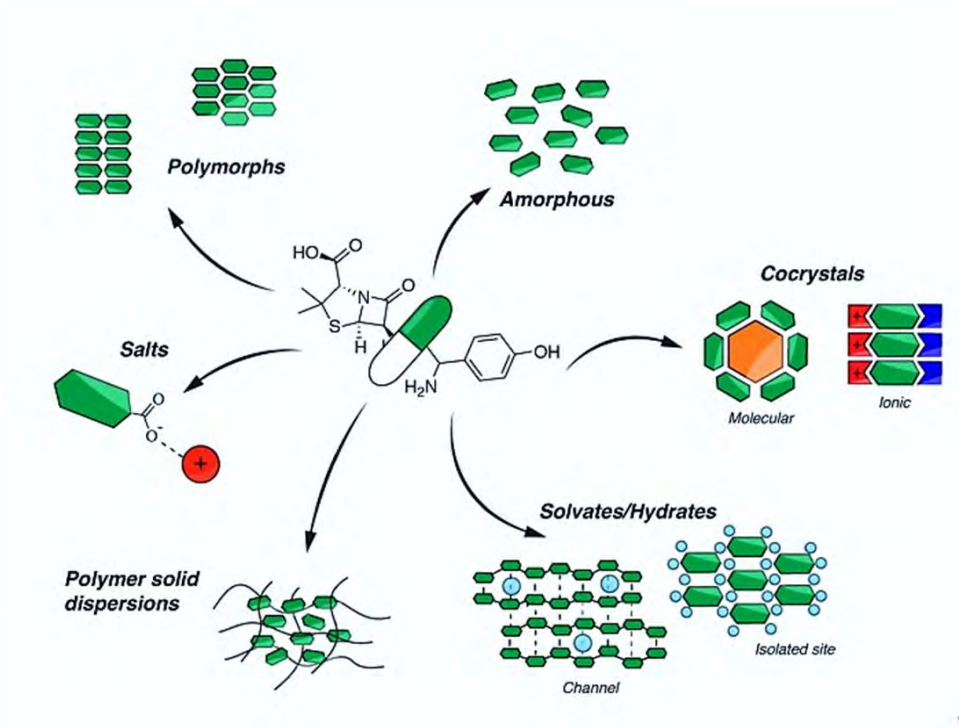

Figure 2.13. Summary of crystal engineering approaches for Solubility enhancement [118]

2.7.10 Nanoformulations for solubility enhancement

Formulations using nanotechnology are a well-studied strategy for improving the solubility of poorly water-soluble pharmaceuticals. This strategy entails diminishing the poorly water-soluble drug particles to the nanometer size range (0.1 nm to 1000 nm), culminating in kinetic and thermodynamic changes to the particles, along with new properties like increased solubility and applications in targeted delivery, controlled or prolonged delivery, and dry powders for inhalation. [119-121]. Several drug nanoformulations have been under clinical investigation or clinically approved in the past decade. Major research efforts have been developing superior nanoformulation technologies, new pharmaceutical materials, and quality control to improve product properties while lowering production costs [122]. Hence, new technological advances and unmet clinical needs are the key driving force for the research and development of nanoformulation strategies [123]. Pharmaceutical nanotechnology-based formulation systems, in the form of nanocrystals/nanosuspensions, nanoemulsions, lipid-based nanoparticles, nanogels and nanomatrices, are well-documented for enhancing the solubility of poorly

soluble drugs. Solubility enhancement by these nanosystems is by the Ostwald–Freundlich [124, 125] equation which simultaneously increases the dissolution rate due to the decreased size and increased surface area.

$$Log\frac{Cs}{C\infty} = \frac{2\sigma V}{2}.303RT\rho r \qquad \textbf{Equation 6}$$

with Cs as saturation solubility, c∞ is the solubility of large particles, σ = interfacial tension, V = atomic volume, R = gas constant, T = absolute temperature, ρ = density of the solid, and r = radius.

Nanoformulations have several advantages over macro-sized systems, such as remarkable thermodynamic stability, high capacity of solubilisation, relatively low viscosity, and capability of undergoing vigorous sterilisation techniques, and due to their hydrophilic nature, they can encapsulate hydrophobic drugs [126, 127].

Different techniques such as media milling, high-pressure homogenisation of aqueous and non-aqueous media, nanoprecipitation technique, polymerisation, and solvent evaporation have been used to develop different nanoformulations. However, every method has its characteristics, advantages, and disadvantages, leading to the creation of different nanosystems with varied sizes, shapes, encapsulations, solubility, and drug release characteristics. The commonly used methods for preparing nanoformulations for solubility enhancement are briefly summarised in **Table 2.14.**

2.7.10.1 Nanocrystals and Nanosuspensions

Nanocrystals are nano-sized drug solid particles made up of 100% API with no carrier material [128]. At the same time, nanosuspensions are coarse biphasic dispersion of finely dispersed insoluble drug particles as the internal phase, suspended in an aqueous vehicle for either oral, topical, parenteral or pulmonary administration [129]. Therefore, nanosuspension technology has emerged as a promising delivery system for hydrophobic drugs [130]. They typically contain drugs and stabilisers, such as hydrophilic polymers and surfactants dispersed in aqueous or non-aqueous media [131, 132].

Nanosuspensions have several advantages, including minimum additives, high drug-loading capability, and scale-up capability [133]. Sharma et al. formulated paclitaxel nanocrystals stabilised by either sodium poly (styrene sulfonate), glycol chitosan, Tween 80, or sodium alginate [131]. Similarly, Soroushnia and Rahim et al. enhanced the solubility and bioavailability of poorly soluble midazolam and glimepiride by the nanosuspension technique [134, 135]. Techniques involving top-down approaches, such as high-pressure homogenisation and media milling or bottom-up approach, such as precipitation, have been used for their preparations.

Nanosuspensions are an outstanding and commercially viable solubility and bioavailability enhancement technique. However, despite their vast applications, the stabilisation capability of the nanosuspensions is still compromised as conserving particle size distribution of substances during prolonged storage in liquid results in challenges such as chemical reactivity, chemical instability, and drug leakage [136].

2.7.10.2 Lipid-based nanosystems

Solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) are primarily two lipid-based nanosystems that attracted much interest in enhancing hydrophobic drug solubility. They offer various advantages, such as easy scale-up, biocompatibility, increased lymphatic transport, and reduced first-pass metabolism. SLNs are the first generation of lipid-based nanoparticles with a mean diameter between 50 and 1000 nm, consisting of an aqueous lipid matrix dispersion, solid at room and body temperature, and stabilised with surfactants. However, their limited drug-loading efficiency due to the partitioning effect, the higher water content of the dispersions and drug expulsion issues during storage are major drawbacks to their use [137].

On the other hand, NLCs are new-generation SLNs made up of a combination of solid and liquid lipids, which provide prolonged drug release, a higher drug-loading capacity, and minimum drug expulsion during storage. The liquid oils in the solid lipid matrix create a less-ordered lipid matrix allowing the

lodging of more cargo molecules [138]. SLNs and NLCs are normally prepared by high micro-emulsification techniques, pressure homogenisation, and displacement [139]. Some studies investigated the potential of SLN and NLC formulations to enhance the solubility of poorly soluble drugs. There have been several reports in the literature on improving the solubility of different poorly soluble drugs such as curcumin, efavirenz, miconazole and glibenclamide through the use of solid lipid nanoparticles [140-143]. Similarly, NLCs have been employed to enhance the solubility of drugs such as olmesartan, medoxomil, fenofibrate, quercetin, and raloxifene [144-147].

Another type of lipid-based nanosystem that has been used for solubility enhancement is Liposomes. Liposomes are spherical vesicles composed of single or multiple phospholipid bilayers enveloping aqueous units that closely resemble the structure of a cell membrane. They range in size from 30 nm to many micrometres [148]. They are highly biocompatible, easily up-scaled to the industry, can entrap both hydrophobic and hydrophilic compounds, resist decomposition of the entrapped molecules, and, when required, release them at the target sites [149]. Active ingredients are incorporated by passive loading, which comprises the integration of the active agent before/or during the manufacturing process using mechanical dispersion, detergent removal or solvent dispersion methods and by active loading involving the production of liposomes containing a transmembrane gradient, having different outside and inside aqueous phases [150]. Researchers are currently using liposomal encapsulation technology for the targeted delivery of bioactive compounds [151]. Conventional liposomes were evaluated for oral bioavailability improvement of a wide range of hydrophobic drugs such as silymarin [152], resveratrol [153] and tacrolimus [154]. Also, Tsai et al. formulated elastic liposomes of Naringenin-a naturally occurring flavonoid, by thin film hydration method and found that cholesterol and Tween 80 had a major impact on the physicochemical properties and permeation capability of Naringenin from elastic liposomes [155]. All the studies, as mentioned earlier, showed augmented solubility and bioavailability by incorporating the drug into liposomes.

2.7.10.3 Nanoemulsions and self-nano emulsifying drug delivery systems (SNEDDSs)

Nanoemulsions are thermodynamically stable translucent and clear dispersions of oil and water stabilised by a surfactant and co-surfactant, having a droplet size of approximately less than 100 nm; also described as ultrafine emulsions, mini emulsions, or submicron emulsions [156]. Nanoemulsions are divided into three (3) based on the composition; oil in water (water is the continuous phase), water in oil (oil is the continuous phase) and bi-continuous (both oil and water are inter dispersed within the system) [157]. They have been reported to enhance the absorption rate of drugs, solubilise lipophilic drugs and improve the bioavailability of drugs. SNEDDSs are mixtures of drugs, oil, surfactant, and co-surfactant that spontaneously form an O/W nanoemulsion upon mixing with water. Lately, intense research has been fixated on nanoemulsions and SNEDDSs because of their unique structure and advantages, such as biocompatible composition, high drug loading capacity, increasing solubility and dissolution rate, protecting drug molecules from enzymatic degradation, ease of preparation and the possibility of large-scale production [158, 159]. SNEDDSs can offer additional advantages, for instance, improved storage stability, possible encapsulation into soft/hard gelatine capsules, and improved palatability [160, 161]. High-pressure homogenisation and micro fluidisation are commonly used for their preparation [162].

Nanoemulsions have been explored to increase the solubility, oral absorption, and anti-malaria activity of artemether, a medication with poor water solubility and GI stability [159]. Another method for incorporating the drug-phospholipid combination into the nanoemulsion system was studied for curcumin [163] and dabigatran etexilate [164]. Similarly, Seo et al. used a ternary phase diagram to describe docetaxel-loaded SNEDDSs consisting of Labrasol®, CapryolTM 90, and Transcutol® HP. Mahmoud et al. described cilostazol SNEDDSs made up of CapryolTM 90, Cremophor® EL, and Transcutol® HP (19.8:30.5:49.7 wt. ratio). The technology enhanced the drug's solubility in water by up to 2000 times. Elgart et al. also investigated the mechanism of SNEDDSs for two BCS class II

chemicals (amiodarone and talinolol) [165], reporting better solubility and permeability relative to plain drugs.

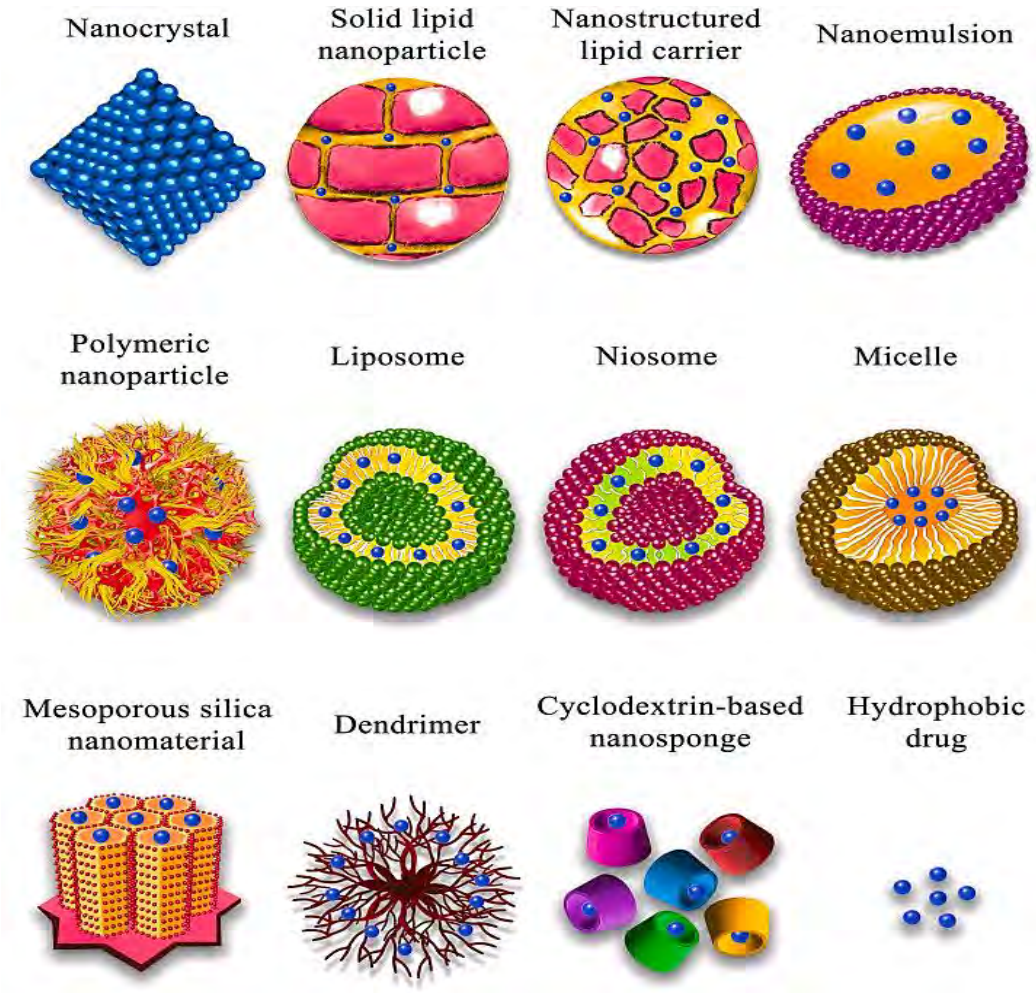

Figure 2.14. Schematic representation of various nanostructures designed for solubility and bioavailability enhancement [161]

2.7.10.4 Nanogels/nanomatrices

Nanogels/nanomatrices, often regarded as next-generation drug delivery systems, are the nano-sized form of hydrogels. They are highly cross-linked nano-sized hydrogel systems that are either monomers or copolymers, which can be either ionic or non-ionic [166, 167]. They are also considered three-dimensional hydrophilic networks that absorb water or physiological fluid in a large amount without undergoing dissolution [168]. Nanogels are very favourable in drug delivery applications due to their

tenable size, biocompatibility, stability, swelling, ease of preparation, hydrophilicity, high drug-loading capacity, and stimuli responsiveness (pH, temperature, light, biological agent) [169]. In addition, they have prolonged serum half-life due to their size and ability to evade renal clearance. Both hydrophobic and hydrophilic drugs can be incorporated into nanogels. Nanogels have applications in several fields, such as diagnostics, sensing, and bioengineering, but their greatest impact has been drug delivery [170, 171]. However, they have disadvantages, such as toxicity traceable to the residual monomer or surfactant in the formulation, most particles being in the micrometre range, and difficult scale-up due to the mean size and weight. The development of nanomatrices has solved these drawbacks.

A typical example of nanomatrices is the dendrimers. Dendrimers are uniformly-dispersed, hyperbranched and three-dimensional nanometric macromolecules in the size range of 1–10 nm. Modifiable surface functionality, large hydrophobic cavity, well-defined molecular weight, high drug-loading ability for host-guest entrapment of bioactive compounds, penetration ability across cell membranes and biological barriers and reproducible pharmacokinetic behaviours are unique advantages of dendrimers for controlled and sustained drug delivery. A dendrimer consists of four main domains: i) a central core, ii) interior layers composed of repeating units attached to the core (generation, G), iii) exterior surface groups, and iv) void spaces [172]. Poly (amidoamine) (PAMAM) dendrimers are the most investigated members of dendrimers in drug delivery systems. Precipitation polymerisation, suspension polymerisation, free radical polymerisation, and emulsion polymerisation have been described for their preparation [170]. Xing et al. have fabricated poly acrylic acid (PAA) poly (N-isopropyl acrylamide) (PNIPAM) based pH/temperature, dual stimuli-responsive hollow nanogels which possessed distinct acid triggered drug release behaviour suitable for stomach-specific drug delivery system [173]. In the same way, the solubility of drugs fenofibrate, olanzapine, apixaban and simvastatin has been enhanced using nanomatrices [174-177].

The mechanism of drug release from nanogels/nanomatrices depends on the monomers and polymers for preparation and the drug-loading method into the nanogels. The matrix size has a distinct role in sustaining drug release, which can either be stimuli mediated or through a simple diffusion mechanism. Drug release mechanisms from the nanogels include erosion of the nanogel matrix due to swelling of nanogels by absorbing water/body fluid, simple diffusion, changes in pH of the surrounding environment, release by displacement through counter ions present in the external environment, and triggered release due to external stimuli such as magnetic field, light, and pressure [103]. Drug-loading into the nanogels is commonly done by covalent interaction or physical entrapment. The physically entrapped drug is released immediately upon swelling of nanogels, while in the case of drug-loaded through covalent/non-covalent interactions, drug release from the nanogel network occurs due to physical and chemical stimuli such as temperature alterations, pH, magnetic field, pressure, ionic strength, electric field and molecular recognition [178].

Kifayat et al. enhanced the solubility of a poorly aqueous soluble drug, olanzapine, and meloxicam, by 38 and 36 folds by encapsulating them in nanogels. The drug's hydrophilic moieties were exposed to an external dissolution environment by the nanogels, while its hydrophobic moieties were masked [179, 180]. Similarly, Asghar and Badshah et al. prepared bi-polymeric nanomatrices through cross-linking polymerisation to enhance the solubility of poorly soluble drugs acyclovir and chlorthalidone, respectively. The polymer in the synthesis offered the aqueous part of the drug to the solubilising solvent, while the lipophilic part was inhibited in the internal part [181, 182].

2.7.10.5 Polymeric nanoparticles and micelle

Biocompatible polymers have been widely used in pharmaceutical research as additives in pharmaceutical formulations and, more recently, nanomedicines to improve the therapeutic effects of powerful pharmaceuticals [183]. Nanosystems formed in an aqueous solution by amphiphilic block copolymers were regarded carriers for hydrophobic bioactive chemicals that were either covalently

connected to polymer chains [184] or non-covalently integrated into the micelles [185, 186]. Because of their desirable characteristics, such as controlled release potential, better stability, minimized cargo degradation, increased cargo solubility, and enhanced bioavailability, the use of block copolymers in the structure of polymeric micelles as therapeutic agents has been widely studied over time [187, 188]. Again, structural factors like the hydrophilic shell help to minimize unexpected drug loss from serum constituents and opsonisation by the complement cascade, resulting in fast drug clearance from blood circulation [189]. The kind and nature of the polymer employed in the formulation and the loaded drug significantly impact these properties. Chitosan, alginate, and gelatine from natural sources, as well as synthetic polymers such as poly (lactic-co-glycolic acid) (PLGA), polylactic acid (PLA), poly (-caprolactone) (PCL), and Polyglycolide, have been thoroughly studied for solubility enhancement [189, 190]. These polymers have been used alone or in combination to improve solubility, transport over the blood-brain barrier (BBB), and target cancer delivery [191]. Polymeric nanoparticles were coated with hydrophilic polymers such as polysorbates [193], PEG [194], and chitosan [195] in various investigations. Polymeric nanosystems with central hydrophobic parts composed of hydrophobic moieties such as poly (ethylene-imine) (PEI), poly (propylene oxide) (PPO), poly (-caprolactone) (PCL), 1, 2-distearoyl-sn-glycero-3-phosphatidyl ethanolamine (DSPE), and outer hydrophilic shell (typically poly (ethylene oxide) (PEO)) can self-assemble in an aqueous environment. Lu et al. created star-block copolymers with a branching PEI core and many grafted poly (-benzyloxy carbonyl-L-lysine) chains as a nanocarrier for indomethacin delivery [196].

Poly D, L lactide-co-glycolide (PLGA) is the most often utilised polymer in creating new carrier systems; it provides excellent drug encapsulation, prolonged drug release characteristics, and biocompatibility [197]. Maity et al. [198] employed PLGA to create NAR-PLGA nanoparticles with a 70% encapsulation effectiveness and a particle size of 129 nm, resulting in the delayed and sustained release. They have also been studied to increase the solubility and bioavailability of a variety of drugs, including paclitaxel [199], resveratrol [200], coenzyme Q10 [201], curcumin [202], and tamoxifen

[203]. Khalil et al., for example, investigated the ability of PLGA and PLGA-PEG nanoparticles (with particle sizes less than 200 nm) to increase the oral administration of curcumin, a BCS class IV compound. Both formulations demonstrated sustained curcumin release (20% in 24 h); however, curcumin release from the PLGA nanoparticles was slower than from the PLGA-PEG nanoparticles, and when compared to the curcumin suspension, the PLGA and PLGA-PEG nanoparticles increased curcumin oral bioavailability by 15.7 and 55.5-fold, respectively. The PLGA and PLGA-PEG nanoparticles revealed 3.5- and 5.4-fold increases in $t_{1/2}$ and 2.9- and 7.3-fold increases in concentration [194]. Along the same lines, fenofibrate nanoparticle formulations comprised of hydroxypropyl-cyclodextrin (HP-CD), polyvinylpyrrolidone (PVP), or gelatine have been observed. These polymers were chosen based on fenofibrate saturation solubility in various polymers' 1% (w/v) solutions. On the other hand, the gelatine formulation had the maximum solubility and sustained fenofibrate release [204]. Other polymers, including poly (n-butyl cyanoacrylate) (PBCA), Eudragit®-E100 poly (methyl vinyl ether-co-maleic anhydride), and (PVM/MA)-graft-HP-CD, have been used to encapsulate the hydrophobic medicines quercetin [193], efavirenz [205], and tacrolimus [206]. Polymeric micelles have also enhanced the solubility of nevirapine [207], paclitaxel [208], and apigenin [209].

Table 3: Summary of Nanoformulation strategies for solubility enhancement, their method of preparation and reported solubility

Type of Nanoformulation	Techniques for preparation	Examples	Reported Solubility (mg/mL)	References
Nanocrystals/ Nanosuspensions	• High-pressure homogenisation • CO_2-assisted in situ nanoamorphisation • Antisolvent precipitation • Spray drying after milling. • Microprecipitation high-pressure homogenisation • Magnetic stirring milling,	Paclitaxel Ritonavir Curcumin Tacrolimus Atovaquone	0.006 0.001 0.006 0.004 0.0008	[131] [210] [211] [212] [132]
Solid Lipid nanoparticles (SLNs) and Nanostructured lipid carriers (NLCs)	• Emulsification and low-temperature solidification, • Solid dispersion effervescent • Emulsification-solvent evaporation • High-shear homogenisation coupled with high-pressure homogenisation	Raloxifene Felodipine Rosuvastatin Resveratrol Rifampicin	0.0006 0.02 0.09 0.07 1.4	[213] [214] [215] [216] [217]

	• Evaporation followed by low-temperature solidification • Sonication			
Liposomes	• Thin film hydration, • Solvent injection • Solution-enhanced dispersion by supercritical fluids • Film deposition on the carrier	Curcumin Coenzyme Q10 Sorafenib	0.006 0.0002 0.002	[218] [219] [220]
Nanoemulsions and self-nano emulsifying drug delivery systems (SNEDDSs)	• Aqueous Titration • High-pressure homogenisation, Sonication • Sonication-absorbing onto the solid carrier	Paclitaxel/Curcumin (SNEDDS) Rebamipide Carvidilol Curcumin Arteether	Approx. 0.006 0.008 0.0006 0.006 0.28	[221] [222] [223] [158] [224]

Nanogels/Nanomatrices **Dendrimers**	• Stirring-solvent evaporation • Sonication-mixing	Simvastatin Albendazole Camptothecin Vardenafil	0.01 0.02 0.51 0.1	[225] [226] [227] [228]
Polymeric Nanoparticles/nanogels	• Nanoprecipitation-evaporation • Sonication-dialysis • Thin film hydration-sonication • Emulsification (with sonication)-solvent evaporation • Spray-drying • Ionic gelation	Quercetin Paclitaxel Nifedipine Amphotericin B Efavirenz Fenofibrate	0.06 0.006 0.02 0.75 0.009 0.25 (at 25 ℃)	[193] [229, 230] [193, 231] [232] [205] [204]

2.8 An Overview of Pyrazolone-based derivatives.

Most novel chemical entities (NCEs) in use or being investigated globally are small-molecule medicines and drug prospects [233]. These molecules, which play important roles in nature, medicines, and technology, are made possible partly by the skill and inventiveness of medicinal and process scientists who synthesise the compounds using an organic chemistry area. As a result, organic synthesis has contributed to the development of polymers, plastics, agriculture, vitamins, cosmetics, pharmaceuticals, diagnostics, and other high-tech sectors. In recent decades, the pharmaceutical industry has focused on identifying new molecules with desirable qualities, concentrating on heterocyclic compounds. Heterocyclic compounds are the biggest class of organic chemicals and have enormous biological and economic value. Pyrazolone [234] is an example of a heterocyclic whose derivatives have found widespread use as both synthetic and natural remedies.

Pyrazolone core is one of the most studied antecedents among varied fused heterocycles, with functions in various pathophysiological circumstances [235]. They are five-membered heterocyclic compounds with one ketonic group and two neighbouring nitrogen atoms and are thought to be pyrazole derivatives with an extra Carbonyl (C=O) group [236]. The discovery of pyrazolone derivatives is considered the pinnacle of medicinal chemistry; various antipyrine analogues have been studied, and precursor designing approaches have developed from structural modification to fragment-based drug design and high-throughput screening at the same time [235]. These compounds can make dyes, pigments, insecticides, and chelating agents. Due to the planar shape of the aromatic heterocycle and the almost universal range of pharmacological activity it exhibits, the pyrazole nucleus has caught the interest of numerous researchers over the years, making it one of the most thoroughly studied pharmacophores in the world [238]. Pyrazolone can substitute many chemical frameworks with similar chemical structures and biological profiles, providing important usefulness for drug design and development. They are also being developed for other pathological screenings, such as analgesic, anti-inflammatory, anticancer, antibacterial, antifungal, antioxidant, antidiabetic, antiviral,

antiproliferative, antitubercular, CNS, and cardiovascular action [239]. Several pyrazole nucleus-containing pharmaceutical medicines are now on the market and in clinical trials. Tepoxalin (NSAIDs), celecoxib (selective COX-2 inhibitor), betazole (analgesic), crizotinib (anticancer), surinaban (treatment for nicotine addiction), difenamizole (anti-obesity), mepiprazole (tranquillizer), CDPPB (antipsychotic), and fezolamide (H2-receptor agonist and antidepressant agent) and more biologically active derivatives have been identified. For example, N-formyl pyrazolines have anticancer and antioxidant characteristics [240], while pyrazolo-pyrimidines have cytotoxic and radical scavenging capabilities [241]. Certain curcumin pyrazole analogues have been shown to reduce oxidative stress-induced PC12 neuronal damage [242], and pyrazole-thiazoles have antibacterial and antioxidant properties [243]. Similarly, Chen, Masih, and colleagues discovered that 3,5-dioxopyrazolidine and 4-(4-fluorocyclohexyl)-piperazine-1-carbonyl)-2,5-dimethyl-2,4-dihydro-3H-pyrazole-3-one might be used to treat SARS-CoV-II infection [244, 245]. Obakachi et al. reported the activity of various new pyrazolone-based derivatives against SARS-CoV-2 and CDK-2 cells using computational studies [246, 247]. Along the same lines, Alfei and Sun et al. reported a possible anticancer agent in 2-(4-Bromo-3,5-diphenyl-pyrazol-1-yl)-ethanol pyrazole derivative and 6-amino-4-(2-hydroxyphenyl)-3-methyl-1,4-dihydropyrano [2,3-c] pyrazole-5-carbonitrile [248, 249].

Despite adequate study on pyrazolones and their biological functions, low water solubility and stability are key limits to their use as pharmaceutical drugs [250]. As a result, we hypothesised in this work that employing the nanoformulation technique to improve the solubility of two new pyrazolone compounds would allow them to be used as medicinal products.

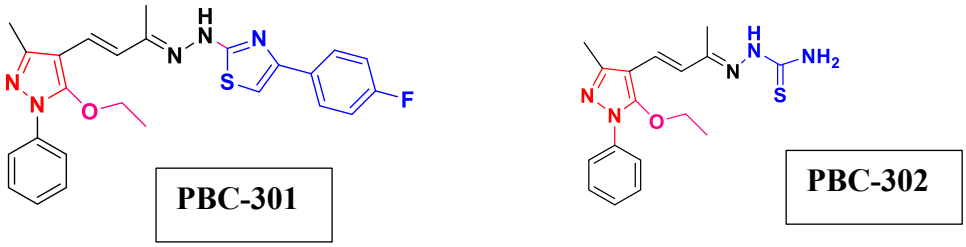

Figure 2.15. Schematic representation of the Novel Pyrazolone-based compounds

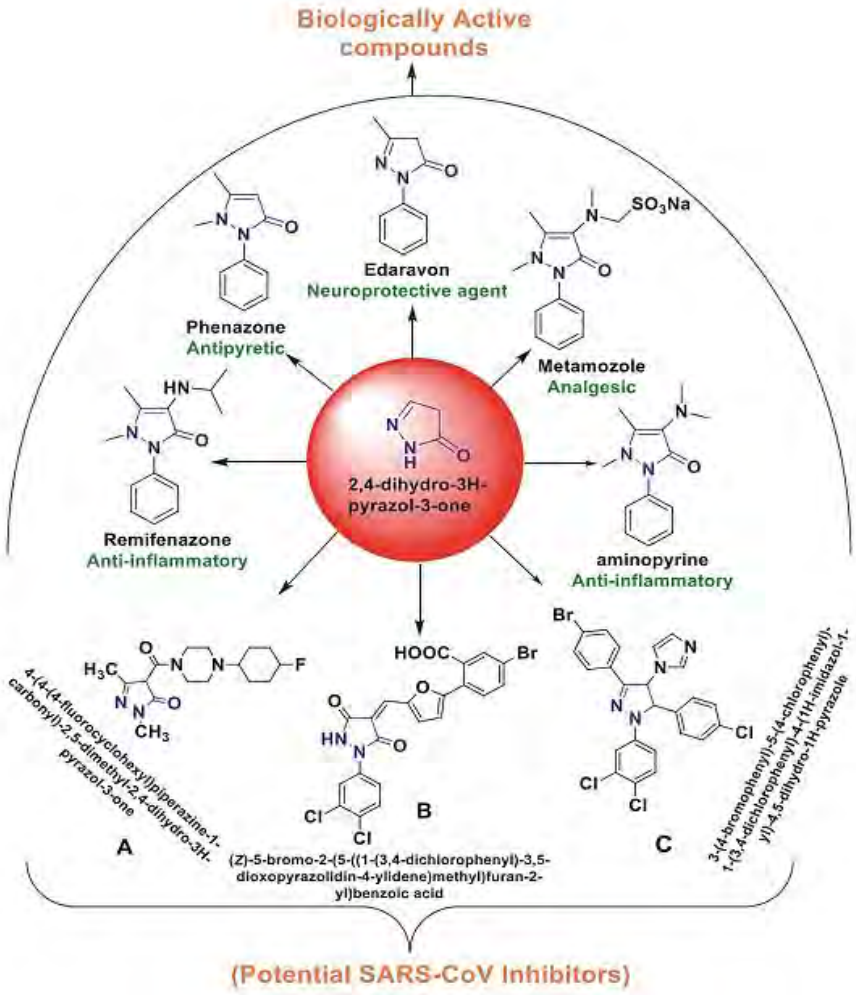

Figure 2.16. Examples of biologically active pyrazolone-based compounds [246]

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CHAPTER 3: EXPERIMENTAL PAPER 1

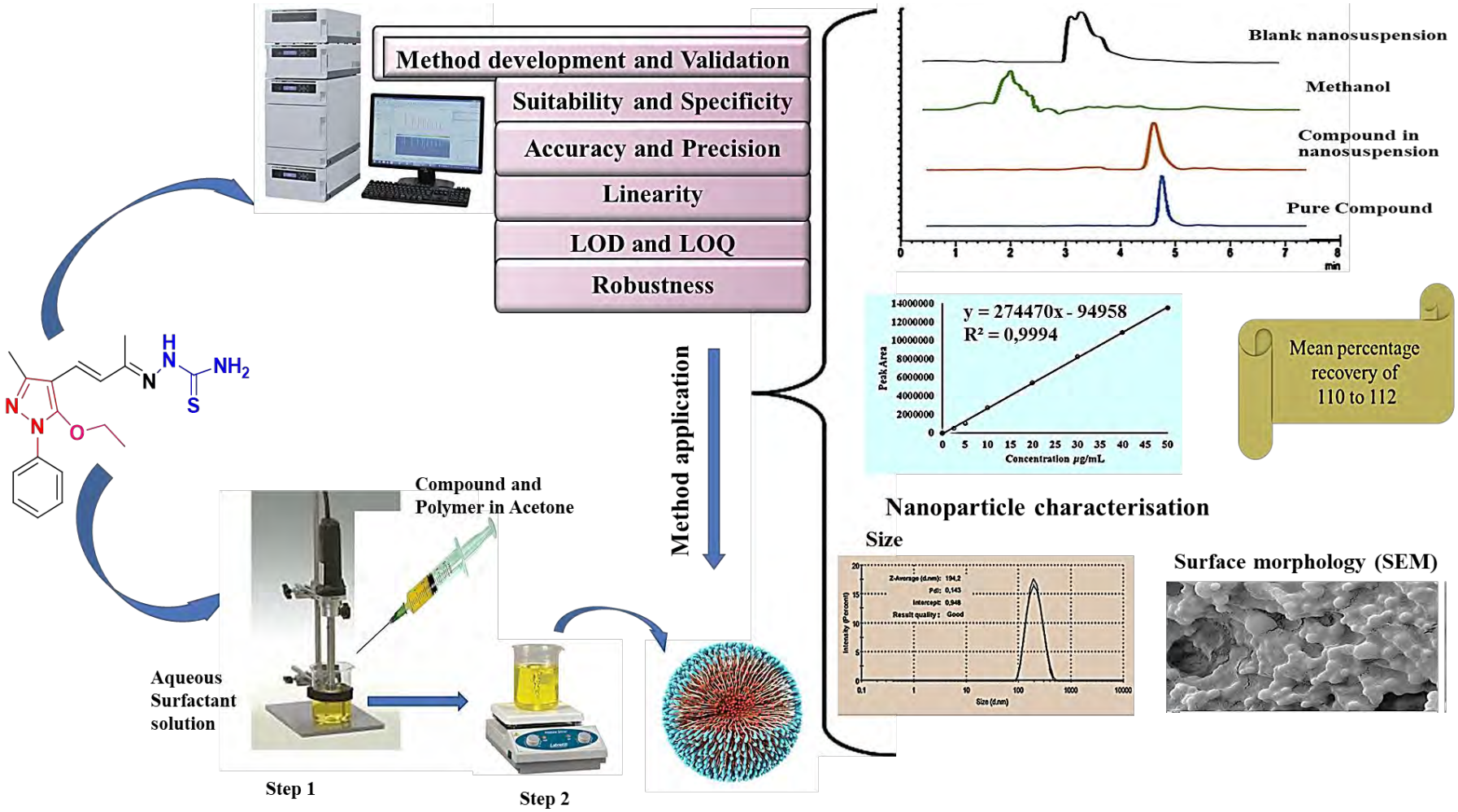
3.1 Introduction

This chapter addressed objective 1 of the study and is a first-authored research paper submitted to the Journal of Pharmaceutical and Biomedical Analysis (3.571 impact factor) and presented here in the required journal format; it describes the development and validation of a simple RP HPLC-PDA method for the detection and quantification of a novel Pyrazolone-Based compound in nanosuspensions.

Author contribution

Igbokwe Nkeiruka- Conceptualisation, Data curation, investigation, Formal analysis, Validation and Writing- Original draft preparation; ***Eman A. Ismail-*** Conceptualisation, Methodology and Writing-Review and editing; ***Vincent A. Obakachi-*** Design and synthesis of the PBC-302; ***Rajshekhar Karpoormath -*** Co-Supervision and ***Mbuso Faya-*** Supervision; writing-review and editing.

3.2 Graphical Abstract



3.3 Submitted Manuscript

Development and Validation of Simple RP HPLC-PDA Method for Detection and Quantification of a Novel Pyrazolone-Based Compound in Nanosuspensions

Nkeiruka N. Igbokwe[1]; Eman A. Ismail[1,2]; Vincent A. Obakachi[1]; Rajshekhar Karpoormath[1]; Mbuso Faya[1]**

[1]Discipline of Pharmaceutical Sciences, College of Health Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban, South Africa.

[2]Department of Pharmaceutics, Faculty of Pharmacy, University of Gezira, Wad Medani, Sudan.

*Corresponding author.

Email address: igbokwenk@gmail.com/ FayaA@ukzn.ac.za

3.4 ABSTRACT

A specific, accurate, and precise reverse-phase high-performance liquid chromatography method was developed and validated to detect and quantify a novel pyrazolone-based derivative in nanosuspensions. Efficient separation and quantification were conducted using Shim-pack GIST C18 (5 μm 150 × 4.6 mm) column, maintained at 25 ℃ with isocratic elution using Acetonitrile and acidified water (0.1% Trifluoracetic acid) (75:25 v/v) at 0.5 mL/min flow rate. The injection volume was 20 μL, and eluents were detected at 333 nm at a retention time of 4.82 mins. Method validation was done following ICH guidelines. Results demonstrated that the developed method is specific, precise, and accurate within the recommended limits. The method showed good linearity with a 0.9994 correlation coefficient over a concentration range of 2.5-50 μg/ml. The method efficiently detected and quantified the novel pyrazolone compound in the nanosuspension. The developed method can be efficiently applied to analyse the compound of interest in pharmaceutical nanoformulations and raw material analysis.

Keywords: Pyrazolone-based, Detection, Quantification, Validation, Method development, Nanosuspension, RP-HP

1.0 Introduction

Small molecule drugs and drug candidates represent the majority of new chemical entities (NCEs) in use or under study worldwide [1]. These molecules are made available in part by the skill and resourcefulness of medicinal and process scientists who synthesise the compounds via a branch of chemistry known as organic synthesis. This represents a unique branch of chemical synthesis involved with the intentional creation of organic compounds, performing vital functions in nature, drugs and technology [2]. For instance, organic synthesis significantly impacts the advancements related to functional polymers, plastics, agriculture, vitamins, cosmetics, pharmaceuticals, diagnostics, and other high-tech industries. It has also permitted and decisively facilitated biology and medicine by providing molecules that serve as biological tools and drugs and, as such, able to address and meet the global challenges identified in the United Nations Sustainable Development Goals (UN SDGs), particularly in Good health and Well-being, zero hunger, Responsible-consumption and Production [3].

In the last two decades, a significant focus has been directed toward discovering novel compounds of desired properties, especially in the pharmaceutical industry. Among these are the heterocyclic compounds representing the largest classical organic chemistry division and are of immense biological and industrial importance. Heterocyclic compounds are cyclic organic compounds with at least one hetero atom in their ring system [4]. Owing to their structural similarities to numerous biological molecules, including vitamins, haemoglobin, RNA, DNA, and hormones, which possess a heterocyclic ring system as their primary framework [5], organic heterocyclic compounds gained momentum for their biological activities and their potential for the treatment of various diseases. They have also found use as additives and modifiers in cosmetics, photography, information storage, plastic industries, agriculture, and pharmacy. A typical example of a heterocyclic compound whose derivatives have found massive applications as both synthetic and natural medicines is pyrazolone [6].

Pyrazolone core is one of the most explored precursors among diverse fused heterocycles, possessing a wide range of biological activities [7]. 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 [8]. Pyrazolone derivatives are a class of heterocyclic compounds found in many drugs and synthetic products (Al-Mutairi et al., 2010); moreover, they have been considered valuable pharmaceutical intermediates. The development of pyrazolone derivatives is regarded as the epitome of medicinal chemistry. Several analogues 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 [7]. Such molecules have the potential to substitute a significant percentage of chemical frameworks with an analogous chemical structure and similar biological profiles, thereby offering significant functionality for drug design and development.

Several research activities have emphasised that pyrazole compounds and their analogues have gained significant momentum in chemical, medicinal, and pharmaceutical research as a structural framework in various drugs [9]. A new pyrazolone compound, edaravone, also known as MCI-186, has been developed as a medical drug for brain ischemia and has also been reported to be beneficial in myocardial ischemia [10]. In addition, 4-(4-(4-fluorocyclohexyl) piperazine-1-carbonyl)-2,5-dimethyl-2,4-dihydro-3H-pyrazole-3-one has been suggested by Masih et al., as a potential lead for therapeutic application to control the inflammatory response in SARS-CoV-II infection [11]. Along the same line, 3,5-dioxopyrazolidine has been reported as a SARS-CoV 3CLpro inhibitor [12]. Interestingly, by using high throughput screening, some pyrazolines, particularly those displaying a 1,3,5-triaryl substitution pattern, have been identified to show activities against SARS-CoV 3CLpro, CoV-229E 3CLpro, CVB3 3Cpro, EV71 3Cpro, and RV14 3Cpro [9]. Recently, stimulated by this study during the outbreak of coronavirus (SARS-CoV-II) disease, Obakachi et al. designed, synthesised and investigated novel pyrazolone-based compounds as inhibitors of SARS-CoV 2 viral entry into the host cells [13]. The inhibitory abilities of the compounds against both spike protein and hACE2 were evaluated using computational techniques. Results of the binding free energy revealed

that several compounds from the series exhibited higher and better binding high affinity to hACE-2 and SARS-CoV-2 Sgp, respectively, compared to the standard drugs MLN-4760 and cefoperazone (CFZ), thus suggesting the pyrazolone-based compounds to be potent blockers of the viral entry into the host cells. In addition to this finding, Obakachi and his co-workers reported that these compounds with high binding energies were poorly soluble, as observed from the ADME simulation studies making their biological applications very limited.

It is previously documented that the number of drug candidates with low solubility has increased and that poor aqueous solubility remains a typical problem for drug candidates in today's pipeline of pharmaceutical development [14-16]. An estimated 75–90% of the drug products in development and 40% of the marketed products are poorly water-soluble [17, 18]. Aqueous solubility is an important property that affects the ease of manipulation during manufacture and testing in the laboratory, the potential for drug absorption after oral administration, and the ability to administer the drug parenterally. Furthermore, a drug molecule must be sufficiently in solution to quantitate meaningful activities in in-vitro assays and achieve adequate absorption for safety and efficacy evaluation in preclinical models and clinical trials. Thus, solubility evaluation has been integrated into the discovery and development process and must be continuously monitored and refined as compounds progress [19].

Along with various conventional approaches introduced for enhancing aqueous solubility, the application of nanotechnology is considered the most convenient. Encapsulation of poorly soluble compounds in appropriate nanoparticles (NP) in the range of 10-1000 nm has been reported to enhance the solubility and bioavailability of the loaded compounds [20]. This was attributed to the small size and increased surface area to volume ratio with better interaction with the surrounding medium [21]. In this approach, a novel, poorly water-soluble pyrazolone-derived compound was formulated as a nanosuspension to improve water solubility and biological activity.

Polylactic-co-glycolic acid- (PLGA) is one of the well-known approved biodegradable and biocompatible polymers widely used as a carrier for protein, vaccine, gene, and peptide delivery [22]. Hydrophobic drugs/bioactive compounds, including natural phytochemicals like curcumin, crocetin coumarin, synthetic bioactive compounds such as novel pyrano [2,3-c] pyrazole heterocyclic [23], and many others have been successfully encapsulated in PLGA nanoparticles to improve solubility and biological applications.

Apart from the physicochemical characterisation of drug-loaded nanoparticles, the determination of drug encapsulation and the drug-loading content rate is considered an essential parameter that could impact the potential of nanoparticles in the prevention and treatment of diseases [24]. Some high-performance liquid chromatographic (HPLC) and spectrophotometric methods have been reported to assay drugs in nanoformulations [25-27]. Driven by this fact, as shown in Figure 1, we proposed, developed and validated a simple RP-HPLC method for the quantification of a pyrazolone-based compound (E-2-E-4-(5-ethoxy-3-methyl-1-phenyl-1H-pyrazole-4-yl)-but-3-en-2-ylidene) hydrazine-1-carbothioamide) as shown in Figure 2 entrapped in PLGA nanosuspensions. The method developed and validated here was used to estimate the novel compound's entrapment efficiency and drug-loading content in the formulated nanosuspensions.

The unsuitability of available quantification methods for similar compounds and the variations in the instrument parameters strongly demanded this work. Also, to the best of our insight, no HPLC-PDA method has been reported to quantitate this novel pyrazolone-based compound, coupled with a need to establish technical specification guidelines to assure the compound’s identity, quality, purity, and strength in its nanoformulations.

The developed method was validated according to the recommendations of the ICH guidelines and updated international convention. The described method’s linearity of response, precision, accuracy, suitability, specificity, and robustness was assessed and validated.

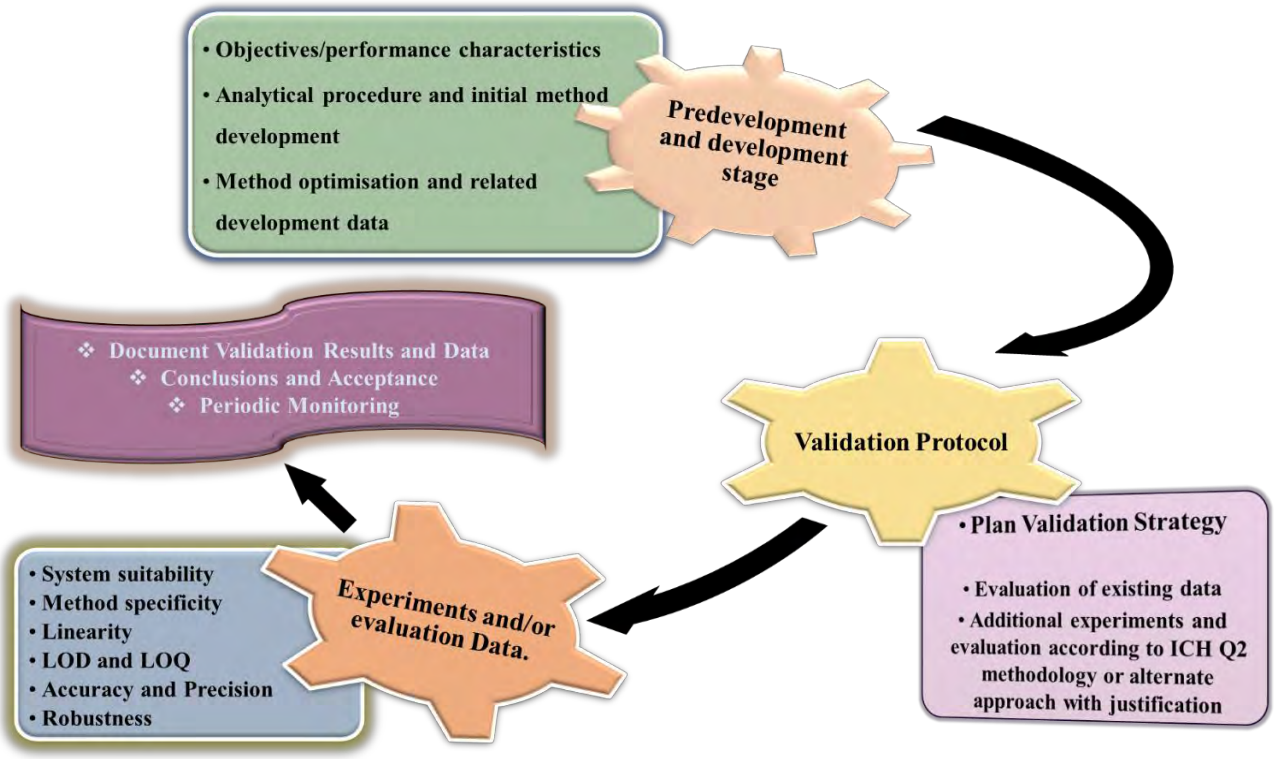


Fig. 1: Schematic diagram showing the validation cycle during a product analysis.

2. MATERIALS AND METHODS

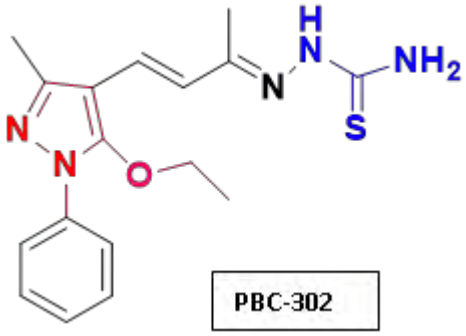


Fig. 2: Novel pyrazolone-based derivative PBC-302.

2.1 Reagents and Chemicals

The model compound was synthesised and characterised as described previously by [13]. Trifluoracetic, HPLC-grade solvents (methanol, acetonitrile) and PBS tablets were purchased from

Sigma–Aldrich. All other solvents are of analytical grade. The compound-containing nanosuspensions and the solutions were stable throughout the experiments and remained stable for a further month.

2.2 Instrumentation and Apparatus

The Shimadzu HPLC system (Kyoto, Japan), fitted with binary high/low-pressure gradient pumps, degasser, PDA detector, and autosampler with LC/GC Solution 5.106 SPI system software, was used to obtain, track and process output chromatogram data. Shim-pack GIST C18 5 μm diameter and size 4.61.D. × 150 mm (Shimadzu). 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-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 Preparation of Mobile Phase.

1 *m*L of Trifluoracetic acid was diluted to 1000 mL with Ultrapure water to prepare the aqueous phase of the mobile phase, then ultrasonicated. The acidified water and acetonitrile were filtered separately through a 0.45 μm membrane filter and degassed. A freshly prepared mobile phase was used for every experiment.

2.4 Preparation of Standard Stock Solution.

The standard stock solution was prepared by weighing accurately 10mg each of and dissolved in 5 mL of methanol in a 10 mL volumetric flask, and the volume was made up to 10 ml with methanol to give a stock concentration of 1 mg/mL. From the above stock solution, 500 μL was taken separately and diluted to 10 to provide a 50 μg/mL concentration. For the calibration curve, concentrations of 50 μg/mL, 40 u μg/mL, 30 μg/mL, 20 μg/mL, 10 μg/mL, 5 μg/mL, and 0.25 μg/mL were prepared by withdrawing 500 μL, 400 μL, 300 μL, 200 μL, 100 μL, 50 μL, 25 μL and 12.5 μL respectively 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.

3. Method Development and Optimisation

The experimental conditions were optimised to achieve good separation and proper chromatographic conditions for analysis. The most common separation conditions include solvent type, sample volume, injection volume, mobile phase composition (ratios, gradient, flow rate), medium pH, column type and column temperature. The experimental conditions for analysis were achieved by a planned/ systematic study of the mentioned parameters.

3.1.0 Chromatographic Conditions

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

3.2.0 Analytical Method Validation

The optimised method was validated according to the ICH Q2 (R1) guidelines [28] to check the method's reliability. The following validation parameters were evaluated:

3.2.1 System Suitability

The system suitability of the optimised method was evaluated by injecting six replicates of a known concentration of the test compound to evaluate whether the method was suitable for its intended use. The Number of Theoretical Plates (NTP), percentage tailing factor, and peak asymmetry were calculated and compared with the recommended limits.

3.2.2 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.

3.2.3 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 analysing standard solutions of the pyrazolone compound in the field of 2.5 to 50 μg/mL and constructing a calibration curve.

3.2.4 Limit of Quantitation (LOQ) and Limit of Detection (LOD)

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

$$LOD = \frac{3.3\varphi}{S} \qquad \textbf{Equation (1)}$$

Then the LOQ is calculated from the following Equation:

$$LOQ = \frac{10\varphi}{S} \qquad \textbf{Equation (2)}$$

Where $\boldsymbol{\varphi}$ is the standard deviation of response and **S** is the slope of the regression line.

3.2.5 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 analysed solutions were prepared using a Blank and stock solution of the tested structure and were done in triplicate.

3.2.6 Precision

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

3.2.7 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/mins, and the percentage TFA used in the analysis, were studied to establish the robustness of the optimised method and their percentage RSD was determined. One factor analysis of Variance (ANOVA) was also carried out using the excel analysis tool kit to further establish variability in the data obtained using the *P* value.

4. Application of the Method for Quantifying Novel Pyrazolone-based Derivative in Nanosuspension

The Nanosuspension was prepared using the nanoprecipitation technique [29] with slight modification. Briefly, 60 mg of PLGA, 1% poloxamer, and 6 mg of the compound were weighed. The 60 mg PLGA was dissolved in 5 mL of Acetone and stirred for two hrs. 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 mins. Then was kept stirring at 600 rpm overnight. A blank was also formulated in this manner. The nanosuspensions were characterised for particle size distribution, zeta potential, and polydispersity index using Zetasizer (Nano ZS, Malvern Instruments, UK).

The total drug content (DL) and the nanosuspension entrapment efficiency (EE) were calculated using the indirect method. Briefly, a 2 ml sample was centrifuged at 12,000 rpm at 4℃ for 30 min, and the supernatant was filtered and analysed 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 mins, then was made up to a volume of 10 mL in a volumetric flask, filtered and analysed using the developed method. The experiment was performed in Triplicates. The Drug content and entrapment efficiency were calculated using equations one ad two below.

$$DL = (W1 - W2)/W \times 100\% \qquad \textbf{Equation (3)}$$

$$EE = (W1 - W2)/W1 \times 100\% \qquad \textbf{Equation (4)}$$

Where W1 was the weight of the total compound in the nanosuspensions and W2 was the weight of the free compound. W was the total weight of the nanoparticles.

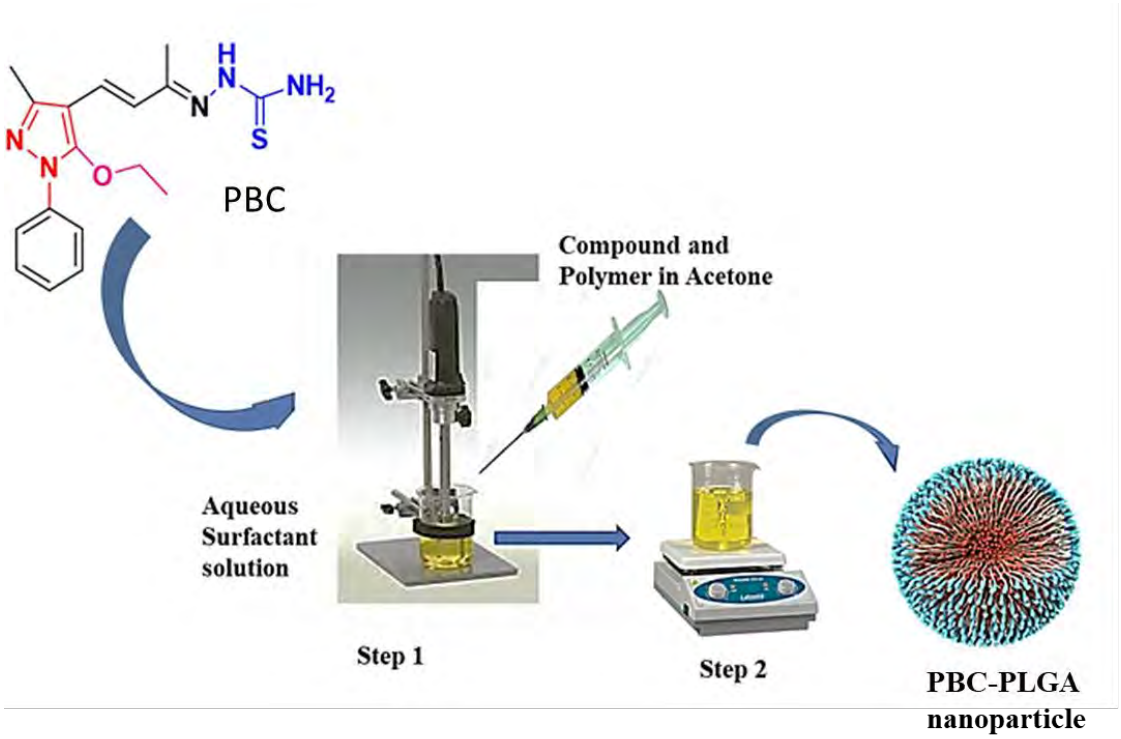


Fig. 3: An illustration of the nanoprecipitation technique of the nanosuspension formulation. The organic phase comprising the compound and polymer in an organic solvent is slowly dropped into the aqueous phase of the surfactant while homogenising.

5. RESULTS AND DISCUSSION

5.1.0 Development of the chromatographic system

Chromatographic separation was accomplished using the Shimadzu HPLC system (Kyoto, Japan with Shim-pack GIST C18 5 μm diameter and size 4.61.D × 150 mm column at 25 ℃. A binary mixture of 70:25 Acetonitrile and acidified water (0.1% TFA) was optimal for isocratic separation of the intended compound at a flow rate of 0.5 mL/min. PDA detection was monitored at a wavelength of 333 nm after scanning. The HPLC elution was completed at below six minutes with a total run time of 10 minutes, and 20 μL was used as an injection volume. These conditions successfully eluted the pyrazolone-based compound with high specificity, as shown in Figure 4. The developed method conditions are summarised in table 1. Method validation was carried out subsequently for linearity, c accuracy, precision, robustness and LOD and LOQ following the recent ICH guideline, 2022.

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
Column type	Shim-pack GIST C18 5 μm, 4.61.D × 150 mm
Injection volume	20 μL
Column Temperature	25 ℃
Retention Time	4.82 min
NTP	2265 (>2000)
% Tailing Factor	1.4% (< 2)

5.2.0 System Suitability

The system suitability test is commonly used to prove that the system perfectly separates the compound of interest with 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. 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 analyse the compound for its intended use. RSD of peak areas obtained from injecting the six samples is 1.19% (≤ 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.

5.3.0 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 [28]. These parameters were assessed by comparing the chromatograms of the pure compound, compound-loaded Nanosuspension and the blank Nanosuspension and are represented in Fig. 4 (D, C and A). As shown in the figure below, the peak purity analysis noted no co-elution and significant interfering peaks. Again, comparing Chromatograms in Fig 4: it can be inferred that the developed method is specific and selective for the test compound.

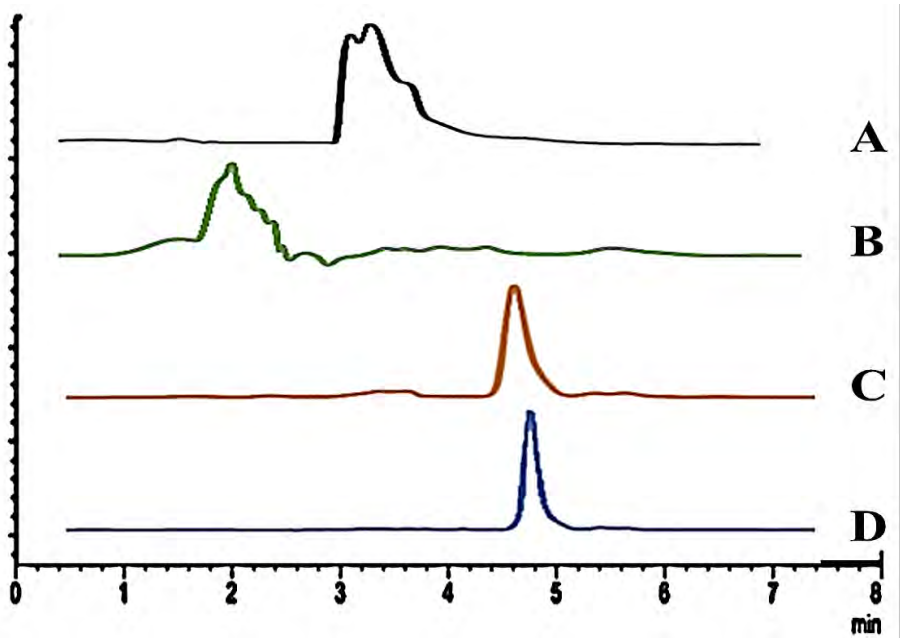


Fig 4: Chromatograms of the blank formulation (A), methanol: solvent for dilution (B), the compound-loaded Nanosuspension (C), and pure compound (D), which further established the specificity of the method and its suitable application in detecting the compound in nanosuspensions.

5.4.0 Linearity

The linearity of the method was observed over a concentration range of 2.5-50 μg/mL. From the constructed calibration curve, as illustrated in Figure 5, the coefficient of determination (R^2) was observed to be 0.9994 showing a linear relationship between the peak area (observed from the chromatogram) and the concentration of the analyte, which ultimately confirms the suitability of this method for the analysis.

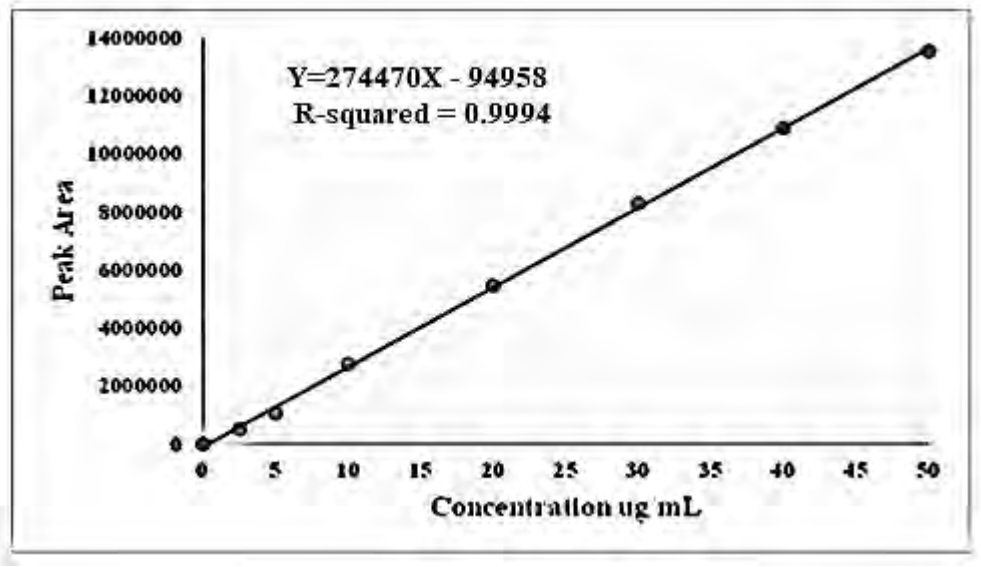


Fig 5: A straight line Curve obtained from the Linearity analysis of the method

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

Table 2: Assessment of the accuracy of the method developed in this study

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

5.6 Precision

Precision is the variability of the results in repeated sample analysis under the same experimental conditions. Intra-day precision of the developed method was evaluated by assaying freshly prepared solutions in triplicate at a known concentration on the same day. Inter-day 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 3. The relative standard deviation (% RSD) obtained for the study is 0.82 and 0.94 (≤ 2%) for repeatability and intermediate precision, respectively, representing excellent precision for the method.

5.7 Limit of Detection and Limit of Quantitation

The calibration curve method was used in determining the limit of detection and quantification. Regression analysis was done on the calibration curve to obtain the standard error of the intercept, which was used to calculate the LOD and LOQ from equations 1 and 2, to be 2.43 μg/mL and 7.38 μg/mL, respectively. The LOD is the lowest concentration from which it is probable to assume the presence of the test compound, while 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 as compared to that reported for similar compound N-Isonicotynoyl-N′-(3-fluorobenzal) hydrazone by [30]

Table 3: Observed Data for Repeatability and Intermediate Precision

Theoretical concentration μg/mL	**Concentrations**		
	Intra Day		**Inter Day**
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

5.8.0 Robustness

Robustness is a parameter that establishes the analytical method's suitability within the intended operational environment. This 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 variation analysis (ANOVA) are represented in table 2 below. All % RSD obtained was within the recommended limit (≤ 2%) except for that obtained in the flowrate analysis, where the % RSD was 9.65 and 6.60 at flow rates of 0.3 and 0.7 mL/min, respectively that for the method to be precise and accurate for the detection of the compound, the recommended flow rate of 0.5 mL/min should be maintained. 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, which indicates that the method is very suitable within its intended operational environment.

Table 4: Results from the method's robustness under different conditions.

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

5.9.0 Application of the Validated Analytical Method

The nanosuspension was prepared and characterised for particle size distribution, zeta potential, and polydispersity index using Zeta Sizer (Nano ZS, Malvern Instruments, UK) to ensure the particles are within the nano range as described in the literature and the surface morphology using a scanning and transmission electron microscope.

The method detected the novel pyrazolone moiety in the nanosuspension by calculating the nanosuspension entrapment efficiency and drug loading capacity. A typical chromatogram of the compound in nanosuspension is depicted in Figure 4. Table 5 shows the results of the physical characterisation of the formulated nanosuspensions, in addition to the encapsulation efficiency and drug loading capacity, which were obtained by applying the validated in the quantification of the test compound in the prepared nanosuspension.

Table 5: Summary of the physical characterisation of the nanosuspension and the % EE and DL

Sample name	Size	PDI	Zeta Potential	Entrapment Efficiency (%)	Drug Loading Capacity (%)
F1	193.1	0.164	13.2	79.33	0.10
F2	193.7	0.139	9.14	80.42	0.10
F3	190.8	0.094	9.98	84.81	0.13

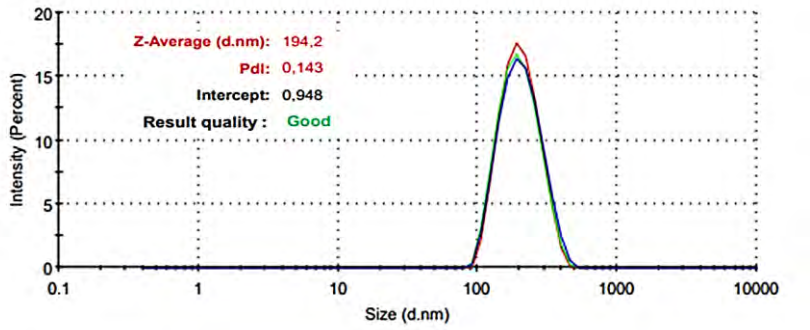


Fig. 6: Size analysis graph for the nanosuspension.

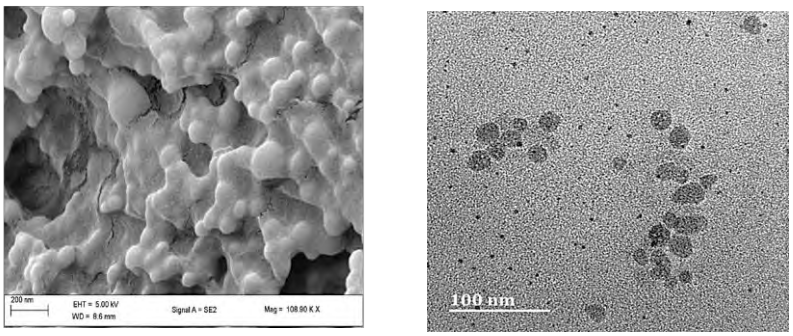


Fig. 7: Surface morphology of the nanoparticles obtained from scanning and transmission electron microscopy (SEM and TEM)

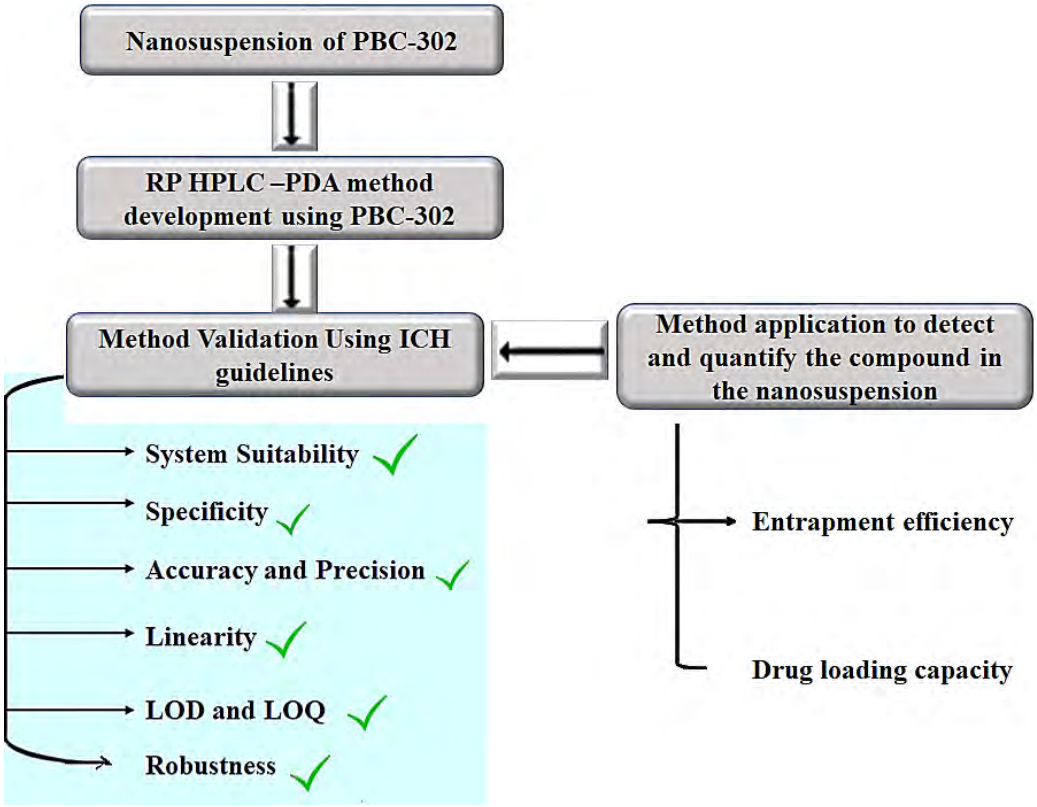


Fig. 8: A concise summary of the workflow of this study.

6. 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 HPLC-UV method is suitable for detecting the pyrazolone derivative in nanosuspensions 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, impurities, and raw material analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank the Discipline of Pharmaceutical Sciences, College of Health Sciences, University of KwaZulu-Natal (UKZN), Durban, South Africa, for providing all the necessary facilities. The authors would also like to acknowledge fellow UKZN colleagues Subashen Naidu and Vishal Bharuth at the MMU department (TEM and SEM analysis).

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CHAPTER 4: EXPERIMENTAL PAPER 2

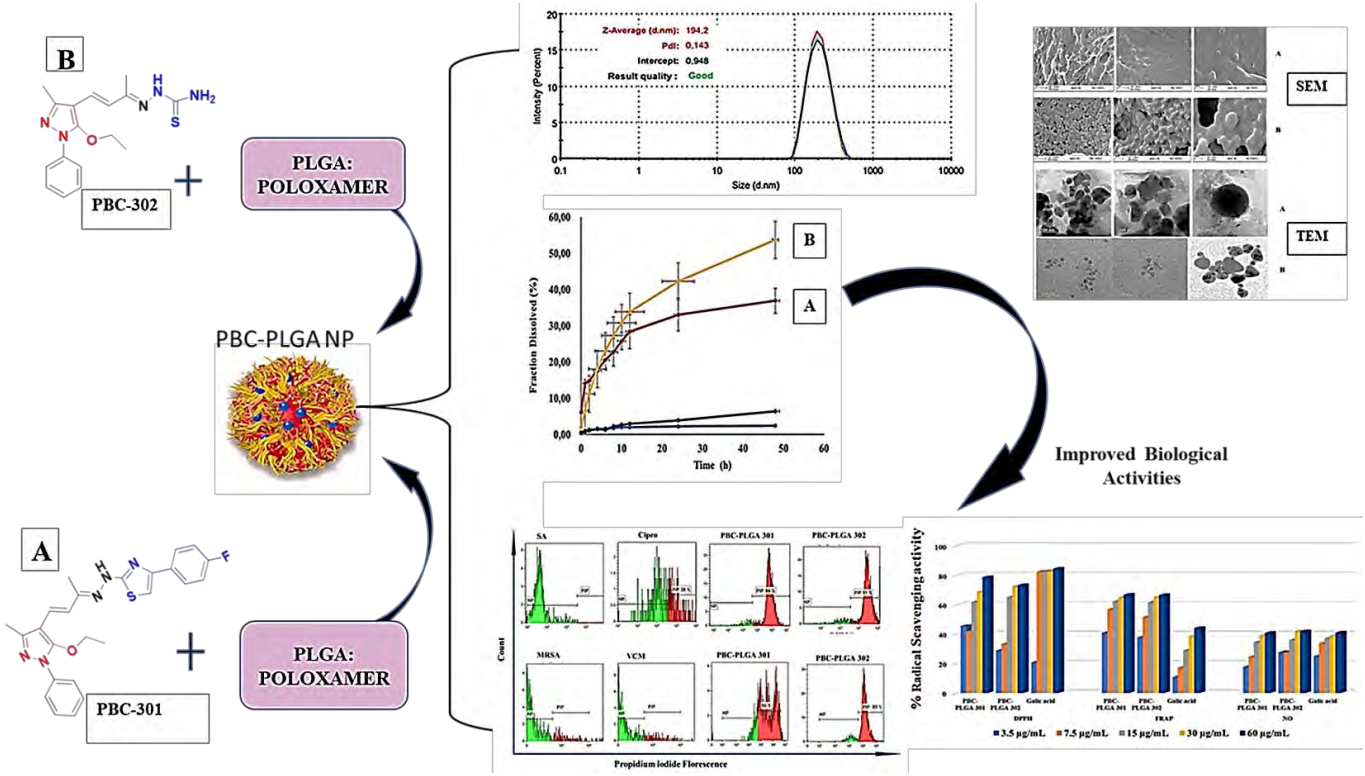
4.1 Introduction

Addresses the aim and objectives 2-4 of the study and is a first-authored research article to be submitted to an internationally recognised journal. This paper reports the encapsulation of two promising novel pyrazolone-based compounds using PLGA and poloxamer to enhance their solubility and biological activities. The research confirmed that the nanoformulation system enhanced the solubility of the pyrazolone-based compounds and significantly improved their antimicrobial and antioxidant activities.

Author contributions

Igbokwe Nkeiruka- Conceptualisation, Data curation, investigation, Formal analysis, Validation and Writing- Original draft preparation. ***Eman A. Ismail-*** Conceptualisation, writing- review and editing, ***Sthabile Mokoena-*** Investigation; ***Blessing Ike-*** Investigation; ***Mohammed Mshelia-***Investigation; ***Vincent A. Obakachi-*** Design and synthesis of PBC-301 and PBC-302; ***Aviwe Ntsethe-*** Flow cytometry analysis; **Rajshekhar *Karpoormath-*** Co-Supervision and ***Mbuso Faya***- Supervision.

Graphical abstract



4.2 Article for Publication

Nanoencapsulation of novel Pyrazolone-based compounds to enhance solubility and biological activity

Nkeiruka N. Igbokwe[1]*; Eman A. Ismail[1,2]; Sthabile Mokoena[1]; Blessing Ike[1]; Mohammed Mshelia[1]; Vincent A. Obakachi[1]; Aviwe Ntsethe[1]; Rajshekhar Karpoormath[1]; Mbuso Faya[1]*

[1]Discipline of Pharmaceutical Sciences, College of Health Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban, South Africa.

[2]Department of Pharmaceutics, Faculty of Pharmacy, University of Gezira, Wad Medani, Sudan.

*Corresponding author.

Email address: igbokwenk@gmail.com/ FayaA@ukzn.ac.za

Abstract

Although pyrazolone-based derivatives have been found to exhibit a variety of biological functions, their poor water solubility and stability are major barriers to their clinical use. Based on this, two pyrazolone-based compounds, PBC-301 and PBC-302, which had previously been reported to be poorly soluble, were encapsulated using PLGA: poloxamer complex to improve their solubility and to investigate the effect of solubility enhancement on their antioxidant and antimicrobial activities. The nanoformulation's activity against the most susceptible bacterial strains, *S. aureus* and *Methicillin-resistant S. aureus*, was measured using the minimum inhibitory and cell permeability assays. The haemolysis experiment validated the nanoformulations' haemocompatibility. Various *in vitro* methods were used to characterise the produced nanoformulations PBC-PLGA 301 and PBC-PLGA 302. The optimised nanoformulations' sizes, PDI, and ZP were 166.6 ± 7.12 nm, 0.129 ± 0.042, -14.14 ± 2.90 mV for PBC-PLGA 301 and 192.5 ± 1.08 nm, 0.132 ± 0.025, -10.77 ± 1.515 mV for PBC-PLGA 302, with encapsulation efficiencies of 84.20 ± 0.930% and 81.5 ± 2.051%, respectively. The chemical release from the nanovesicles was maintained, with PBC-PLGA 301 and PBC-PLGA 302 achieving a cumulative release of around 37% and 53% in 48 hours, respectively. The biological activity experiments revealed that the nanoformulations outperformed the non-encapsulated PBC 301 and PBC-302. The antibacterial activity demonstrated that the compound-loaded nanovesicles outperformed the conventional antibiotic vancomycin and the non-encapsulated compound against the two gram-positive bacteria *S. aureus* and *Methicillin-resistant S. aureus*. On the other hand, the cell penetration experiment demonstrated that the compound-loaded nanovesicles obtained better than 90% propidium iodide penetration for *S. aureus* (translating to cell death) at the stated MIC well, whereas 86% and 89% cell penetration was achieved for *Methicillin-resistant S. aureus*.

Furthermore, the nanoformulations showed increased radical scavenging activity in a concentration-dependent manner, with PBC-PLGA 301 showing the strongest antioxidant activity against DPPH, FRAP, and nitric oxide when compared to the conventional antioxidant-gallic acid and non-encapsulated compounds. This study discovered that increasing solubility might greatly increase biological activity. As a result, nanoformulations of these pyrazolone-based derivatives have the potential to be employed as therapeutic agents to battle bacterial infections as well as other oxidative stress-related diseases such as cancer and hepatic and vascular disorders.

Keywords: Pyrazolone-based, Solubility, Nanoparticles, antioxidant, Flow cytometry, antibacterial

1. Introduction

Over the last two decades, a significant focus has been on discovering novel compounds of desired properties, especially in the pharmaceutical industry. A focus is driven by the dire need to develop efficacious therapies for treating diseases that qualify as having "unmet medical needs" and are potentially life-threatening [1]. However, the discovery and development of a new drug entity (NDE) to become a clinical and marketable drug is a complex, costly and time-consuming process. Poor aqueous solubility remains a typical problem for drug candidates in today's pharmaceutical development pipeline [2-4], and the number of drug candidates defined as having low solubility has increased in recent years. An estimated 75–90% of the drug products in development and 40% of the marketed products are poorly water-soluble [5, 6]. Further establishing that poor solubility is a huge obstacle in drug development.

The poor solubility of novel drugs presents a challenge for developing successful drug formulations, affects biological assay performance in the drug discovery stage and causes poor bioavailability at the clinical stage, affecting the ease of manipulation during manufacture and testing in the laboratory, the potential for drug absorption after oral administration, and the ability to administer the drug parenterally. Limitations in solubility can impair data quality on both *in vitro* assays and *in vivo* toxicity assessments since toxicological studies usually require higher dose exposure than pharmacological or pharmacokinetic studies to assure their safety. On the other hand, limited solubility in the test medium may yield invalid information on the drug's properties *in vitro*, especially in cell culture assays (efficacy, membrane permeation properties, and genotoxicity) required to establish the efficacy and safety of the drug. Thus, a drug molecule must be sufficiently in solution to quantitate meaningful activities in in-vitro assays and achieve adequate absorption *in vivo* for safety and efficacy evaluation in preclinical models and clinical trials.

Again, the solubility of a drug is a critical determinant of its dissolution rate. Therefore, limited dissolution resulting from low solubility primarily leads to low bioavailability of orally administered

drugs [7], culminating in the drug's limited therapeutic potential, thereby leading to therapeutic failure and overall poor clinical outcomes.

Furthermore, at the drug development stage, the solubility of active pharmaceutical ingredient (API) is a very important factor, especially in areas that concern dosage form development and selection for clinical trials, designing experiments to identify potential salt forms, cocrystal forms, polymorphic forms, solvates and hydrates, analytical methods development and drug-manufacturing strategies. Based on these highlights, it is pertinent to integrate solubility evaluation into the discovery and development process, and it must be continuously monitored and refined as compounds progress [8].

Therefore, it is ideal that new chemical entities (NCEs) should have sufficient solubility to ensure that adequate drug exposures can be confidently predicted and achieved in preclinical pharmacokinetic, efficacy and toxicology studies [9]. Several approaches/techniques have been reported in the literature to improve aqueous solubility, dissolution rate and the bioavailability of drug substances with poor solubility. These may include and are not limited to micronisation, supercritical fluid (SCF) process, nanocrystals [10], cyclodextrins complexes [3, 11], nanoparticles and nanosuspensions [12], salt formation [13], amorphous solid dispersions [14], micro-particles [15], polymeric microneedles [16], and self-emulsifying drug delivery system (SEDDS) [17].

Along with various conventional approaches introduced for enhancing aqueous solubility, the application of nanotechnology is considered the most convenient. Encapsulation of poorly soluble compounds in appropriate nanoparticles (NP) in the range of 10-1000 nm has been reported to enhance the solubility and bioavailability of the loaded compounds [18]. Also, nanoparticulate systems have received growing attention among researchers in the last three decades to develop formulations for poorly water-soluble drugs. They are known to improve the inherent properties of drug molecules by recuperating solubility, thus improving drug efficacy and pharmacoeconomics [19]. Hence, nanosystems such as nanocrystals/nanoparticles, nanoemulsions, solid-lipid nanoparticles, polymeric

nanoparticles and nanogels/nanomatrices are well-described in the literature for enhancing the solubility of poorly soluble compounds [19, 20]. However, amongst these, polymer-based systems are the most widely used of all the nanosystems owing to their ease of preparation, long-term stability, less toxic properties and ability to efficiently encapsulate hydrophobic drugs to improve solubility and bioavailability [19, 21].

Three decades ago, this ushered in the era of nanosystems formed by amphiphilic block copolymers in an aqueous solution as carriers for poorly soluble therapeutic [22]. However, over time, the applications of copolymers in the design of polymeric nanoparticles and micelles as therapeutics have been widely studied [23, 24]; owing to their desirable properties such as controlled release tendency, greater stability, minimised cargo degradation, increased cargo solubility, and improved bioavailability. These polymers/copolymers may include chitosan, alginate, gelatine, poly (lactic-co-glycolic acid) (PLGA), polylactic acid (PLA)), poly (Ɛ-caprolactone) (PCL), and Polyglycolide have been comprehensively reviewed by for solubility enhancement [25, 26] among which poly (lactic-co-glycolic acid) (PLGA) is the most commonly used.

Poly (lactic-co-glycolic acid) (PLGA) is a synthetic biodegradable polymer having, in addition to the advantages above, other attractive properties such as biodegradability and biocompatibility, drug targeting, the ability to protect loaded drugs from chemical instability, the possibility of better interaction with biological materials achieved by improving surface properties [27, 28]. PLGA is also approved by US FDA (United States Food and Drug Administration), and EMA (European Medicine Agency) as safe for parenteral administration [29] and some PLGA-based depot formulations have been approved by the FDA for medical use. Examples of drugs based on the PLGA copolymer are Eligard®, used in the treatment of prostate cancer symptoms[30], and Bydureon®, approved to improve glycaemic control when combined with diet and exercise in type 2 diabetes [31]. Also, the literature revealed the enhancement of solubility of several natural and synthetic bioactive compounds such as quercetin [32], resveratrol [33], curcumin [34], and brucine [35] using PLGA polymeric

nanoparticles. Several methods have been applied for PLGA nanoparticle development/preparation. These include nanoprecipitation [36], solvent evaporation [37], dialysis [38], and salting out [39]. All these properties inspired the use of PLGA as a choice polymer in this study.

Pyrazolone is a five-membered lactam ring containing two nitrogen and one ketonic group in its structure. They are a derivative of pyrazole possessing an additional carbonyl (C=O) group. Pyrazolone derivatives are a vital class of heterocyclic compounds, having found use as precursors for dyes, pigments, pesticides and chelating agents, besides finding applications in the extraction and separation of various metal ions. They are also a significant component of drugs and synthetic products possessing analgesic, antioxidant, antimicrobial, antifungal, anti-inflammatory, anti-tumour/anti-cancer, gastric secretion stimulatory, antidepressant, antidiabetic, antiviral, and anthelmintic activities. Due to their numerous biological activities in humans, pyrazole derivatives were thought alkaloids [40]. The pyrazole nucleus possesses almost all types of pharmacological activities owing to the planar structure of the aromatic heterocycle [41] and, throughout the years, has attracted the attention of many researchers, making it one of the most explored pharmacophores in the world [42]. Pharmacological agents currently marketed containing the pyrazole nucleus includes and are not limited to Tepoxalin (nonsteroidal anti-inflammatory drugs (NSAIDs), celecoxib (a selective COX-2 inhibitor), betazole (analgesic), crizotinib (anti-cancer), surinaban b (a treatment for nicotine addiction), difenamizole (anti-obesity), mepiprazole (tranquillizer), CDPPB (antipsychotic), and fezolamide (an H2-receptor agonist and antidepressant agent), further confirming the overwhelming pharmacological potential of the pyrazole nucleus [41, 42]. A recently developed pyrazolone compound, edaravone, also known as MCI-186, is used as a medical drug for brain ischemia and has also been reported to be helpful in myocardial ischemia [43]. 4-(4-(4-fluorocyclohexyl) piperazine-1-carbonyl)-2,5-dimethyl-2,4-dihydro-3H-pyrazole-3-one has been suggested by [44] as a potential lead for therapeutic application to control the inflammatory response in SARS-CoV-II infection. 3,5-dioxopyrazolidine has been reported as a SARS-CoV 3CLpro inhibitor [45]. Several previously synthesised novel pyrazolone-

based compounds evaluated using computational techniques showed that several compounds from the series exhibited higher and better binding high affinity to hACE-2 and SARS-CoV-2 Sgp, respectively, compared to the standard drugs MLN-4760 and Cefoperazone (CFZ) further suggesting the antiviral activity of pyrazolone moieties [46]. Compound 7b (PBC-301) has also been reported to possess a significant binding affinity to CDK-2 cells, suggesting anti-cancer activity using molecular simulations [47]. In addition to this finding, the compounds were observed to possess poor aqueous solubility. For this reason, the compounds were not further investigated for other biological activity because even if they were found to exhibit *in vitro* biological activity, their clinical application would remain limited unless water-soluble formulations of the compounds were developed.

At the time of this assay, only three studies existed concerning the nano-encapsulation of three of the numerous reported bioactive pyrazolone-based derivatives with poor aqueous solubility [48-50]. However, the two studies concern a nanotechnology application to enhance water solubility [48, 50]. Alfei et al. encapsulated 2-(4-Bromo-3,5-diphenyl-pyrazol-1-yl)-ethanol pyrazole derivative (BBB4) in a biodegradable non-cytotoxic cationic dendrimer (G4K). The obtained nanoparticles (BBB4-G4K NPS) showed good drug loading (28.8 ± 1.2%), satisfying encapsulation efficiency (39.0 ± 1.6%) and a biphasic quantitative release profile governed by first-order kinetics after 24 h with a 105-fold higher solubility compared to the pristine form BBB4. On the other hand, Sun and his co-workers encapsulated 6-amino-4-(2-hydroxyphenyl)-3-methyl-1,4-dihydropyrano [2,3-c] pyrazole-5-carbonitrile (AMDPC) derivative in poly (ethylene glycol) methyl ether- block-poly (lactide-co-glycolide) (PEG-PLGA), obtaining nanoparticles which gave clear water solutions at 0.05 mg/mL compared to the pristine AMDPC at the same concentration. However, despite having high entrapment efficiency of 64.3 ± 7.89%, the obtained nanoparticles showed a low drug loading capacity of 1.28 ± 0.46%.

Given this scenario, to solve the solubility drawbacks with two novel pyrazolone-based compounds (PBC-301 and PBC-302), previously synthesised by Obakachi et al., the compounds were encapsulated

using a biodegradable and biocompatible PLGA with Poloxamer. We further characterised these PBC-PLGA nanoparticles and conducted *in vitro* antimicrobial, antioxidant activity testing and cell penetration assay using the flow cytometer to establish the effect of solubility enhancement on the compounds.

2. Materials and Methods

The novel pyrazolone-based derivatives were synthesized by Obakachi and co-workers (University of Kwazulu-Natal) (Figure 1), and all information on the synthesis and characterisation has been reported [46]. PLGA 50:50, poloxamer-188 (Sigma-Aldrich), PBS tablets (Sigma-Aldrich), methanol (HPLC grade), acetone (HPLC grade), MiliQ water, sodium lauryl sulphate, ciprofloxacin (Sigma-Aldrich-St Quentin Fallavier, France), iodonitrotetrazolium chloride (INT) (Sigma-Aldrich), dimethyl sulfoxide (DMSO) (Sigma-Aldrich), propidium iodide kit (Biocom Africa), Mueller Hinton agar and Mueller Hinton broth (Sigma-Aldrich). All reagents and solvents were analytical grades. All solvents were purified by standard procedures, whereas organic reagents were used without further purification.

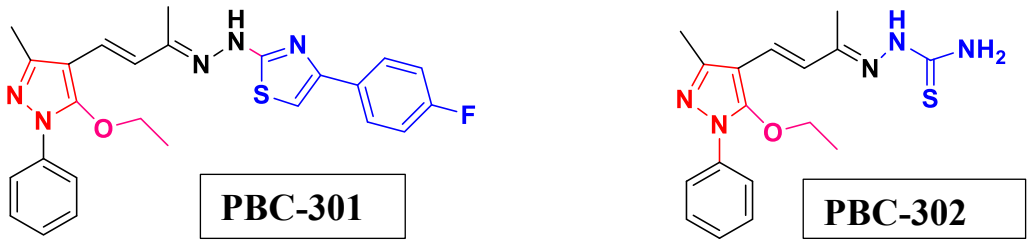


Figure 1. Structure of the novel pyrazolone-based compounds PBC-301 and PBC-302

2.1 Preparation of PBC-PLGA Nanoparticles

The nanosuspension was prepared using the nanoprecipitation Technique [51] with slight modification. Briefly, 60 mg of PLGA, 1% poloxamer, and 6 mg of the compound were weighed. The 60 mg PLGA was dissolved in 5 mL of acetone and stirred for two hrs. The Pyrazolone-based compound (PBC) was dissolved in 500 μL of suitable organic solvent, mixed with the PLGA solution

under Stirring, and then added in drops to 10 mL poloxamer in water under sonication for 5 mins at 30% sonication power. Then was kept stirring at 600 rpm overnight. A blank was also formulated in this manner. Each sample was prepared in triplicate.

2.2 Physicochemical characterisation of PBC-PLGA nanoparticles

2.2.1 Identification and quantification of the Pyrazolone-based compounds

Identification and quantitation of the pyrazolone compound were achieved using the Shimadzu HPLC system (Kyoto, Japan), fitted with binary high/low-pressure gradient pumps, degasser, PDA detector, and autosampler with LC/GC Solution 5.106 SPI system software. Efficient separation and quantification were carried out using Shim-pack GIST C18 (5 μm 150 × 4.6 mm) column, maintained at 25 ℃ with isocratic elution using Acetonitrile and acidified water (0.1% Trifluoracetic acid) with 20 μL injection volume. The mobile phase ratios were 85:15 for PBC-301 and 75:25 for PBC-302, and the flow rates were kept at 1 mL/min and 0.5 mL/min at a wavelength of 203 nm and 333 nm, respectively. The regression equation for the calibration curve for the pyrazolone-based compounds was Y= 65356X-24472 and Y=274470X-94958, and the linearity correlation coefficient R^2 = 0.999 and 0.9994, respectively.

2.2.2 Determination of the Particle Size, PDI and charge of the formed PBC-PLGA nanoparticles.

Dynamic light scattering (DLS) was used to evaluate the (Size, PDI, and ZP) of PLGA-NP at 25 °C using a Zeta sizer (Nano ZS, Malvern Instruments, UK). The samples were diluted with milli-Q water until the scattering intensity was within the sensitivity range of the instrument, and then they were analysed. The average and standard deviations of the three measurements were reported.

2.2.3 Determination of entrapment efficiency (EE) and drug loading capacity (DL)

The nanoparticles were collected by centrifugation at 12000 rpm at 4 ℃ for 30 min. The amount of free compound in the supernatant was measured by High-performance liquid chromatography (HPLC-

PDA) to determine the entrapment efficiency [52]. For the drug content, 1 mL of methanol/ acetone was added to 500 μL of nanosuspension and kept under sonication for 15 mins, then made up to a volume of 10 mL in a volumetric flask, filtered and analysed using the described method above. The experiment was performed in Triplicates. The drug content and entrapment efficiency were calculated using equations one ad two below.

$$Total\ drug\ content\ recovered\ from\ Nanosuspension = \left(\frac{mg}{mL}\right)\frac{Amount\ of\ the\ drug\ in\ a\ nanosuspension - Unentrapped}{The\ total\ Weight\ of\ the\ Nanoparticles} \quad \textbf{Equation 1}$$

$$Entrapment\ efficiency = \frac{Amount\ of\ drug\ \ in\ nanosuspension - Unentrapped}{Total\ Amt\ of\ the\ drug\ in\ nanosuspension} \times 100 \quad \textbf{Equation 2}$$

2.2.4 Differential scanning calorimetry (DSC)

DSC was used to investigate the thermal profiles of bare Pyrazolone-based compounds, P-188, PLGA, physical mixtures, and lyophilised PBC-PLGA NPs (Shimadzu DSC-60, Japan). In brief, 2 mg samples were put in an aluminium pan and sealed with a crimper before being heated to 300 °C at a continuous rate of 10 °C/min under a constant nitrogen flow of 20 mL/min using an empty pan as a reference.

2.2.5 Transmission electron microscope

Transmission electron microscopy (Jeol, JEM-1010 (Japan)) was used for the morphological studies (TEM). The PBC-PLGA nanosuspensions were properly diluted before being put on the surface of a copper grid. Before measuring, the surplus material was blotted off using filter paper, which was then dried at room temperature and dyed with a 2% uranyl acetate solution [53]. All photos were taken with a 100 kV accelerating voltage.

2.2.6 Scanning electron microscope (SEM)

Morphology of the PLGA-PBC nanoparticles was observed by the light electron microscope (SDP TOP, CX 40, Ningbo Sunny Instruments Co., Ltd) and the Field emission scanning electron

microscope “FESEM, JEOL JSM-6700”. Nanoparticle suspension was dropped onto the copper tape and air-dried, then sputtered by gold for 120 s, and visualisation was done at a voltage of 5 kV and a current of 10 A. The SEM micrographs were elaborated by the (Image J 32) image processing program.

2.2.7 *In vitro* drug dissolution Studies

The dialysis bag diffusion technique was used to study the *in vitro* release of pyrazolone-based chemicals from PLGA nanoparticles at 37 2 °C. In a nutshell, 2.0 mL of the PBC-PLGA nanoformulations were placed in a cellulose dialysis bag (cut off 10,000 KDa, Sigma Aldrich) and immersed in 20.0 mL of pH 7.4 phosphate buffer solution and 0.3% Sodium Lauryl Sulphate (SLS) in the recipient compartment (50 mL falcon tube) and incubated at 100 rpm in an orbital shaker. SLS was used in PBS to keep the sink condition and give aqueous phase solubility for the chemicals. At predetermined time intervals of 0, 2, 4, 6, 8, 10, 12, and 24, 2 mL samples from the receiver compartment were removed and replaced with the fresh dissolution medium of the same volume. The PBC content of the release samples was evaluated using the HPLC-PDA technique previously reported. All experiments were carried out in triplicate, and average values were computed. The graphic below depicts the release profiles. The data were analysed with Excel's built-in DD-solver.

2.3 Biological Activity Testing

2.3.1 Determination of bacterial susceptibility

INT colorimetric assay (Eloff, 1998; Mativandlela et al., 2006) was conducted in clear, sterile 96-well microtiter plates (Corning Life Sciences, Acton, MA, USA) to assess the minimal inhibitory concentrations (MICs) of the pyrazolone based compounds (PBC 301 and PBC 302), their nanoformulations (PBC-PLGA 301 and PBC-PLGA 302), vancomycin, ciprofloxacin against two gram-negative bacteria, viz: *Pseudomonas aeruginosa* (PA) ATCC 27853, *Escherichia coli* (EC) ATCC 25922 and Two gram-positive bacteria, viz: *Staphylococcus aureus* (SA) ATCC 25923 and *Methicillin-resistant staphylococcus aureus* (MRSA) ATCC 10069. Briefly, 100 µl/well of MHB was added, followed by 100 µl of the prepared test samples and the controls added to the first well. Serial

dilutions of the samples were carried out in the MHB broth and incubated with 100 µL/well of inoculum (5 x 10^5 CFU/mL) prepared in MHB for 18 hr in a shaking incubator at (100 rpm) 37 °C. The MICs of test compounds were determined after 18 hr of incubation at 37 °C, following the addition of (40 µL) of 0.2 mg/mL INT and incubation at 37 °C for 30 min.[54]. Ciprofloxacin was used as drug control for PA, EC, and SA, whereas vancomycin was used for MRSA. Viable bacteria reduced the colourless dye to pink. The minimum inhibitory concentration was the lowest sample concentration that prevented this change after incubation and exhibited complete inhibition of microbial growth.

2.3.2 Cell membrane penetration assay using Flow cytometry.

The capacity of the nanoformulations PBC-PLGA 301 and PBC-PLGA 302 to permeate MRSA and SA membranes was evaluated using flow cytometry. In brief, MRSA and SA cells generated according to the previously established bacterial susceptibility technique were extracted after 24 hours of incubation and treated with Propidium iodide dye (5 µL) for 30 minutes at room temperature [55]. 50 µL of each sample combination was put into sample vials, each with 350 µL of sheath fluid, and vortexed for one minute. The cell-penetrating efficacy of the samples was subsequently determined by FACScan measurement of PI influx into bacterial cells. A 488 nm laser was used to stimulate the PI fluorescence, which was then captured using a 617 nm bandpass filter (red wavelength). The negative control was untreated MRSA cells. Flow cytometry was performed using the DxFLEX (Beckman Coulter Life Sciences, USA) equipment. The sheath fluid flow rate was set to 16 mL/min, while the sample flow rate was set at 30 µL/min. The flow cytometer software Kaluza-2.1 was used to obtain data with fixed cells (Beckman Coulter USA). For fluorescence-activated cell sorting (FACS) analysis, the voltage settings were 731 (forward scatter FSC), 538 (side scatter SSC), and 444 for PI. The bacteria were first gated using forward scatter, then cells of the right size were gated, and at least 10,000 cells were collected in duplicate for each sample. Furthermore, in SSC investigations, the detection threshold was set at 1,000 to minimise any background signals from particles smaller than the bacteria.

2.3.3 *In vitro* haemolysis study

The proportion of haemolysis was calculated using a previously reported method [56]. In brief, freshly obtained sheep blood was cleaned three times with autoclaved phosphate buffer saline (PBS, pH 7.4) before being centrifuged for 15 minutes at 3500 rpm. PBS was used to dilute the blank formulation and PBC-PLGA (301 and 302) to concentrations ranging from 0.05 to 0.5 mg/ml for each sample. The RBC suspension (0.2 ml) was mixed into 1.8 ml of each sample and incubated for 30 minutes at 37 °C. The materials were then centrifuged at 3000 rpm for 10 minutes. Spectrophotometric readings of the supernatant of each sample at different concentrations were taken to determine haemoglobin release. To obtain 0% and 100% haemolysis, 0.2 ml of RBC suspension was added to 1.8 ml PBS and distilled water. The degree of haemolysis was calculated using the equation below

$$Haemolysis\ (\%) = \frac{(Abs - Ab0)}{(Abs100 - Abs0)} \times 100 \qquad \textbf{Equation 3}$$

Where ABS100 and ABS0 are the absorbances of the solution at 100% and 0% haemolysis, respectively.

2.3.4 Radical scavenging activity of the bare novel PBCs and PBC-PLGA Nanoparticles

2.3.4.1 DPPH scavenging activity

One hundred µL of each sample at different concentrations of 3.5, 7.5, 15, 30 and 60 µg/mL for the nanoformulations and 15, 30, 60, 120 and 240 15-240 µg/mL for the free compounds was incubated with 50 µL of 0.3 mM DPPH (2,2- diphenyl-1-picrylhyrazyl) in methanol (solution) and placed in the dark for 30 mins at ambient temperature. The absorbance was then read at 517 nm against a blank sample of DPPH solution.

The scavenging activity was calculated using the equation below:

$$DPPH\ radical\ Scavenging = (Ac - As)/Ac) \times 100 \qquad \textbf{Equation 4}$$

Where Ac represents the absorbance of the control, and As is the absorbance of the sample.

2.3.4.2 FRAP Activity

The total reducing power of the PBC and PBC-PLGA nanoparticles was measured using the FRAP method of Oyaizu [57] with slight modifications. Briefly, 1 mL of each test sample (concentrations 3.5-60 15-240 µg/mL and 15-240 15-240 µg/mL) for the PBC-PLGA nanosuspension and bare PBC, respectively, were incubated with 1 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 1% potassium ferricyanide at 50 ºC for 30 min. After that, 1 mL of 10% trichloroacetic acid was used to acidify the reaction mixtures. Then, 1 mL of the sample was mixed with an equal volume of distilled water and 200 µL of 0.1% $FeCl_3$. The absorbance of the resultant solution was read at 700 nm in a spectrophotometer. Antioxidant activity was calculated using the equation below:

$$\%\ FRAP\ radical\ scavenging = \frac{The\ absorbance\ of\ the\ test\ Sample}{Absorbance\ of\ control} \times 100 \quad \textbf{Equation 5}$$

2.3.4.3 Nitric oxide (NO) radical inhibitory activity

At physiological pH, sodium nitroprusside could create much nitric oxide (NO), reacting with oxygen to form nitrite ions. This capability forms the basis of this test [58]. The test was performed by incubating 500 µL of 10 mM sodium nitroprusside in sodium phosphate buffer (pH 7.4) and 500 µL of test samples at various concentrations (15-240 µg/mL) for 2 h at 37 °C. The reaction mixture was then treated with 500 L of Griess reagent. The absorbance at 546 nm was measured to detect a chromophore produced by the interaction of nitrite and sulphanilamide. The percentage inhibition of NO emitted was calculated by comparing the absorbance of a prepared control to the percentage inhibition of NO released (10 mM sodium nitroprusside in phosphate buffer). The experiment was carried out in triplicate, and the scavenging capacity of the test sample was estimated using the formula below:

$$\%\ scavenging\ activity = \frac{The\ absorbance\ of\ control - Absorbance\ of\ test\ Sample}{Absorbance\ of\ control} \times 100 \quad \textbf{Equation 6}$$

2.4 Data Analysis

Experimental data were presented as mean ± standard deviation (SD) and analysed using the Excel Analysis toolkit. The one-way ANOVA defined statistical significance as a p-value of < 0.05.

3. Results and Discussions

These pyrazolone-based analogues are novel synthesised molecules with poor solubility limiting their biological applications. However, considering the numerous pharmacological applications of the pyrazolone nucleus, we attempted to develop effective PBC-encapsulated PLGA nanoparticles to solve this solubility issue. We further characterised these PBC-PLGA nanoparticles and carried out *in vitro* antimicrobial, antioxidant activity testing and cell penetration assay using the flow cytometer to establish the effect of solubility enhancement on the compounds.

3.1 Preparation of PBC-PLGA nanoparticles

Choosing a nanoparticle preparation method for the adequate encapsulation of hydrophobic compounds involves selecting the suitable polymer composition, stabilizer, solvent, drug solubility and technique [59]. Several methods have been explored for encapsulating bioactive compounds into polymeric nanoparticles; however, the choice of method is firmly dependent on the nature of the compound and polymer—the necessity to solubilize the chosen chemicals compelled their formation into nanoparticles. Poly (lactic-co-glycolic acid) polymer (PLGA) was employed to effectively encapsulate pyrazolone-based chemicals due to its solid-state solubility and biocompatibility. The use of PLGA nanoparticle formation has improved the solubility of hydrophobic medicines such as quercetin, paclitaxel, and rifampicin [60-62]. The nanoprecipitation approach mentioned in the procedure section was used to create the PBC-PLGA nanoparticles. In this case, the spontaneous diffusion between the organic and aqueous phases drives the creation of nanoparticles. Because of adjustable formulation parameters, this process demonstrates a bottom-up way of creating particles with the required characteristics [63, 64].

Knowing that physical properties such as solubility, thermal strength, viscosity, crystallinity, mechanical strength, and degradation rate are affected by the molecular weight of the polymer [65], a low molecular PLGA 50/50 was chosen as the carrier for the PBC. The choice of solvent was carefully considered because it influences the size of the nanoparticles, polydispersity, and amount of drug entrapped. This study chose acetone as an organic solvent for PLGA and PBC. The sonication strength, duration, and stirring speed were changed to optimise the nanoparticle formulation. Furthermore, to pick a suitable stabiliser that could successfully maintain the stability of nanoparticles, the impact of several surfactants, polyvinyl alcohol, poloxamer-188, and span 80 on the physicochemical attributes was investigated. The optimum size, PDI, and Zeta potential were achieved with a sonication time of 5 mins at 30% and a stirring speed of 600 rpm with 1% poloxamer-188 as the stabiliser.

3.2 Size, PDI, Zeta potential and Surface Morphology of nanoparticles of PBC-PLGA

The physicochemical parameters of the developed PBC-PLGA nanoparticles are presented in Table 1, the distribution curves for both the size and zeta potential are depicted in Fig. 2-3, and the diagrams for SEM and TEM surface morphology in Fig 4 and 5, respectively.

Particle size is recognised to be an important component in particle cellular absorption and intracellular trafficking, making tiny particles more efficient than their micron-sized counterparts in medication delivery to infected loci [52]. The size of nanoparticles dictates where they are targeted within the body. Particles with sizes ranging from 100 to 200 nm are appropriate for long-term circulation and efficient transfer across biological membranes. Nanoparticles larger than 200 nm are effectively phagocytosed and mostly transported to the liver and spleen [67]. The size of the NPs used locally in orthopaedic surgeries is critical regarding the region they can cover and the depth to which they can penetrate the substrate. The nanoparticle suspensions displayed monodisperse size distributions (PDI < 0.15) with an intensity-averaged diameter determined by DLS of < 200 nm the PBC-PLGA nanoparticles (Z-Ave = 166.6 ± 7.12 nm and 192.5 ± 1.08 nm) for PBC-PLGA 301 and PBC-PLGA 302 respectively. Thus, they are suitable for a parenteral application, easy penetration across

membranes, targeted delivery and sustained circulation when administered. Even though the sizes obtained in this study appeared more significant than the reports from Alfei and Sun [48, 50] on the nanoformulations of some pyrazolone-based derivatives (68.16 ± 0.67 and 112.1 ± 10.7), they are very much in agreement with the observed sizes (100-250 nm) for most PLGA nanoparticles [28, 68].

The PDI, which ranges from 0 to 1, is used to determine particle homogeneity. When a sample's value approaches 0, it becomes exceedingly homogeneous [69]. As a pointer to uniform particle distribution, samples with homogeneous and equally sized particles would have a lower PDI value, whereas samples with a more diverse range of particle sizes would have a higher PDI value [70]. A minimum PDI is required to maintain a continuous and regulated medication release rate. The PDI of all the nanoparticles varied from 0.12 to 0.14, indicating a homogeneous particle size distribution, as shown in (Table 1). Zeta potential (ζ) is a pointer to the stability of water-based formulations, determined *via* the mobility of charged particles monitored by an electrical potential. In general, particle charge is the determining factor, and a ZP of $> +30$ mV and < -30 mV is ideal for the physical stability of any nanosuspension [71]. It is also a measure of the amount of charge repulsion/attraction between particles, which is important in phenomena such as dispersion, flocculation, or aggregation [72], and the zeta potential values can be positive, neutral, or negative depending on the polymer and surface modification [73]. Nanoparticles with a zeta potential between -10 and $+10$ mV are considered neutral, while nanoparticles with a zeta potential greater than $+30$ mV and lower than -30 mV are considered strongly cationic and strongly anionic, respectively [74]. The surface charges of nanoparticles substantially impact their interaction with cells and uptake. Positively charged nanoparticles allow for greater internalisation due to ionic interactions between positively charged particles and negatively charged cell membranes [75, 76]. As a result, they can escape from lysosomes and demonstrate perinuclear localisation after being absorbed. Negatively and neutrally charged nanoparticles, on the other hand, tend to co-localise with the lysosome [77]. The zeta potentials of the PBC-PLGA nanoparticles (-14.14 ± 2.900 mV and -10.77 ± 1.515 mV) in this study

were negatively charged and consistent with the literature [78, 79]. These zeta potentials are comparable to the result (−16.87 ± 1.10 mV) obtained in the nanoencapsulation of AMPDC-a pyrazolone derivative using PLGA/PEG [50]; however, Alfei and his co-workers observed a positive Zeta potential (+ 28.9 mV) with dendrimer encapsulation of a poorly soluble bioactive Pyrazole derivative. The negative charge on the nanoparticles may be attributed to the PLGA used in the formulation, even though the adsorbed poloxamer could be a contributing factor, as it has been previously reported that the adsorbed poloxamer is capable of altering the physicochemical parameters of nanoparticles [80, 81] and responsible for decreasing zeta potential [82, 83]. So, these ZP values confirmed the nanoparticle to be strongly anionic and capable of co-localising with lysosomes. The values also confirmed the physical stability of the samples.

To also confirm the formation of NPs, SEM and TEM images were recorded and depicted in Fig 4 and 5. SEM and TEM images of PBC-PLGA 301 and PBC-PLGA 302 confirmed that the synthesized nanoparticles are discrete and spherical with a smooth surface having a

Table 1: Optimised Size, PDI, Zeta Potential, Entrapment Efficiency and Drug Loading Capacity of the PBC-PLGA nanoformulations.

Sample name	Size (nm)	PDI	ZP	EE (%)	DL (%)
PBC-PLGA 301 NPS	166.6 ± 7.12	0.129 ± 0.042	-14.14 ± 2.900	84.20 ± 0.930	0.30 ± 0.000
PBC-PLGA 302 NPS	192.5 ± 1.08	0.132 ± 0.025	-10.77 ± 1.515	81.5 ± 2.051	0.11 ± 0.012

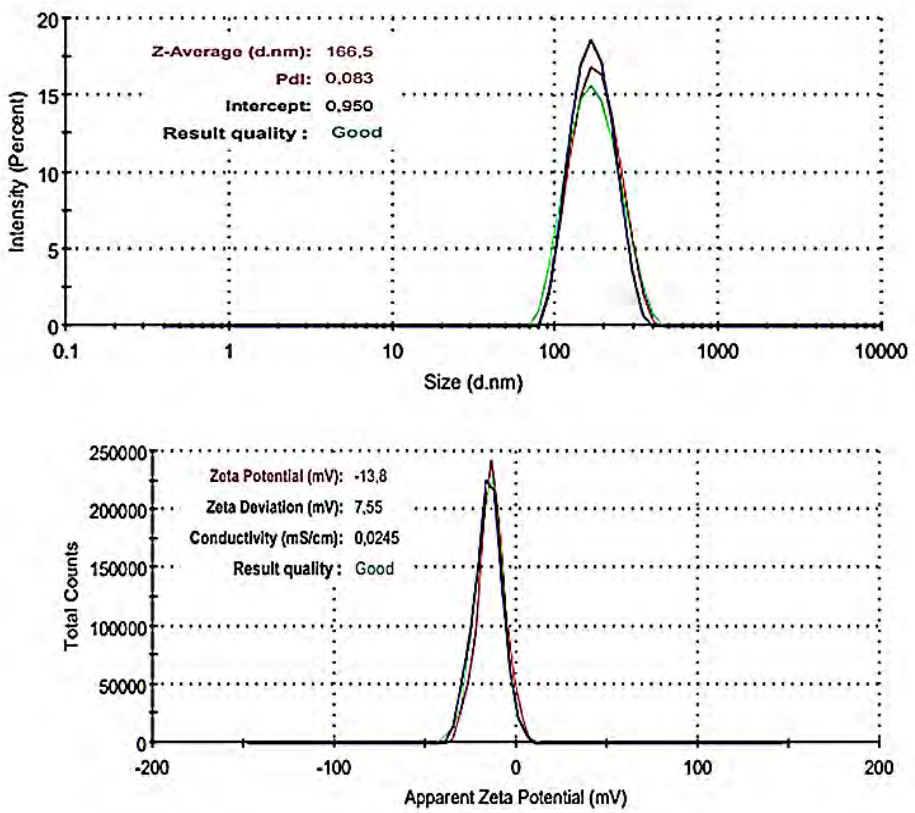


Figure 2: PBC-PLGA 301 Size and Zeta potential distribution curves

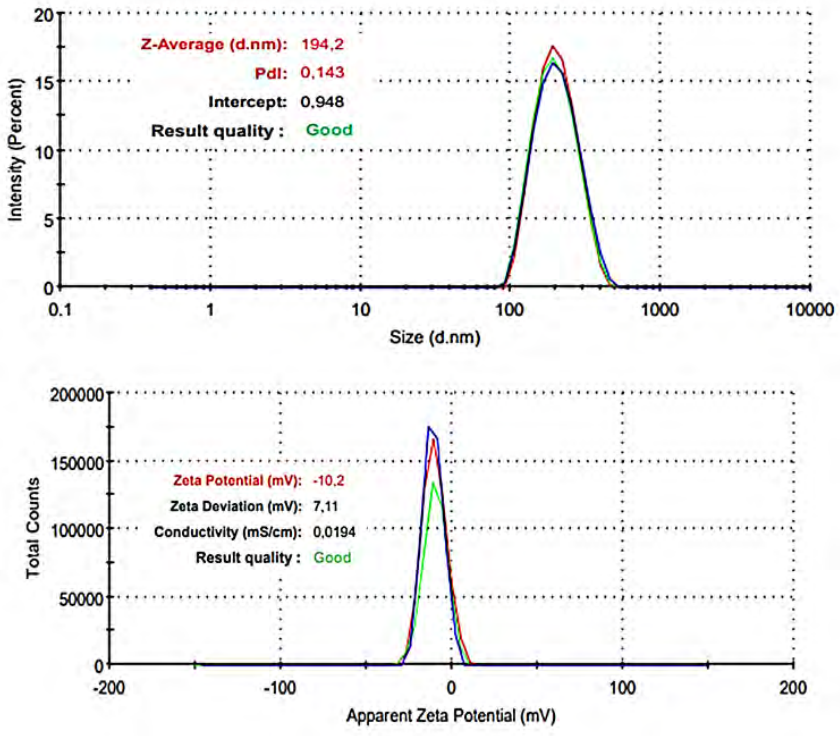


Figure 3: PBC-PLGA 302 Size and Zeta potential distribution curves

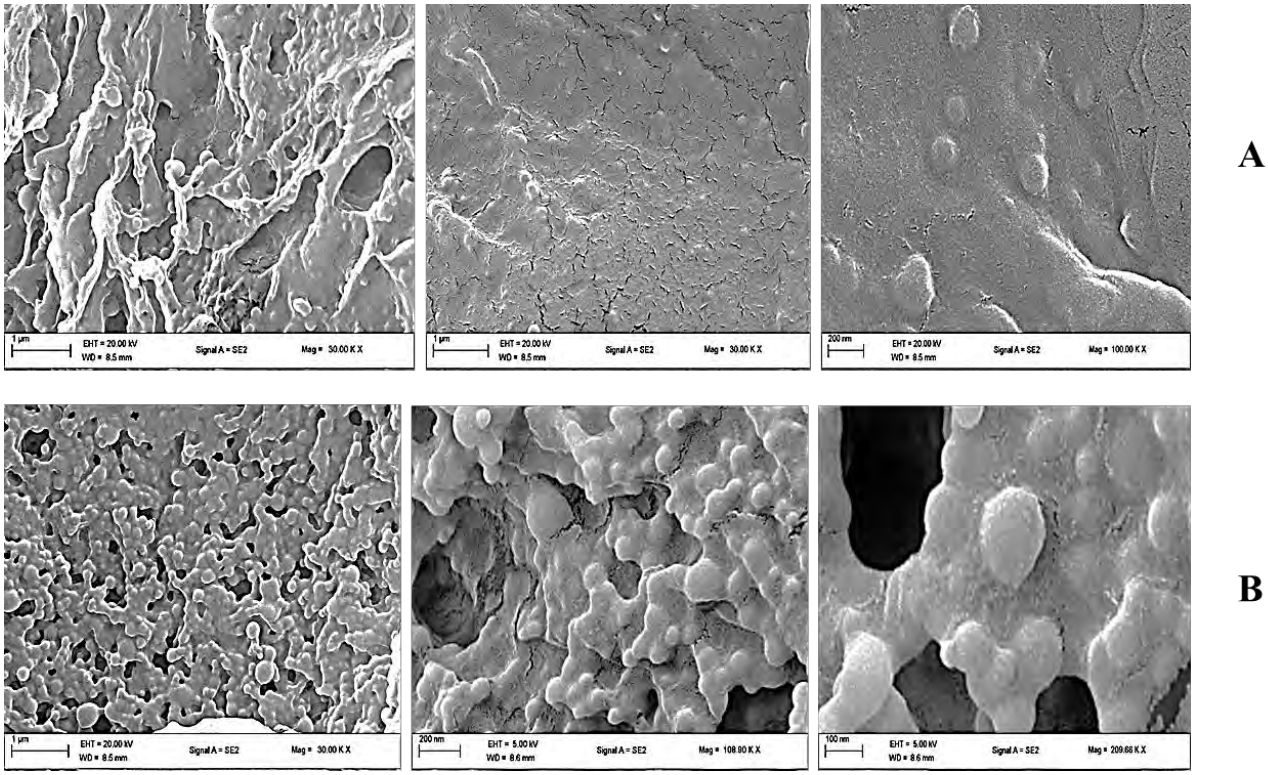


Figure 4: SEM diagrams of the optimised nanoparticles: PBC-PLGA 301 (A) and PBC-PLGA 302 (B)

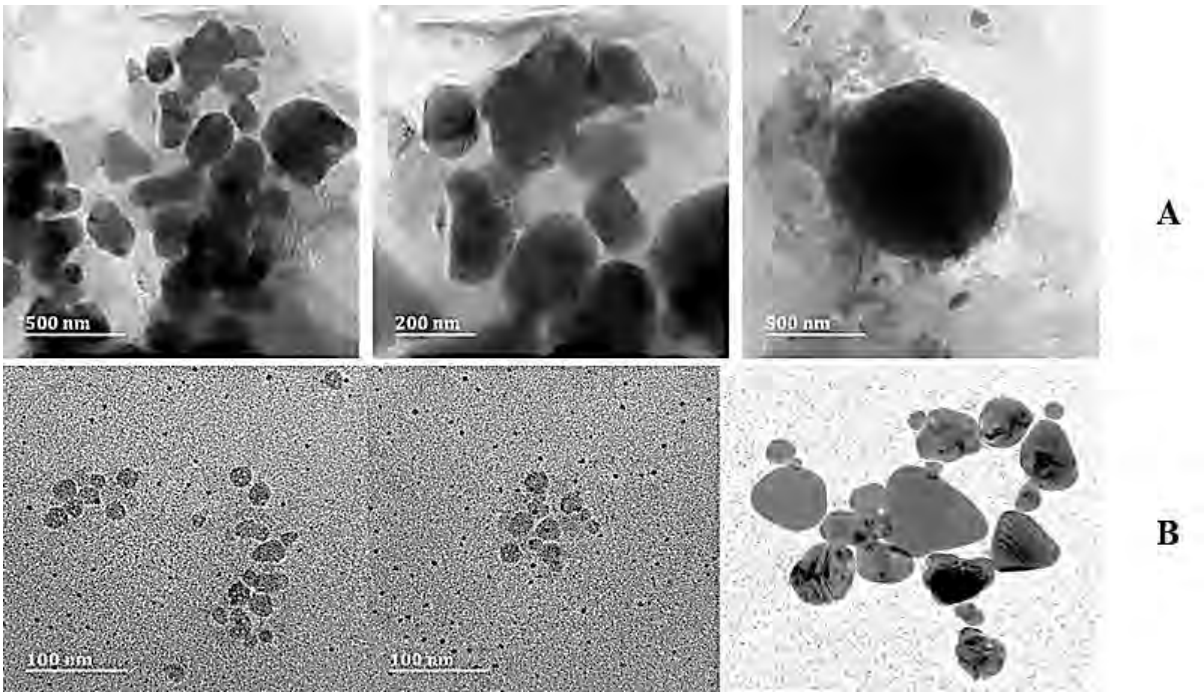


Figure 5: TEM diagrams of the optimised nanoparticles: PBC-PLGA 301 (A) and PBC-PLGA 302 (B)

3.3 Entrapment Efficiency and Drug Loading Capacity

The stated HPLC technique was used to measure entrapment efficiency and drug capacity, and results were derived using equations 1 and 2. Table 1 summarises the findings. All particle systems have two important parameters: entrapment efficiency and drug-loading capacity. The proportion of PBC encapsulated in the particles in relation to the total amount of the chemical employed in the preparation is denoted by EE. As a result, it indicates the method's yield for the active material. Drug leakage from nanoparticles caused by an erroneously designed production method, formulation parameters, or drug degradation during the preparation process can all affect EE [84, 85].

On the other hand, the Loading capacity (LC) is the ratio of the drug loaded in the nanoparticles to the total mass. LC is not only associated with the dosage of the drug but also with its dissolution profile and nanoparticle degradation. The molar mass, chain architecture, and character of the polymer end groups influence the LC of PLGA derivatives [60]. The polymer encapsulates the drug mainly through non-covalent hydrophobic interactions in the nanoprecipitation method. It is reported that most PLGA nanoparticle systems have relatively low drug loading (< 10%), and developing methods to increase it remains a challenge [86].

In our work, a low molecular weight PLGA (50:50) was deployed to encapsulate the pyrazolone-based compounds. PBC-301 and PBC-302 were effectively incorporated into the nanoparticles with high cumulative efficiencies of 84.20 ± 0.930% and 81.5 ± 2.051%, respectively, which is in line with and even higher than several other reports in the literature on PLGA nanoparticle carriers. For example, average encapsulation efficiency of 60-70% for drugs like estradiol and xanthones and 6-90% for dexamethasone and paclitaxel has been reported [25]. However, encapsulation efficiencies of 39.0 ± 1.6% with dendrimer and 64.3 ± 7.89% with pegylated PLGA nanoparticles were reported for similar pyrazolone-based compounds; indicating that the pyrazolone-based compounds in this study were well encapsulated using PLGA. Despite having high encapsulation efficiency, the nanoparticle system presented with low loading capacities of 0.30% and 0.11% for PBC-103 and PBC-302, respectively.

This Poor drug loading may be due to a minimal swelling of the PLGA macromolecular chains in aqueous media to entrap the compound.

Low loading capacity in the range of 0.1 to 1 mg per 100 mg nanoparticles has been previously reported for nucleic acid-encapsulated PLGA nanoparticles [78].

3.4 *In Vitro* drug-release study

The release of the pyrazolone-based compounds from the PBC-PLGA nanoformulations was investigated using 7.4 PBS at 37 ℃ for 48 hours (h), and the cumulative fraction released/dissolved presented in Fig. 6. The drug release from polymer-modified nanoparticles is a complicated process. Drug release from PLGA nanoparticles is a complex process affected by many factors, such as polymer degradation, molecular weight, the binding affinity between the polymer and drug, rate of diffusion, protected layer stability, and the drug's physicochemical properties.

Compared with the non-encapsulated compounds (PBC-301 and PBC-302), a biphasic release pattern was observed with the PBC-PLGA NPs: an initial fast release over the first 10 h followed by a slow, sustained release over a prolonged time (48 h). Only 2.5 and 6.4% of non-encapsulated compounds were dissolved in 48 h. However, approximately 37% and 53% of the compounds were released from the nanoparticles simultaneously. Prolongation in release time is attributed to the slow degradation of PLGA, indicating that PBCs release from nanoparticles largely depends on the rate of its diffusion from the polymer surface/matrix and bulk erosion/swelling of the polymer [87]. The extended-release pattern observed in this study can also be due to the strong hydrophobic interactions of compounds with PLGA, which further prohibited a rapid release of the compounds from nanoparticles.

In addition, the dissolution pattern of the nanoparticles revealed a burst release during the first 10 hours of the research, followed by a lag phase of relatively gradual release, as has been widely described in the literature concerning PLGA nanoparticles [50, 61, 88]. This first burst impact is attributable to the quick release/dissolution of medications adsorbed to the surface and those positioned near the surface of the nanoparticles and can be effective in suppressing the advancement of a disease state in a short

time. According to the release curves, PBC-301 released slower than PBC-302 in both free-drug and their respective nanoparticle suspensions, which might be due to PBC-301's poor water solubility in contrast to PBC-302. Similarly, the cumulative percentage dissolved for PBC-PLGA 301 was greater in the first three hours of the experiment than for PBC-PLGA 302, owing to barer compound adsorption on the surface of nanoparticles. However, the cumulative percentage dissolved for PBC-PLGA 301 from the nanoparticles was lower at 48 hours than that of PBC-PLGA 302, indicating that PBC-302 dissolution was enhanced.

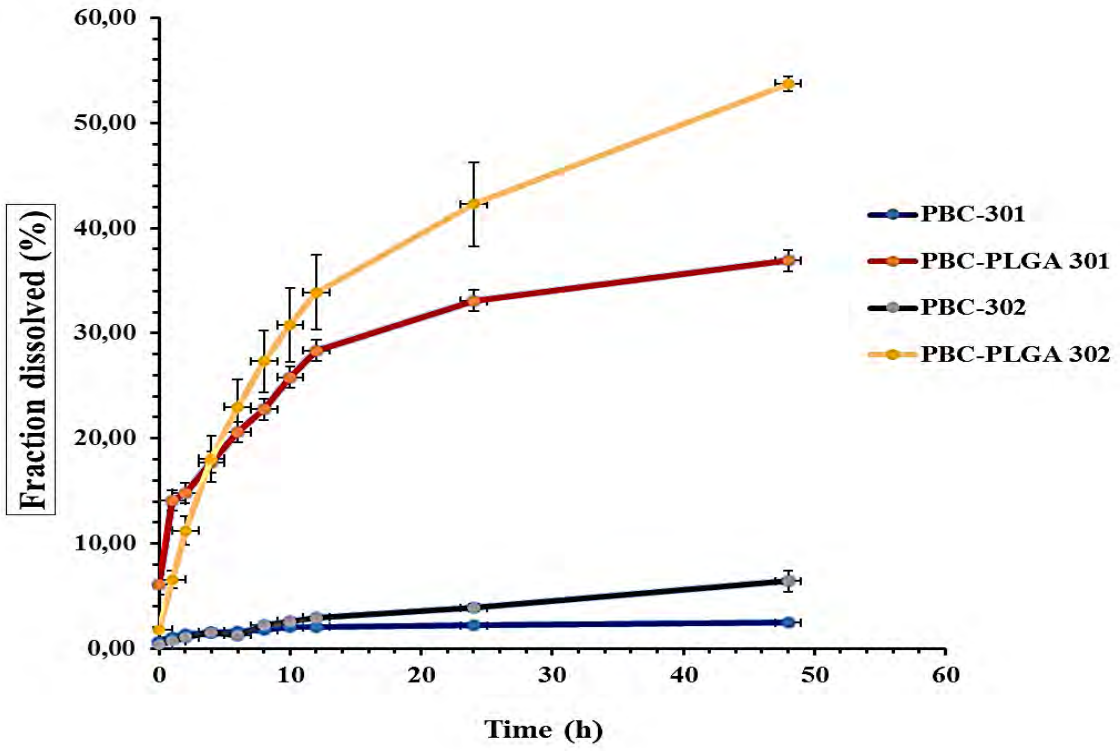


Figure 6: Comparison of cumulative percentage fraction dissolved from PBC-PLGA 301 and PBC-PLGA 302 nanoformulations with the Bare compounds PBC-301 and PBC-302 (Mean ± SD, n=3)

3.5. Differential scanning calorimeter (DSC)

The samples were DSC analysed to look for structural interference between the polymer and the medication and to look for the creation of solid-state complexes. The DSC results acquired in this investigation demonstrated that the PBC was successfully encapsulated within the carrier polymer

(CS). Individual peaks of each chemical (the bioactive ingredient and the polymer) can be seen in the DSC thermogram if the encapsulated bioactive material is not fully integrated into the encapsulating polymer [89]. The absence of a PBC peak in the PBC-PLGA nanoparticles thermogram (Fig. 7 A and B) indicates that the PBC is in the disordered-crystalline phase. Thus, the absence of typical melting and crystalline peaks in the thermograms of PBC-PLGA nanoparticles indicates that PBC has been encapsulated in the PLGA/poloxamer complex.

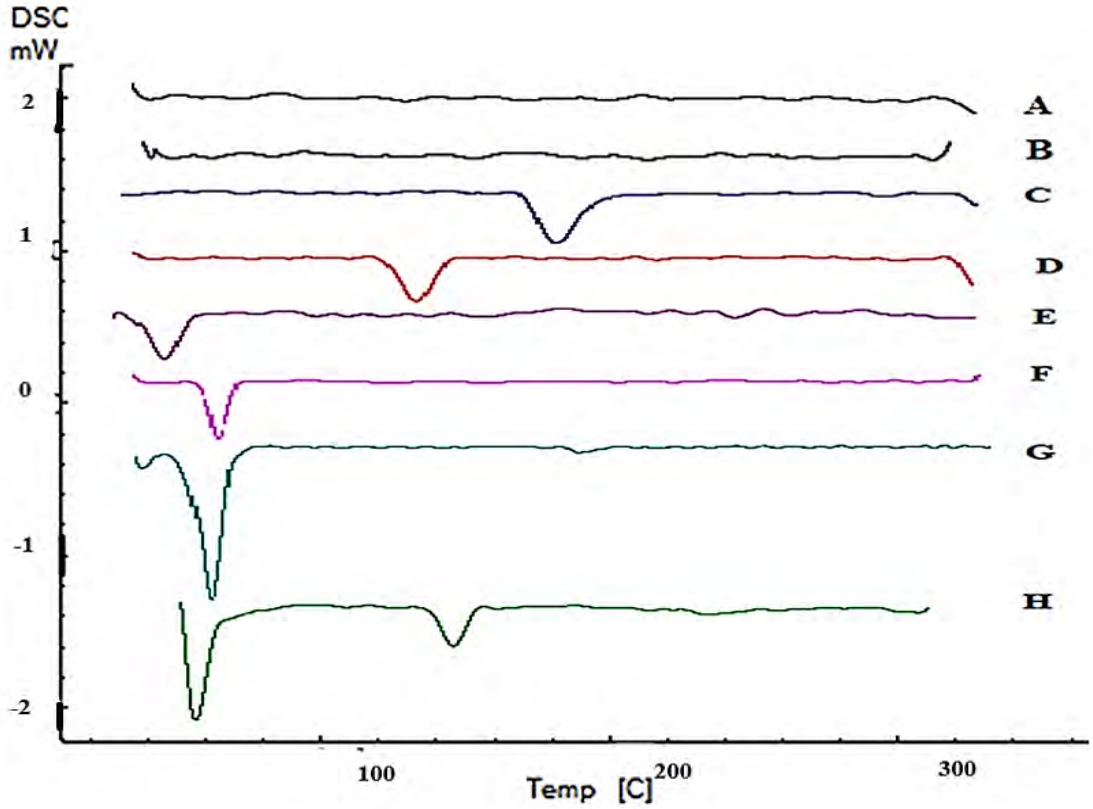


Figure 7: DSC thermogram of the Bare compounds, Excipients, and Lyophilised nanoparticles. A-PBC-PLGA 301, B-PBC-PLGA 302, C-PBC-301, D-PBC-302, E-PLGA, F-Poloxamer, G-PBC-301 physical mixture, PBC-302 physical mixture.

3.6. Antimicrobial activity

The non-encapsulated pyrazolone-based compounds and their nanoformulations were tested for antibacterial activity against *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus* and *Methicillin-resistant staphylococcus aureus*. Ciprofloxacin was used as a standard drug for PA, EC,

and SA, whereas vancomycin was used for MRSA in the study. The minimum inhibitory concentration (MIC) values used to evaluate the antimicrobial activities of the test compounds are summarised in Table 2, and higher MIC values indicate low antibacterial activity. Table 2, representing the MIC values, shows that the PBC-PLGA 301 and PBC-PLGA 302 nanoformulations had better antibacterial activity against *E. coli, S. aureus and* MRSA than the non-encapsulated PBC 301 and PBC-302. The MIC obtained for MRSA was comparable to the standard drug vancomycin, thus proposing the nanoformulations (PBC-PLGA 301 and PBC-PLGA 302) to be better targeted against MRSA. However, all the test samples showed minimal activity against *P. aeruginosa* at 62.5 µg/mL for both non-encapsulated PBCs, 92 µg/mL for PBC-PLGA 301 and 61 µg/mL for PBC-PLGA 302. Studies have shown that antimicrobial activity depends on the type of cell membranes of the bacteria, the core moiety of the drug, and the substituted functional groups. Similar **c**ompounds containing the pyrazole/hydrazo-/thiazole moiety have been previously found to possess antibacterial activity [90-92]. The MIC values obtained from the non-encapsulated compounds, when compared to their nanoformulations, can be inferred that enhancing the solubility of the compounds resulted in improved antimicrobial activity against *E. coli, S. aureus and* MRSA, which is in line with the previous report by Alfei and Sun et al. on bioactivity being improved by solubility enhancement [48, 50]

Table 2: Minimum inhibitory concentration (MIC) of the non-encapsulated PBC and their nanoformulations (µg/mL)

Organisms	**Test Samples**					
	PBC 301	**PBC 302**	**PBC-PLGA 301**	**PBC-PLGA 302**	**CIPRO**	**VCM**
P. aeruginosa	62.5	62.5	92	61	0.49	-
E. coli	125	125	15.25	30.5	0.49	-
S. aureus	62.5	62.5	11.25	7.63	0.49	-
MRSA	125	31.25	0.95	1.406	-	3.9

3.7 Bacterial Cell membrane permeability

MRSA and SA bacterial cells were incubated with the nanoformulations (PBC-PLGA 301 and PBC-PLGA 302) and the standard drugs (ciprofloxacin and vancomycin) for 24 hours. The PI dye was utilised to test the cell permeability of the nanoformulations, which resulted in cell death. The PI dye is an impermeant membrane dye that is often avoided by live cells. It attaches to double-stranded DNA by intercalating between base pairs, and its presence implies cell death or a loss of membrane integrity. Ciprofloxacin was employed as a control for SA and VCM and as a control for MRSA to demonstrate cellular uptake or cell killing in this investigation. VCM degrades the cell wall's integrity, increasing PI permeability and absorption. SA and MRSA samples were collected from wells 6 and 5, respectively. The data was subsequently recorded and analysed using the flow cytometer software Kaluza-2.1 (Beckman Coulter USA). The bacterial cell was treated with nanoformulations and conventional control medicines, which resulted in a change in PI fluorescence, as seen in Figure 8. The PBC-PLGA nanoformulations demonstrated greater cell membrane permeability than the conventional medicines Ciprofloxacin and Vancomycin. Furthermore, the nanoformulations demonstrated greater than 90% permeability into SA compared to MRSA, which is significantly less than 90% for PBC-PLGA 302 and 86% for PBC-PLGA 301. These findings imply that nanoformulations have greater penetration than normal medicines and might be potential antibacterial agents.

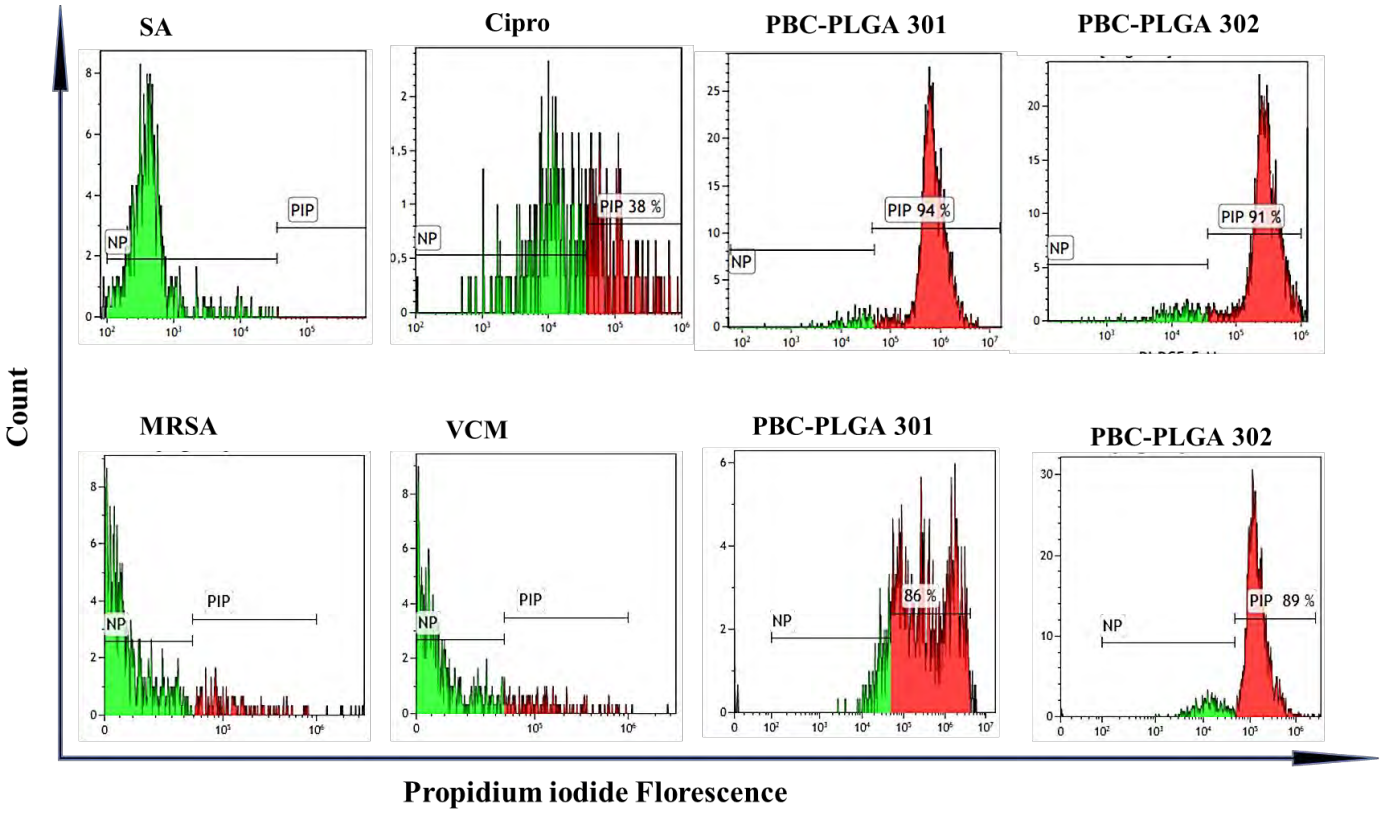


Figure 8. Propidium dye uptake vs cell counts: Green shows untreated SA/MRSA (Not PI permeable); Red represents the proportion of absorption in the population following Cipro, VCM, PBC-PLGA 301, and PBC-PLGA 302 incubation.

3.8 *In vitro* Haemolysis study

The haemolysis test is one of the tools that pharmaceutical scientists utilise to represent the toxicity of formulations in blood circulation. As a result, haemolysis is stated to have happened when RBCs break down, releasing the contained haemoglobin into the surrounding medium. As a result, a nanoformulation must interact with bloodstream components as little as possible while remaining stable over time. The "American Society for Testing and Materials (ASTMF 756-00, 2000)" classified materials into three categories: haemolytic (haemolysis greater than 5%), mild haemolytic (haemolysis between 2% and 5%), and non-haemolytic (below 2%) [52, 93]. The *in vitro* haemolysis investigation for the PBC-PLGA nanoformulations was performed in this work using sheep blood, and the average haemolysis is shown in Figure 9. According to the graph, PBC-PLGA 301 is non-haemolytic (less than

2%), showing that the formulation is extremely haem compatible. The PBC-PLGA 302 formulation, on the other hand, demonstrated mild haemolysis, with average haemolysis ranging between 2 and 5%, suggesting that it is mildly compatible with blood components. A comparable study on PLGA: poloxamer mix nanoparticles found less than 1% haemolysis [94]. As a result, the minimal interaction found with PBC-PLGA 302 with organic components of the blood may be related to other parameters, such as particle type and concentration, as stated by Kim et al. [95]. However, coating these particles with hydrophilic polymers, such as PEG, may improve their biocompatibility [95].

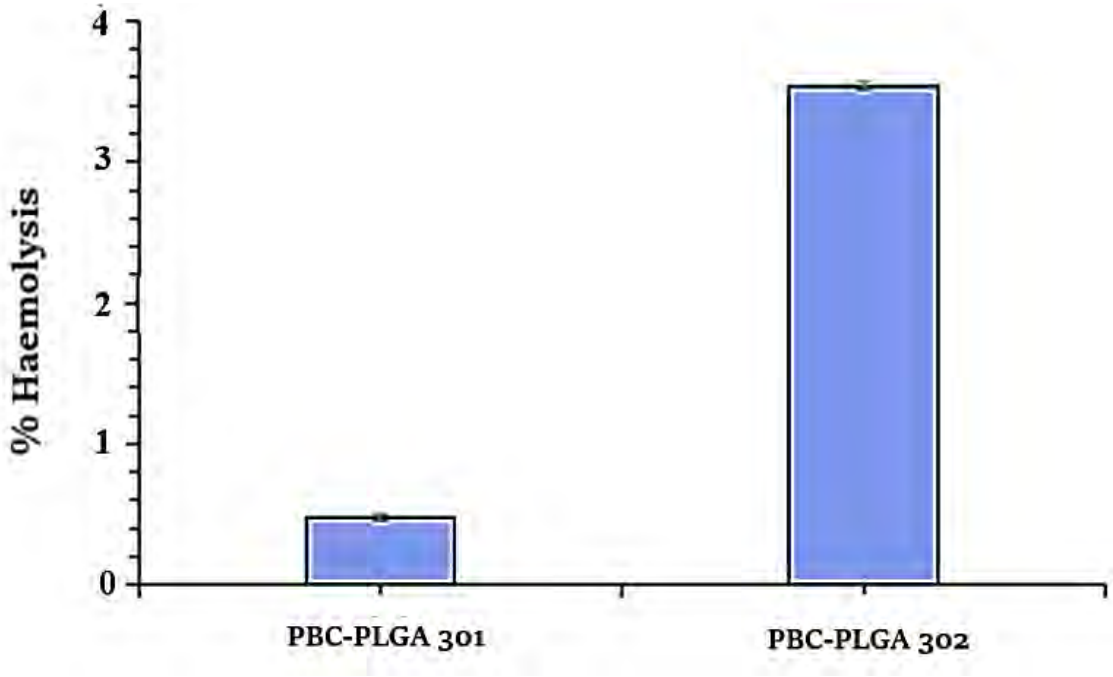


Figure 9. Percentage Haemolysis of PBC-PLGA nanoformulations.

3.9 Radical scavenging activity

Free radicals are highly reactive and unstable molecules that induce cellular damage in live cells and can cause DNA damage and strand break [96]. These free radicals have been linked to a variety of disorders, including hepatic and vascular disease, inflammatory diseases, rheumatoid arthritis, oxidative stress, cancer, and ageing. Significant progress has recently been achieved in the study of free radicals and the creation of antioxidants. Antioxidant molecules prevent free radicals from stealing electrons from another molecule, preventing molecule destabilisation and oxidative damage- a function

accomplished by providing an electron to the free radical without destabilising themselves, thereby terminating the free radical chain reaction [97]. Using Gallic acid as the reference, the DPPH, Ferric reducing antioxidant power (FRAP), and Nitric oxide (NO) scavenging activities of free PBC and PBC-PLGA nanoparticles were investigated in this work. Data are presented as mean ± SD.

Results obtained in this study indicated different DPPH, FRAP, and NO scavenging rates of the tested pyrazolone-based compounds and their optimised nanoparticles (Fig 10 and 11), respectively, with the tested components showing a dose-dependent radical scavenging activity. The novel pyrazolone-based compounds exhibited moderate scavenging activity of less than 40% with DPPH, with Gallic acid outperforming the compound at the different concentration ranges. However, there were no substantial differences in the scavenging activity for the different compounds and Gallic acid for the FRAP and NO assay, even though activity was less than 20% at all test concentrations with NO. IC50 values (Table 3) obtained in the three assays further established that PBC-301 has better FRAP scavenging activity than the Standard antioxidant and a better antioxidant when compared to PBC-302. Both compounds contain the pyrazolyl-thiazole moiety and hydrazo linkage previously implicated in antioxidant activity [98-101], suggesting that their radical scavenging activity could result from these moieties.

Figure 11; illustrates the PBC-PLGA nanoparticles' antioxidant efficiency in the assays. In the DPPH assay, the PBC-PLGA 301 and PBC-PLGA 302 nanoparticles at higher concentrations of 15-60 µg/mL exhibited solid antioxidant activity (70-80%) compared to the standard gallic acid, which is not so at lower concentrations. However, PBC-PLGA 301 is a better DPPH scavenger than PBC-PLGA 302. The nanoparticles showed better Ferric-reducing ability at all test concentrations than Gallic acid in the FRAP assay. Also, the antioxidant power of the PBC-PLGA 301 and 302 nanoparticles are comparable at all test concentrations as no significant difference was observed, and both attained a maximum scavenging activity of 70% at the highest test concentration. While the test samples indicated strong antioxidant power with DPPH and FRAP assays, they appeared to have moderate NO

scavenging activity (maximum of 50%) in a dose-dependent manner. There was no significant difference in the NO scavenging activity of both PBC-PLGA nanoparticles and the antioxidant standard Gallic acid. The IC50 values of the nanoparticles in the different assays (Table 3) further substantiated the percentage scavenging activity in Fig. 10 and 11. Generally, the nanoformulations showed improved and better antioxidant activity compared to the free compounds, as an approximately three-fold increase in percentage scavenging activity was observed in all assays.

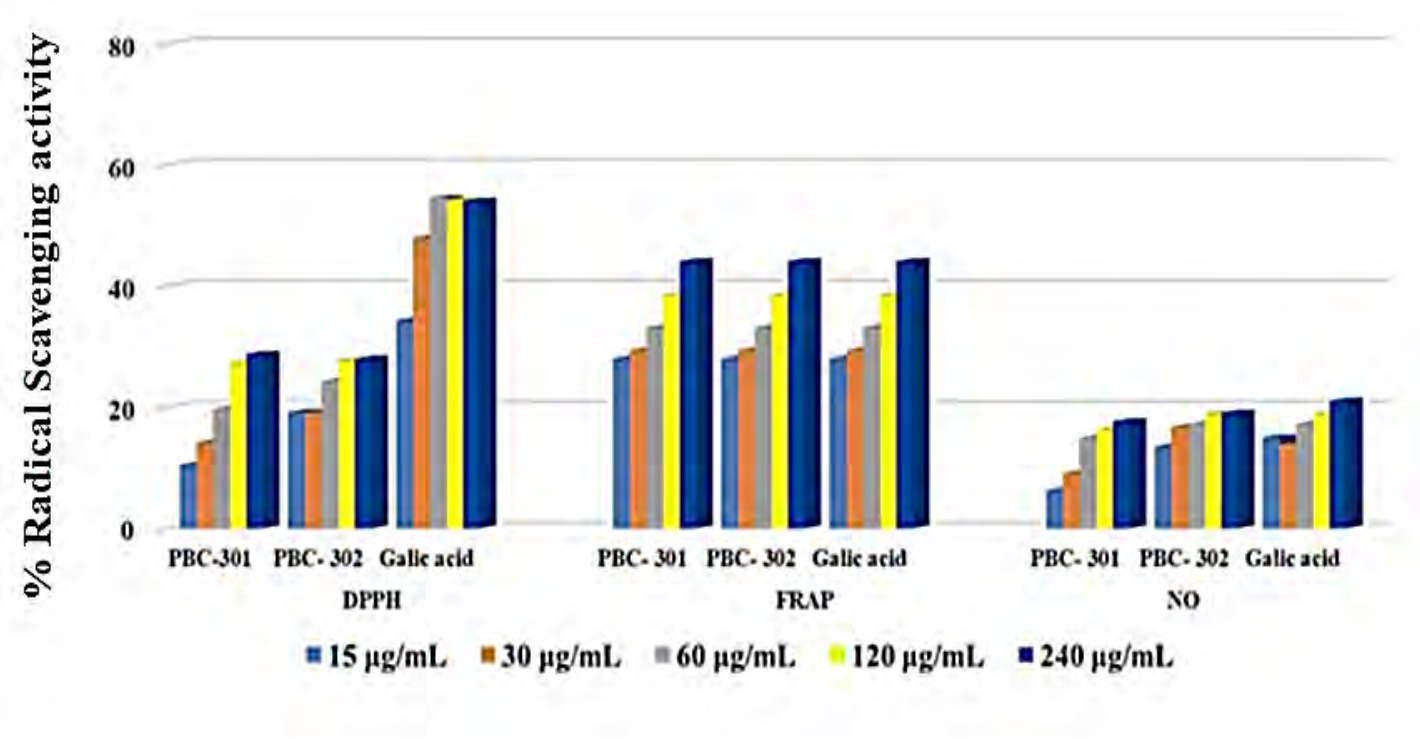


Figure 10: Radical scavenging activity of novel PBC-301 and PBC-302 against DPPH, FRAP and NO

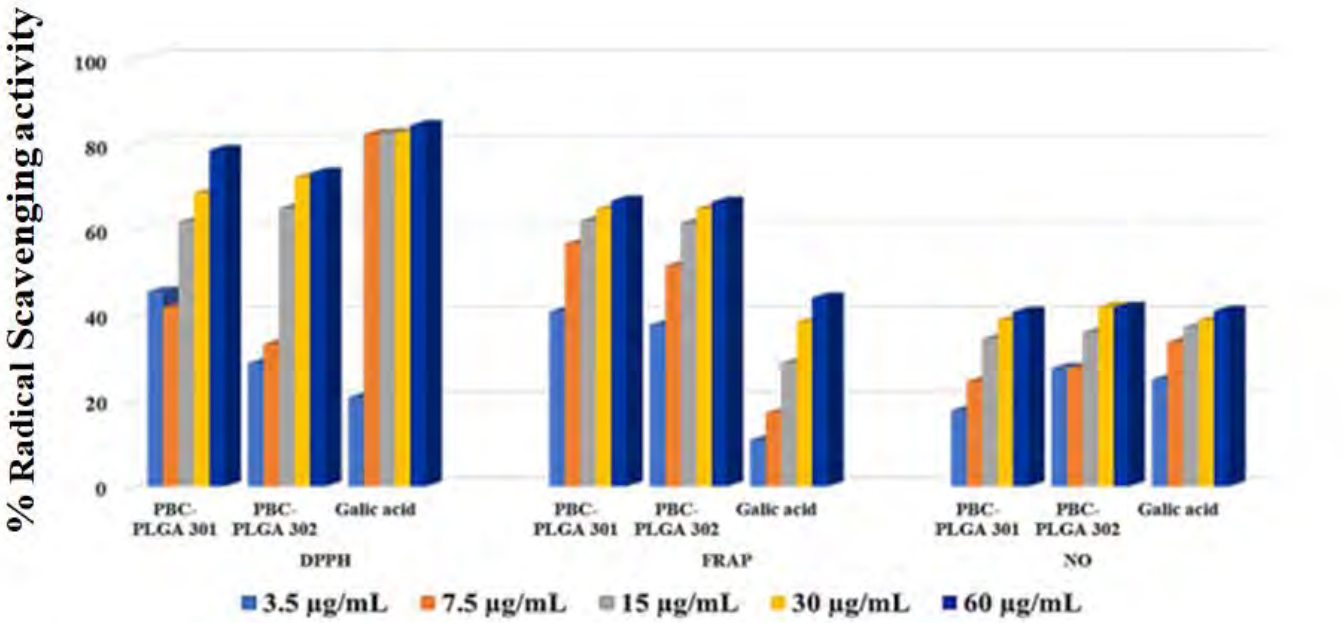


Figure 11: Radical scavenging activity of novel PBC-PLGA 301 and PBC-PLGA 302 against DPPH, FRAP and NO

Table 3: IC50 values of biological activities exhibited by the bare pyrazolone-based compounds and their nanoparticles on different antioxidant parameters

Biological activity	PBC-301	PBC-302	Gallic acid	PBC-PLGA 301	PBC-PLGA 302	Gallic acid
DPPH	8.99	13.03	3.2	2.00	2.64	1.38
FRAP	4.6	15.04	6.72	1.62	2.08	5.51
NO	15.4	27.73	22.17	6.08	6.48	6.99

4.0 Conclusion

Poor solubility is a major setback in the drug development process. In this study, nanoformulations of two novel pyrazolone-based compounds were developed and optimised to enhance solubility and biological activity simultaneously. The optimised nanoformulations showed enhanced solubility with

better-improved antimicrobial and antioxidant activity compared to the bare compounds. The nanoformulations showed highly significant cell permeability (greater than 85%) for both SA and MRSA culminating in cell death, hence suggesting them as bactericidal agents. The haemolysis assay also indicated that PBC-PLGA 301nanosuspension is non-haemolytic (less than 2%) and very haem compatible whereas PBC-PLGA 302 nanosuspension, showed mild haemolysis therefore should be used with caution when administered intravenously. With these promising results, the pyrazolone-based derivatives' nanoformulations can be exploited as potential pharmaceutical agents to fight bacterial infections and other diseases triggered by oxidative stress, cancer, hepatic and vascular diseases.

Declaration of Competing Interest

The authors state that they do not have any known competing monetary interests or personal ties that may have influenced the work disclosed in this study.

Acknowledgement

The authors thank the University of KwaZulu-Natal (UKZN) College of Health Sciences, the UKZN Nano drug delivery unit, the Microscopy and Microanalysis Unit, the Department of Human Physiology, and the Flow Cytometry Research Laboratory.

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CHAPTER 5: CONCLUSION

5.1 Introduction

The pharmaceutical industry focuses on developing efficacious therapies for potentially life-threatening diseases, turning the searchlight onto the heterocyclic compounds, especially the pyrazolone nucleus. The pyrazolone nucleus, often referred to as the "multipurpose molecule," is well studied for its numerous pharmacological activities such as antibacterial, antioxidant, analgesic, anti-inflammatory, anticancer, antifungal, antidiabetic, antiviral, antiproliferative, anti-TB, CNS, and cardiovascular effect. However, pyrazolone-based compounds' poor solubility and stability have consistently limited their pharmaceutical usefulness. The poor solubility of new drug entities not only affects the ease of handling during manufacture but also affects *in vitro*/*in vivo* biological activity assays and clinical use of the drug, thereby leading to prolonged time and increased cost in drug discovery and development. Attempts to resolve this led to the development of different strategies to enhance the solubility of these poorly soluble entities. Among the various described approaches for solubility enhancement, the nanoformulation approach is considered the most convenient owing to its cost-effectiveness, remarkable thermodynamic stability, high capacity of solubilisation, relatively low viscosity, the capability of undergoing vigorous sterilisation techniques, and the ability to encapsulate hydrophobic drugs due to their hydrophilic nature. Therefore, the specific aim of this research was to prepare a nanoformulation of two novel pyrazolone-based compounds to enhance their solubility and biological activity simultaneously.

The main conclusions generated from the research data are summarised below:

- A specific, accurate, and precise reverse-phase high-performance liquid chromatography method was developed and validated following ICH guidelines to detect and quantify the novel pyrazolone-based derivatives in nanosuspensions. Results demonstrated that the developed method is specific, precise, and accurate within the recommended limits. The method showed good linearity with a

0.9994 correlation coefficient over a concentration range of 2.5-50 μg/ml. The method efficiently detected and quantified the novel pyrazolone compound in the nanoformulation.

- PLGA: Poloxamer complex was employed to formulate the PBC- loaded nanosystems. Size, PDI and ZP of the optimised nanoformulations were 166.6 ± 7.12 nm, 0.129 ± 0.042, -14.14 ± 2.90 mV for PBC-PLGA 301 and 192.5 ± 1.08 nm, 0.132 ± 0.025, -10.77 ± 1.515 mV for PBC-PLGA 302 with the encapsulation efficiency being 84.20 ± 0.930% and 81.5 ± 2.051% respectively. Further characterisation of PBC-PLGA nanoparticles by DSC showed that no new chemical compound was formed during formulation and that the PBCs were converted into an amorphous form in the formulation.
- The compound release from the nanovesicles followed a sustained release pattern, with PBC-PLGA 301 and PBC-PLGA 302 attaining a cumulative release of approximately 37% and 53% in 48 hours compared to the bare compounds, which attained less than 5% release in the same period.
- The biological activity assays showed a better-enhanced activity with the nanoformulations compared to the non-encapsulated PBC 301 and PBC-302. *In vitro* antibacterial activity revealed that the compound-loaded nanovesicles have better activity against the two gram-positive bacteria *S. aureus* and *Methicillin-resistant S. aureus* compared to the standard drug vancomycin and the non-encapsulated compound. On the other, the cell penetration assay further revealed that the compound-loaded nanovesicles achieved greater than 90% propidium iodide penetration (translating to cell death) at the reported MIC well for *S. aureus* while showing 86% and 89% cell penetration for *Methicillin-resistant S. aureus.* Also, the nanoformulations showed improved radical scavenging activity in a concentration-dependent manner, with PBC-PLGA 301 exhibiting the best antioxidant activity against DPPH, FRAP and nitric oxide compared to the standard antioxidant-gallic acid and the non-encapsulated compounds.

Therefore, the findings of this study confirmed that the nanoformulation approach is a convenient and valid means of enhancing the solubility of poorly soluble drug candidates. Also, enhancing the

solubility of a poorly soluble drug candidate can improve the biological activity, hence its pharmaceutical applicability.

5.2 Significance of the findings in the study

The nanoformulation approach of solubility enhancement was successfully employed to address the poor solubility associated with two novel pyrazolone-based compounds. The significance of the findings in this study includes the following:

New Pharmaceutical products: PBC-PLGA 301 and PBC-PLGA 302 were formulated with improved solubility and biological activities, enabling their possible pharmaceutical and biological applications. Also, these novel compound formulations are potential pharmaceutical products to combat drug resistance and serve as new drug entities for effective therapeutic outputs.

Improved patient therapy and disease treatment: The PBC-PLGA nanoformulations exhibited significant antibacterial and antioxidant activities. Therefore, there is a foreseeable transition of these formulations to clinical usage, especially in infectious diseases, cancer therapy, and other diseases triggered by oxidative stress.

Creation of new knowledge to the scientific community: This study and its findings have contributed several ways to the pharmaceutical sciences knowledge database. These include the following:

- New knowledge was generated by identifying methods of solubility enhancement, preparation, and characterisation of the PBC-PLGA nanoformulations, which can serve as a basis for selecting techniques for solubility enhancement, increasing the pharmaceutical applications of poorly soluble drug candidates and possibly reduce the cost and time of drug development.
- The developed method can be effectively applied to analyse the compound of interest in pharmaceutical nanoformulations, raw material analysis, and detect impurities. The method validation report can also serve as a template for other researchers working on similar projects.

- Formulation parameters and processes of the PBC-PLGA nanoformulations were identified using various experimental techniques.
- The in-depth antimicrobial and antioxidant testing and flow cytometry cell permeability assay showed improved biological activities of the compounds after solubility enhancement. Also, the haemolysis study showed the biosafety of the formulations. Therefore, the information will help researchers working on similar compounds better predict their proposed studies' outcomes.

Stimulation of new research: The search for new and improved therapy is imminent. Therefore, this research findings can provide potential research directions to explore

i. The development of nanoformulations of other poorly soluble pyrazolone-based derivatives accelerates the discovery and development of new drugs for therapy.
ii. The possibility of conducting other biological *in vitro/in vivo assays.*
iii. Further biological activity testing against cancer, HIV, Diabetes, and inflammation can be undertaken.

5.3 Recommendations for future studies

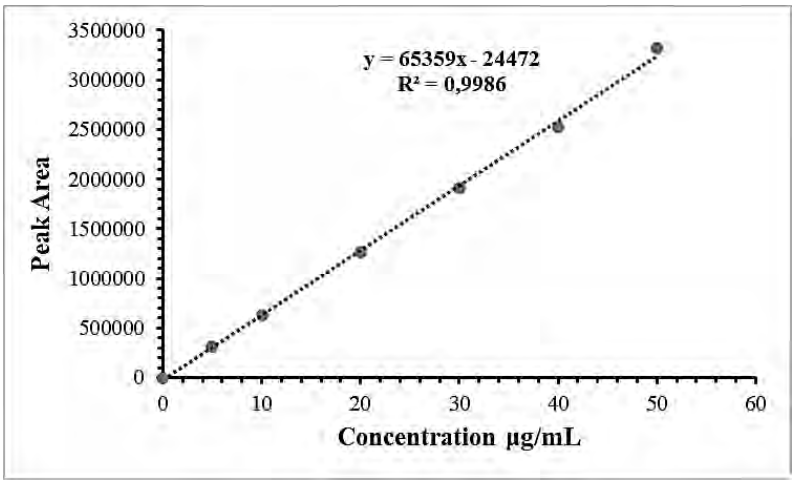
Although this approach has demonstrated the simultaneous enhancement of solubility and biological activities of the novel pyrazolone-based compounds, additional studies are necessary to improve their formulations to ensure eventual regulatory approval for patient use.

The following studies are proposed:

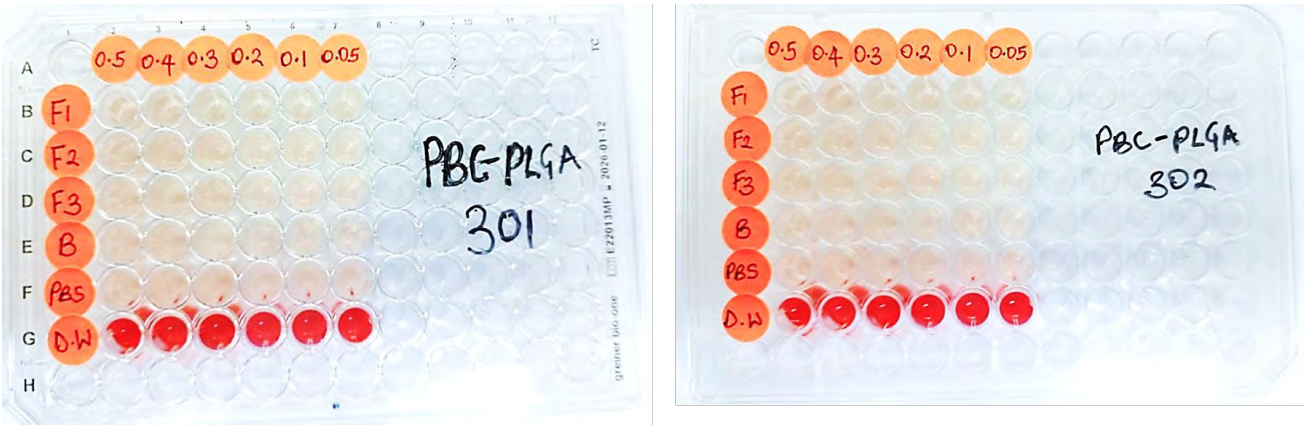
- The next phase would be to formulate other poorly soluble pyrazolone-based derivatives using this simple technique, study the effect of surfactants in the market and explore other formulation parameters to enhance their water solubility and activity further.
- Further in silico modelling and simulation studies are required to understand better molecular interactions of the PBC-PLGA 301 and PBC-PLGA 302 nanoformulations with the SA and MRSA bacteria.

- A qualitative analysis of the bacterial cell protein degradation can be performed using the SDS-PAGE technique to understand the mechanism of antibacterial action of PBC-PLGA 301 and PBC-PLGA 302 nanoformulations. Also, western or dot blot analysis can quantitatively determine a specific protein.
- Further biological activity testing of the nanoformulations against cancer, HIV, diabetes, inflammation, and other diseases triggered by oxidative stress can be conducted.
- To establish their safety levels, other biosafety assays, such as cytotoxicity and genotoxicity assays, must be conducted.
- *In vivo* acute, intermediate, and long-term toxicity studies models can be performed to determine the full toxicological profile of the material and the formulations reported in this study.
- *In vivo* IV infection model, bioavailability, and pharmacokinetic studies followed by clinical trials on both the developed nano-systems could be performed to achieve approval for market introduction.
- Long-term stability studies using ICH conditions to assess optimised formulations' physical and chemical stability must be undertaken to confirm their shelf life.

APPENDIX



Appendix A. Calibration Curve for PBC-PLGA 301 analysis



Appendix B. Haemolysis images for PBC-PLGA 301 and PBC-PLGA