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“Development of Amorphous Ternary Solid Dispersions of a Novel Anti-Tubercular
Indole-2-Carboxamide Derivative with Curcumin/Clofazimine for Tuberculosis
Treatment”

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A THESIS

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Abstract

This research presents novel amorphous ternary (drug-drug-hydrophilic polymer) solid dispersions to simultaneously enhance the aqueous solubility of incorporated poorly soluble drugs. A highly potent novel anti-tubercular drug (i.e., Indole-2-carboxamide derivative (North 2)) was combined with an antioxidant (i.e., curcumin) and an anti-tubercular drug, clofazimine. Binary and ternary amorphous dispersions of North 2-Curcumin and North 2-Clofazimine were prepared with hydrophilic polymers, namely polyvinylpyrrolidone (PVP), Eudragit® EPO® (EPO), and hydroxypropyl methylcellulose (HPMC), at 1:1w/w (for binary solid dispersions) and at 1:1:2 w/w/w (for ternary solid dispersions) ratios. The binary and ternary solid dispersions and their respective physical mixtures were characterized for their crystallinity by X-ray diffraction (XRD) and differential scanning (DSC). Infrared spectroscopy (IR), solution nuclear magnetic resonance (NMR), and molecular modeling studies (docking studies using MOE with the MMFF94x forcefield) were utilized to understand the interaction between compounds and polymers. In the case of ternary solid dispersions, North 2: CUR: EPO at 1:1:2 w/w/w, North 2: CUR: HPMC at 1:1:2 w/w/w, and North 2: CUR: PVP at 1:1:2 w/w/w, North 2:Clofazimine:EPO at 1:1:2 w/w/w were confirmed to be amorphous by XRD and DSC. Significant peak shifts indicating molecular interactions such as hydrogen bonding were observed in the IR data of binary and ternary dispersions compared to physical mixtures, indicating potential North 2- polymer, Curcumin-polymer, and Clofazimine-polymer interactions. These interactions mainly involved carbonyl group of both compounds, indole NH of North 2 and Phenolic OH of Curcumin and NH amide of clofazimine with the polymer chain. These interactions were confirmed by solution NMR and molecular

modeling studies in case of North 2-Curcumin and EPO combination. For North 2-Curcumin, the dissolution studies showed that the concentrations of the compounds released from all the ternary solid dispersions were significantly higher than their physical mixtures. Out of the three hydrophilic polymers, EPO enhanced the solubility by 55 and 59 times for North 2 and CUR, respectively, within 12 hours. For North 2-Clofazimine, amorphous ternary solid dispersion of North 2:Clofazimine:EPO (1:1:2) found to be enhance the solubility of North 2 and clofazimine by 84 and 47 folds as compared to physical mixtures . For North 2-Curcumin and North 2:clofazimine:EPO ternary solid dispersions, XRD stability data of the ternary solid dispersions showed that they were stable over 90 days at room temperature.

Preface

Publications:

1. Shambhavi Borde, Dhirender Singh, Navneet Sharma, Duneshe Kumari, and Harsh Chauhan, Book chapter “*Solid Dosage Forms: Formulations and Characterizations*” in *Handbook of Space Pharmaceuticals*, Springer, The Language of Science, December 2018
2. Shambhavi Borde, Harsh Chauhan, Pooja Hegde, E. Jeffrey North, Pavan Prathipati, ‘*Development and characterization of ternary amorphous solid dispersions of novel anti-TB agent with anti-oxidant with polymer to improve the apparent aqueous solubility of the combination*’ Manuscript under submission process, October 2018

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with hydrophilic polymers for enhancement of aqueous solubility and stability'

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4. Shambhavi Borde, Harsh Chauhan '*Development and Characterization of Amorphous Formulation of Combinations of Poorly Soluble Compounds for Oral Delivery*' Best Poster Presentation Award Recipient, Albert's day, Creighton University, Nebraska, March 2017

Oral Presentations:

1. Shambhavi Borde, '*Development of amorphous ternary solid dispersion of novel anti-tubercular agent, indole-2-carboxamide derivative with clofazimine and curcumin for the combination therapy of tuberculosis with the enhancement of solubility of compounds*' 2nd Place winner, 3 Minute Thesis competition, organized by Midwest Association of Graduate Schools held at Creighton University, January 2019
2. Shambhavi Borde, '*Combination anti-TB therapy with enhanced aqueous solubility: Amorphous Ternary Solid Dispersions*' 2nd place winner, 3-minute presentation at Creighton's 2nd Annual Elevator Talk Competition hosted by the Phi Sigma Biological Honors Society and the Biology Club, April 2017

Dedicated to my family

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List of Abbreviations

µg	Microgram
µm	Micrometer
C	Celsius
Cur	Curcumin
CFZ	Clofazimine
North 2	N-(1-adamantyl)-1H-indole-2-carboxamide
DSC	Differential Scanning Calorimetry
FDA	Food and Drug Administration
FTIR	Fourier Transform Infrared Spectroscopy
G	Gram
HPMC	Hydroxy Propyl Methyl Cellulose
Hr	Hour
IUPAC	International Union of Pure and Applied Chemistry
M	Molar
MDSC	Modulated Differential Scanning Calorimetry
Mg	Milligram
Min	Minute
mL	Milliliter
Ng	Nanogram
Pka	Acid dissociation constant
PM	Physical Mixture
RPM	Rotations per Minute
SD	Solid Dispersion
TSD	Ternary Solid Dispersion
ATSD	Amorphous Ternary Solid Dispersion
TPM	Ternary Physical Mixture
EPO	Eudragit® EPO®
PVP	Polyvinylpyrrolidone
TB	Tuberculosis
SEM	Scanning Electron Microscopy

TGA	Thermo Gravimetric Analysis
UV	Ultraviolet
Vis	Visible
XRD	X-ray Powder Diffraction
°	Degree
Θ	Theta
LC-MS/MS	Liquid Chromatography -Mass Spectroscopy-Mass Spectroscopy
HPLC	High Performance Liquid Chromatography
QC	Quality Control
ICH	International Conference of Harmonization

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1 Introduction

1.1 Tuberculosis:

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*M. tb*), has existed for millennia and remains a major global health problem (Global Tuberculosis Control: A Short Update to the 2009 Report WHO, 2009) (WHO Report 2010 Global Tuberculosis Control, 2010). It causes health related issues in millions of affected people each year. In fact, for the past 5 years, tuberculosis has been one of the top 10 causes of death worldwide, ranking above HIV/AIDS as one of the leading causes of death from an infectious disease (Global Tuberculosis Report, 2016). According to most recent data in WHO report, in 2017, 10.0 million people were infected with TB, and 1.3 million HIV-negative people died from the disease and additional 300 000 deaths from TB among HIV-positive people (Global Tuberculosis Report, 2017). Current TB treatment comprises of a six-month minimum treatment with the administration of first-line agents such as isoniazid, rifampicin, pyrazinamide, ethambutol and streptomycin, and second line agents such as fluoroquinolones, aminoglycosides and others. Drug-resistant TB continues to be a public health crisis (Global Tuberculosis Report, 2017) (Bailo, Bhatt, & Anisa, 2015). Worldwide in 2017, 558 000 people (range, 483 000–639 000) developed TB that was resistant to rifampicin (RR-TB), the most effective first line drug, and of these, 82% had multidrug-resistant TB (MDR-TB). Among cases of MDR-TB in 2017, 8.5% were estimated to have extensively drug-resistant TB (XDR-TB) (Global Tuberculosis Report, 2017). MDR-TB is caused by strains of *Mycobacterium tuberculosis* that are resistant to at least rifampicin and isoniazid, two key drugs in the treatment of the disease (Martin, 2014). About 1.7 billion people are estimated to have a latent TB infection and are thus

at risk of developing active TB disease during their lifetime, this is about 23% of the world's population (Global Tuberculosis Report, 2017).

North 2: Recently, indole-2-carboxamides (IC) were successfully studied and evaluated for their anti-mycobacterial potency. These compounds were found to be active against mycobacteria at low concentrations (N.D. Franz, 2017). Out of series of compounds; North 2; N-(1-adamantyl)-1H-indole-2-carboxamide was found to be highly potent against mycobacteria and non-cytotoxic against THP-1 cells. However, the compound was found to have poor pharmacokinetic profile, mainly due to poor aqueous solubility. (A.N. Pandya, 2019) (Lun, et al., 2013) This compound can be classified in the Class II drugs. (N.D. Franz, 2017). (Figure 1.1)

Curcumin: curcumin, a known antioxidant, is an active component of the spice turmeric. It is (diferuloylmethane) a β -diketone constituent that is extracted from rhizome of *Curcuma longa* Linn. Curcumin possesses multiple beneficial properties, for example, anticarcinogenic properties, anti-inflammatory properties etc. Curcumin is classified into the BCS Class II drugs and needed to be worked on its solubility (Figure 1.1). (Zang, 2014), (Aanandhi, 2014) (Huang, 1997).

Clofazimine: Clofazimine is the prototype riminophenazine antibiotic. It was originally described in 1957. It is classified as Class III drugs in which both the solubility and permeability is poor. (Figure 1.1) (BARRY, et al., 1957). This molecule is very active against *Mycobacterium tuberculosis*, including multidrug-resistant mycobacterial strains (Reddy VM, 1999). Clofazimine has not been reported as active against other bacteria hence it is considered as narrow spectrum an anti-tubercular agent (I. Chopra, 1998).

This research is focusing on the two novel formulations one being combination of

compounds, belonging to Class II, North 2, Curcumin, and Clofazimine (**Figure 1.1**). The aim of this research is to develop ternary amorphous solid dispersions of all three compounds for the simultaneous enhancement of solubility of two compounds.

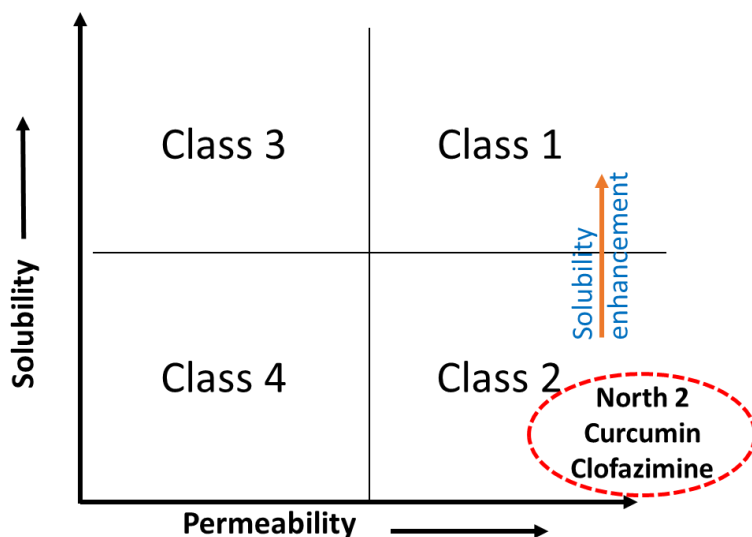


Figure 1.1: BCS classification based on solubility and permeability, North 2, curcumin, and clofazimine used in the project belong to Class II due to poor solubility

1.2 Poor aqueous solubility:

Poor solubility of the drugs has become a challenge for drug development. Most drugs, more than 40%, fail during drug development stages, due to poor aqueous solubility (Al-Kassas, 2017). Poorly water-soluble drugs, especially BCS class 2 drugs, which have low solubility and high permeability are of major interest for research and industrial approach. There have been many conventional methods for increasing the solubility of these compounds, which include use of solubilizing agents, salt formation, use of complexing agent, etc. However, all these approaches possess limitations caused by the side effects

of co-solvents, salt formation, addition of large amount of excipients, etc. (Aanandhi, 2014) (Huang, 1997). Other, more successful methods of increasing solubility include formation of liposomes (Li, 2014), inclusion of cyclodextrins (Tonnesen, 2002), emulsions, solid dispersions, etc. (Al-Kassas, 2017).

1.3 Significance of solubility for oral delivery:

The most commonly employed and convenient route of drug deliver is oral route because of its ease of administration, high patient compliance, less sterility constraints, cost effectiveness and flexibility in the dosage of drugs (Yellela, 2010). But the success of the oral route is dependent on the bioavailability, which is further dependent on various factors like permeability, aqueous solubility, dissolution rate, metabolism, susceptibility to efflux mechanism. The chances of achieving the desired concentration of the drug in plasma is dependent on the solubility of the drug and to achieve this (Vemula, 2010), poorly water soluble drugs often administered in the high doses which leads to adverse effects of the medication. The drug must be solubilized to be absorbed into the system and the poorly water-soluble drugs have slow absorption leading to less or varying bioavailability. Overall, improvement of solubility for optimum bioavailability after oral administration remains a significant challenge (Savjani, 2012).

1.4 Solubility v/s bioavailability:

Water soluble drugs: provided that the permeability and metabolic inertness is sufficient for the drug, most water-soluble drugs can penetrate the biological membrane while

passing through GI tract and can be available for their therapeutic effect (Lipp, 2013). In contrast, poorly water-soluble drugs will not be available for their therapeutic effect as they cannot cross the biological membrane because of the low solubility (Lipp, 2013). (Figure 1.2).

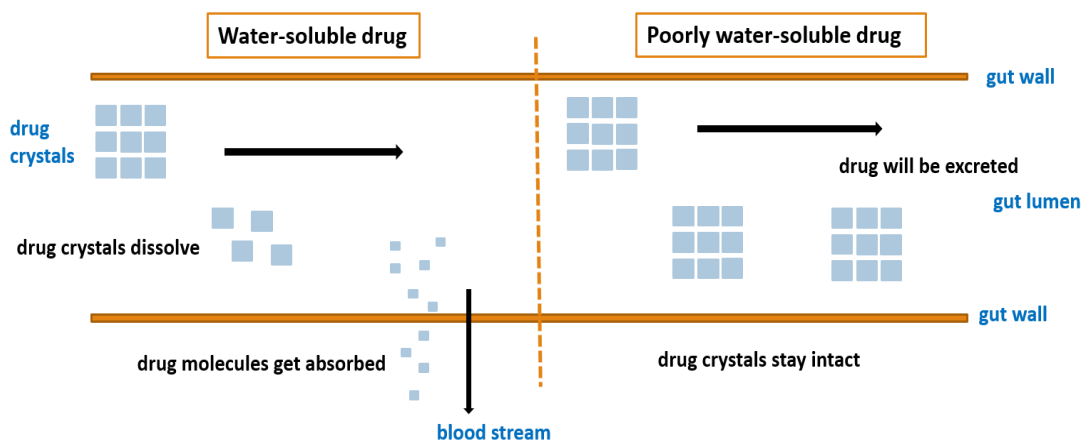


Figure 1.2: Dissolution and absorption of water soluble and water insoluble drugs in the gut (Lipp, 2013)

There have been many studies that try to enhance the solubility of these drugs by using various ways. There have been many conventional approaches for increasing the solubility of these drugs, which include use of solubilizing agent, salt formation, use of complexing agent, etc. But all these approaches are now limited due to side effects of co-solvents, salt formation, addition of large amount of excipients, etc. (T., 1996) (Johnson, 2003). There are other methods of increasing solubility which include, liposomes (Matloob, 2014), inclusion of cyclodextrins (Tonnesen, 2002), emulsions, solid dispersions, etc. (Al-Kassas, 2017). All these methods are used for enhancement of solubility of drugs with success.

1.5 Amorphous solid dispersions (ASDs):

ASDs consist of drug molecules dispersed in hydrophilic amorphous polymeric carriers. Drug stabilization is a consequence of factors such as intermolecular interactions, the anti-plasticization effect exerted by the polymer, physical barriers to the crystallization process (local viscosity), and reduction in the drug's chemical potential (D Prasad, 2016). The role of the polymeric carrier is not limited to stabilization, but also mechanisms responsible for improved dissolution rate and absorption. Hydrophilic carriers such as poly(vinylpyrrolidone) (PVP), poly-(1- vinylpyrrolidone-co-vinyl acetate) (PVP VA64), and hydroxypropyl methylcellulose (HPMC) are highly water soluble and enhance water uptake into the solid dispersion matrix. Carriers also play a crucial role in maintaining supersaturation and precipitation inhibition *in vivo*, which is widely accepted as critical to improving solubility in the GI tract (F. Meng, 2015). Another mechanism responsible for improved solubility is reduced particle size, resulting in increased surface area. In an ideal case (i.e., molecular dispersion), the surface area available for dissolution is the maximum since the drug size is reduced to (almost) a single molecule. This is not always the case and the active pharmaceutical ingredient (API) distribution within the carrier matrix becomes inhomogeneous, leading to drug-rich and polymer-rich regions (Al-Obaidia, 2011) (F. Meng, 2015). Since drug polymer miscibility is crucial for solid dispersion stabilization, phase separation can promote API crystallization (F. Meng, 2015). Therefore, every effort should be made to produce miscible solid dispersion systems and protect them from drivers of phase separation, such as high temperature, humidity, and mechanical stress.

1.6 Advantages of amorphous solid dispersion:

How the solid dispersions are beneficial can be explained by using the terms of enthalpy energy. These three terms are crystal packing energy, cavitation energy and solvation energy and these quantities mainly govern the solubility of the solid compound (Lipinski, 2012).

$$S = f(\text{Crystal Packing Energy} + \text{Cavitation Energy} + \text{Solvation Energy})$$

Crystal packing energy: energy necessary to disrupt the crystal lattice and to remove isolated molecules. Cavitation energy: energy required to disrupt water in order to create the cavity to host the solute molecule. Solvation energy: release of energy as favorable interactions is formed between the solvent and the solute. This crystal packing energy is larger than the other two energies and amorphous solid dispersion is formulated to minimize this energy by disrupting the crystal lattice in the dosage form. By doing this, in conjugation with hydrophilic polymers, apparent solubility can be increased by up to 10,000 folds (Hancock, 2000) (Miller, 2012). And in terms of Nernst-Brunner equation, faster resolution is due to drastic increase in saturation solubility (jermain, 2018).

There are some other mechanisms which can further contribute to solubility by reduced particle size resulting in increased surface area. And molecular dispersion is an ideal case, since the drug size is reduced to (almost) a single molecule and hence the surface area available for dissolution is the maximum. However, usually this is not the case for all the conditions, the active pharmaceutical ingredient distribution becomes inhomogeneous in the polymer matrix and forms the drug rich and polymer rich regions in the system. And this kind of phase separation can promote to the recrystallization of the drug which makes

the drug-polymer miscibility a crucial for solid dispersion stabilization. Therefore, every effort should be made to produce miscible solid dispersion systems and to prevent the crystallization of the drug in the system. (Lipinski, 2012).

Formulating a solid dispersion of these poorly soluble drugs has been a successful method for enhancement of solubility and dissolution in solution state. There are several studies which suggest that amorphous solid dispersion (ASD) of these drugs will result in enhancement of solubility (Tonnesen, 2002) (Meng F. , 2015). Binary amorphous solid dispersion of curcumin with polymers like Eudragit was prepared and proved to be more soluble than pure curcumin (Baldwin, 2015). In addition, curcumin formulated with EPO and HPMC showed enhancement in dissolution of curcumin for one hour and six hours, respectively (Meng F. , 2015). It has also been reported that enhancement of curcumin solubility by formulating with amorphous solid dispersions ASD also results in bioavailability of curcumin in rat models.

1.7 Binary solid dispersions and ternary solid dispersions:

Binary solid dispersions are formulations of drugs with a suitable polymer in which the drug is molecularly dispersed in the polymer matrix. The basic concept is to convert crystalline drugs into an amorphous form by mixing with an amorphous polymer, but this technique often results in the instability of the formulation after longer storage (D Prasad, 2014). By adding a third component to the system, this problem can be overcome (**Figure 1.3**). This third component can be another polymer, surfactant, any water-soluble excipient, another compatible drug, carrier, or water itself, which will result in enhanced solubility

and eventually increased stability of the formulation. TASDs have a number of advantages over binary solid dispersions based on mechanism, drug loading, and stability, which will be covered in detail in this study.

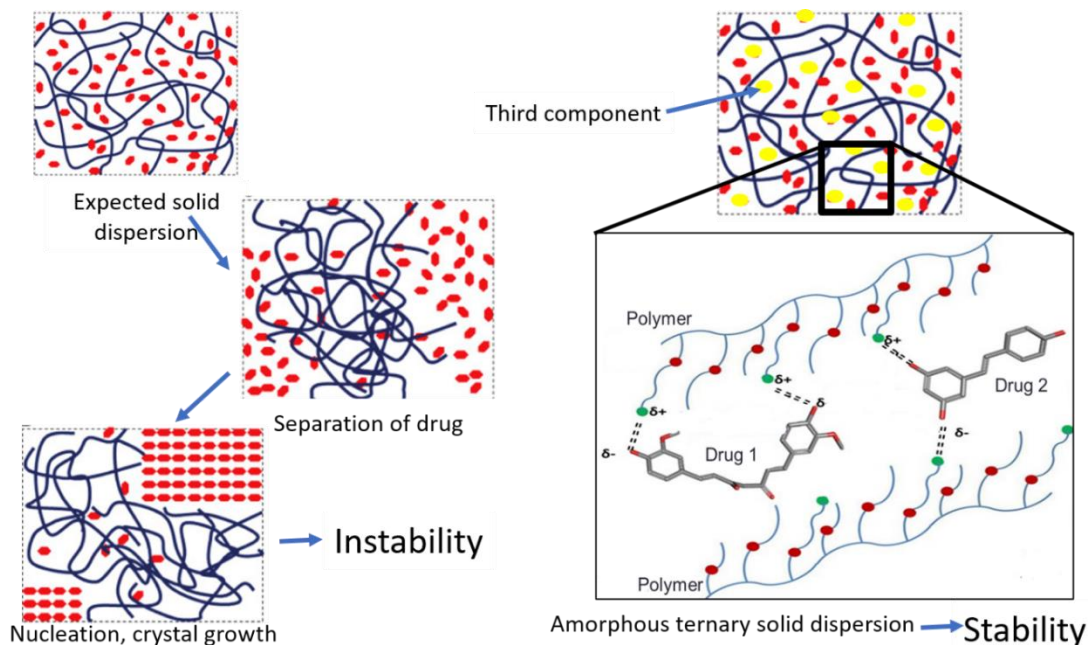


Figure 1.3: Challenge associated with ASD and the proposed solution

1.8 Classification:

TASD can differ based on the different components of the system such as drug, polymer, surfactant, etc. Accordingly, they are classified into 5 types:

1.8.1 Drug-Surfactant-Polymer (D-S-P):

In this ternary solid dispersion, poorly water-soluble drug is combined with a suitable surfactant and is dispersed in the polymer matrix. In some cases, the surfactant added acts

as a polymer in a synergistic way or it gets coated on the drug and improves dissolution in the solution. The detailed mechanism is explained in this review.

1.8.2 Drug-Water Soluble Excipient-Polymer (D-SE-P):

In this case, the water-soluble excipient is used as a carrier for the drug in a polymer matrix. This third component plays significant role in enhancement of dissolution. This method is also used to make the drug stable at a particular pH; as further discussed in this review, this T ASD shows hydrogen bonding between the drug and excipients, which results in stabilization of formulation and improved solubility.

1.8.3 Drug-Polymer-Polymer (D-P-P):

This type of T ASD is the most common and has a simple mechanism of the synergistic effect of polymers used in the formulation, which leads to a formulation with improved solubility and stability. This method gives scope to increase drug loading in the formulation, which is a current topic of interest for scientists.

1.8.4 Drug-Drug-Polymer (D-D-P):

Two drugs that have an interaction with each other are selected for this type of formulation. The intermolecular interaction between two poorly water-soluble drugs and adsorption of polymer on the surface of drugs or a drug's interaction with the polymer result in a decrease in activation energy of the formulation for crystallization, leading to a stable formulation. This reduces the desolvation energy of drug and improves its solubility and stability.

1.8.5 Drug-Excipient-Carrier (D-E-C):

In this technique, a special carrier is used instead of a polymer; this special carrier mainly includes adsorbents. The drug is dispersed in the water-soluble excipient and then is allowed to adsorb on the surface of the adsorbent. This leads to a highly stable formulation; this method is mainly used in dispersible formulations.

1.9 Mechanisms of Solubility and Stability Enhancement in TASD:

1.9.1 Drug-Surfactant-Polymer:

Many studies have shown the mechanism of molecular interactions for polymer-drug solid dispersions, but literature regarding the mechanism involved in polymer-surfactant-drug interactions is still limited. There are some studies that discuss the interaction of polymers and surfactants based on the presence of ionic regions in both compounds. In one comparative study between the interaction of nonionic and anionic surfactants with polymers, the complexes of nonionic polymers and anionic surfactants were more effective in the encapsulation of drugs and their dissolution in solutions, whereas a nonionic surfactant promoted the crystallization of drugs in solution state (Qi, 2012). The basic mechanism of the interaction between polymers and surfactants is based on the adsorption of surfactant molecules on polymer chains; this adsorption is dependent on the concentration of both compounds, this is explained in. When the amount of surfactant is below CAC, there is no adsorption of the surfactant on the polymer; however, as soon as the concentration of the surfactant increases, it starts to adsorb on the polymer chains. When the concentration of the polymer is lower than that of the surfactant, there is intramolecular adsorption of surfactant clusters on the polymeric chains in which one

surfactant cluster is present on one polymer chain, which may result in shrinkage of the polymer chain. In contrast, if the concentration of the polymer is greater, intermolecular interactions occur, in which a single surfactant cluster is shared by more than two polymer chains, which results in increased viscosity and greater stability of the solid dispersion (Nilsson, 1995). This does not happen when the surfactant is nonionic because it can't form ionic bonds with the reactive regions of the polymer chains. This interaction is based on the degree of redistribution (adsorption), the shape and size of clusters that are formed, and the capacity of these clusters to solubilize the drug (Nilsson, 1995). However, the selection of a surfactant for this type of ternary system is critical. It is known that the molecule with the lower weight and low T_g increases molecular mobility and, as a result, increases the rate of crystal growth. Water is a good example, since it enhances the crystal growth of amorphous felodipine (Rumondor, 2009) and amorphous indomethacin (Andronis, 1997) due to its low T_g. Some studies have shown that, if a surfactant is miscible with the compound of interest (drug), the rate of crystal growth will increase as it depresses the T_g of the system (I., 2013). In 2014, Frank *et. al.*, suggested that use of surfactant in ASD increases the permeability of the drug along with the dissolution. They studied the drug ABT-102 by formulating the solid dispersion with polymer and three surfactants and observed that supersaturation phase in dissolution leads to enhanced permeability of the drug (Kerstin J. Frank, 2014). ABT-102 ASD was also studied for the effect on permeation, apparent solubility, and molecular solubility and detailed mechanism responsible can be obtained in the research performed by Kristin Frank (Kerstin J.Franka, 2012).

Formulation Considerations and Challenges: The main challenge for this type of T ASD is to select the proper surfactant-polymer combination. As it is discussed above, the

polymer should be non-ionic and the surfactant should be anionic, as it is required to have interaction between the polymer and the surfactant for better viscosity and stability of the formulation. The formulation should be highly selective for the ionicity of both compounds.

1.9.2 Drug-Water Soluble Excipient-Polymer:

Sometimes the drugs blended with a polymer exhibit some limitations, such as insolubility at a particular pH. To overcome this challenge, various studies are done. In such cases, substances that are soluble in water are used to enhance the stability and solubility of amorphous dispersions and ternary dispersions are prepared. The mechanism for the stability of such T ASD involves the ionic or hydrogen bonding between the water-soluble polymer, such as EPO, and a water-soluble third excipient (saccharin), and another molecular interaction between the poorly soluble drug and the polymer. These two interactions in dispersion stabilizes the system at varied pHs (Kenjirou Higashia, 2015). However, this is not possible for every combination of water-soluble excipients and water-soluble polymers, so one has to look for the chemical structure and stability to predict the interaction between the compounds. In some cases, such as HP-beta-CD, it has good complexing capacity and the ability to modify the chemical, physical, and even biological properties of many hydrophobic compounds, but due to its large molecular weight and low water solubility, scientists are trying to find approaches to reduce the concentration of HP-beta-CD in the formulation (Loftsson, 1996) (Loftsson T. , 2007). Similarly, l-arginine was found to be the best third water-soluble component for the enhancement of solubility and stability of glyburide by acting in synergistic manner with HP-beta-CD in a solid dispersion system. This effect was found to be a result+ of simultaneous salt formation and arginine

interaction with the hydrogen bond system of the host (Singh, 2012). **Formulation Considerations and Challenges:** The important challenge is to select the perfect combination of excipient and polymer for the critical behavior of their interaction. If they don't react with each other at all, the formulation will not be stable. Another challenge is the solubility of the water-soluble excipient being used in the formulation. Also, the dose of the drug cannot be varied to a large extent.

1.9.3 Drug-Polymer-Polymer:

The idea of the amorphous solid dispersion starts from the dispersion of the API and polymer to enhance the solubility of the crystalline drug. However, some studies have suggested that the addition of a third polymer to the binary API/polymer system will lead to better stability of the formulation. This stability depends on the structure and chemistry of the two polymers and the API in the system, as well as how they react to each other to enhance the stability (D Prasad, 2016) (D Prasad, 2014). This technique suggests adding a third, bridging polymer to the binary solid dispersion of API and polymer; this third polymer has the capacity to form H-bonds (or any other stable interaction) with both the API and the polymer. For example, the solid dispersion of griseofulvin and PVP was unstable because of the lack of non-covalent bonding between them (R.Nair, 2001); however, by adding the third polymer PHPMA, which can form H-bonding with the griseofulvin and PVP, a more stable system of ternary solid dispersion can be formed (Al-Obaidia, 2011). The main reason for this is the miscibility of PHPMA in PVP (Kuo, 2004). In conclusion, there will be a synergistic effect of two polymers in ternary systems and a small number of polymers can be used for ternary solid dispersions.

Formulation Considerations and Challenges: This technique is basic in this field and many advancements have been studied for better options. The main challenge is the concentration of polymers: sometimes a large quantity of polymer is required in comparison with the drug, which affects oral doses.

1.9.4 Drug-Drug-Polymer:

In this system, two poorly soluble drugs are dispersed in one polymer. This ternary solid dispersion system is still being studied for the stability and solubility of the two drugs. In these solid dispersions, two drugs are selected that can have synergistic effects or can be co-administered in fixed dose combination. Initially co-amorphous systems of only two poorly water-soluble APIs (i.e., darunavir and ritonavir) were studied, but they showed no enhancement in stability or dissolution after preparing the amorphous system, but the addition of a third compound, such as cyclodextrin, which formed complexes with the APIs, enhanced the stability and solubility of the combination of drugs (Nguyen DN, 2014). Sometimes, two drugs behave better than the ternary solid dispersions because they can have chemical interactions within them, and they don't need the polymer or other carrier for better dissolution. Researchers have studied the combination of poorly water-soluble drugs such as naproxen: indomethacin (K, 2011), ritonavir:indomethacin (Dengale SJ, 2014), and simvastatin: glipizide (Löbmann K, 2012). In these combinations, no intermolecular interactions were observed and they showed dissolution of one drug over the other, except for the combination of naproxen: indomethacin, which showed intermolecular interactions and an increase in dissolution rates of both drugs (K, 2011). The fixed-dose combination of ezetimibe and lovastatin was prepared with the hydrophilic polymer Soluplus® and tested for enhancement in dissolution. It has shown great stability

due to the hydrogen bond interaction between the drugs and the polymer and high dissolution of up to 92% and 83%, respectively, in 5 minutes (Riekes M. K., 2016). In some studies, they have compared the FDC of class 2 drugs with the marketed preparations. The FDC of weak base pioglitazone and weak acid glimepiridine and the other combination of simvastatin and ezetimibe were prepared with the Scopulus® and HP-beta-Cd and proved to have better dissolution than the marketed preparation. The precipitation inhibition in this case is based on the ability of polymers, like Scopulus®, to adsorb on the drug surfaces and inhibit nucleation and crystal growth (Augustijns, 2009). CD, in contrast, improves the solvation of drug molecules and increases the activation energy for desolvation during crystal growth. Hence, by decreasing the degree of supersaturation and decreasing the energy for crystallization, Scopulus® and CD, respectively, stabilize the ternary solid dispersion system (Taupitz, 2012). **Formulation Considerations and Challenges:** The main challenge for this type is the combination of drugs. Sometimes drugs acting in a similar mechanism can affect each other's activity. The interaction between the drugs should be studied in minute detail because, as in the example discussed in the mechanism section, one drug can affect the dissolution, and the final formulation would result in an enhancement of solubility and dissolution of only one drug over the other. In this type, the drug-drug interaction is important for the most accurate prediction of the formulation's final performance; preliminary studies are required for each combination of drugs.

1.9.5 Drug-Excipient-carrier:

Some ternary solid dispersions include a drug, a carrier for the drug, and any other excipient that may help the system to stabilize for a longer period. In the case of dispersing granules,

an adsorbent is used for the stability of formulation. In this system, the drug forms hydrogen bonds with the adsorbent, hence improving the stability and dissolution in the solution state. When the drug is soluble in a dispersing carrier, there is a flux developed toward the surface of an adsorbent to increase H-bonding from the molecularly dispersed state; hence, during storage, the flux creates the gradient for the reaction and H-bonding keeps increasing with time, which results in high stability of the system and dissolution in the solution state (Gupta, 2002). This situation is opposite when the drug is less soluble in the dispersing carrier and the system is not stable during longer storage (Gupta, 2002). Another example is the enhancement of tibolone by preparing the solid dispersion with PVP and silica. In this case, there were chemical interactions between the drug and PVP or silica that helped the stabilization of the system and the dissolution in the solution state (Papadimitriou, 2009). **Formulation Considerations and Challenges:** The foremost challenge for this type is the solubility of a drug in the dispersing carrier on which the H-bonding is dependent. The second challenge is the chemical structure of the adsorbent and carrier for the drug to predict the proper interactions between the systems.

1.10 Fixed dose combinations:

Many different types of fixed dose combinations are commercially available for oral, parenteral and inhalation formulations. According to FDA, top five therapeutic areas for approved FDCs are infections, cardiovascular, pain, allergies and hormones. Majority of them are composed of two drugs. (Desai, 2013). Many studies are done for the binary solid dispersions although ternary solid dispersion of two poorly water-soluble drugs and carrier is still in very initial phase of research. And this rarely explored research can be used to

enhance the solubility of the combination of the drugs. In FDCs, one drug can be combined with the another to improve the safety of the treatment. For example, misoprostol is combined with NSAIDs to reduce gastro-irritation of diclofenac. FDCs mainly reduces the adverse effect of the medication. Disadvantage of FDCs includes the size of tablet for pediatric and elderly patients to swallow, as FDC contains multiple drugs in one tablet. For example, for type 2 diabetes, metformin with the dose range of 500 mg–2000 mg. When any other antidiabetic agents are combined with metformin, the tablet size can be too big to swallow easily (Desai, 2013).

Table 1.1: Examples of drug-drug-polymer type ATSD

FDC	Carrier	Solubility	Stability	Ref
Pioglitazone and glimepiride	Soluplus or HP-beta-CD	100% dissolution	30 Days	(Taupitz, 2012)
Ezetimibe and simvastatin	Soluplus or HP-bet-CD	65% of ezetimide and 85% dissolution of simvastatin	30 Days	(Taupitz, 2012)
Ezetimibe and lovastatin	Soluplus	Dissolution improved by 18 folds for ezetimide and 6 folds for lovastatin	Not specified	(Riekes M. K., 2016)

1.11 Advantages of drug-drug-polymer type of ATSD for FDCs:

In this ATSD combination of two poorly drugs can be administered (fixed dose combinations), which is not possible in other types of ATSD. This system is capable of improving the dissolution of two drugs simultaneously, as the drug-drug interaction may add to the stability of formulation. Immediate enhancement of solubility is possible as the drugs are directly converted to amorphous form and will be available for absorption after oral route. In this thesis, novel indole-2-carboxamide derivative, North 2 is formulated in two different amorphous ternary solid dispersions, one with anti-oxidant, curcumin and another with anti-tubercular drug, clofazimine.

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2 Combination of North 2, anti-TB compound and curcumin, anti-oxidant combination

Curcumin, a known antioxidant, is an active component of the spice turmeric. It is (diferuloylmethane, **Figure 2.1**) a β -diketone constituent that is extracted from rhizome of *Curcuma longa* Linn. Curcumin possesses multiple beneficial properties, for example, anticarcinogenic properties, anti-inflammatory properties etc (Zang, 2014), (Aanandhi, 2014), (Huang, 1997). Curcumin is found to play an integral role as a protective agent against oxidative stress associated with first line drugs such as isoniazid, rifampicin etc. in anti-TB therapy (Li, 2014), (Singh, 2012). For example, first step of current TB treatment involves the administration of isoniazid, which forms free oxygen radicals as a part of its mechanism of action. These free radicals target the bacterial pathway but are also known to cause side-effects by affecting the host cells. Hence, an anti-oxidant is commonly administered as a protective agent along with isoniazid. Although, beneficial it leads to increase in pill burden (Li, 2014).

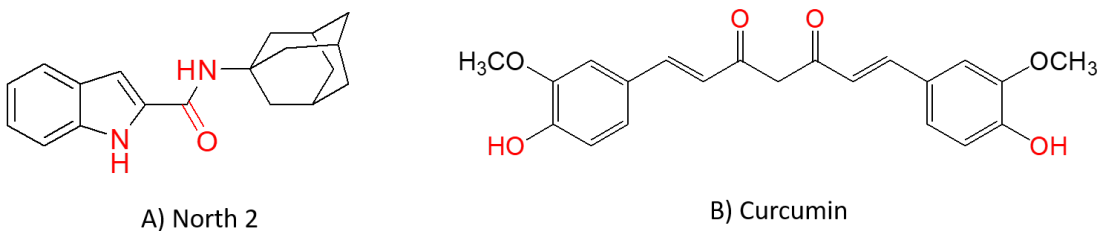


Figure 2.1: Chemical structure of A) North and B) Curcumin

Overall, a formulation containing North 2 and curcumin, will be a significant addition to current TB treatment since North 2 has anti-mycobacterial potency against drug resistant TB and curcumin can protect against free radical formation associated with the first line drugs in anti-TB treatment. Also, curcumin is reported to have anti-TB activity and hence

this formulation may have an additive effect against the TB bacteria (Baldwin, 2015). However, both North 2 and curcumin have very poor water-solubility ($<1 \mu\text{g}/\text{mL}$) (N.D. Franz, 2017) and they can be classified as class II compounds in biopharmaceutical classification system (BCS). Increase in number of pharmacologically active BCS class II compounds, has become a challenge for drug development (Al-Kassas, 2017). There have been many conventional methods for increasing the solubility of these compounds, which include use of solubilizing agents, salt formation, use of complexing agent, etc (Kumar,2011). However, all these approaches possess limitations such as toxicity after the use of co-solvents, increased powder loading, hygroscopicity and reduced shelf life after salt formation, addition of large amounts of excipients Kumar,2011), (Tonnesen, 2002). Other, more successful methods of increasing solubility include amorphous solid dispersions (Kumar,2011), formation of liposomes (Al-Kassas, 2017), inclusion of cyclodextrins (Tonnesen, 2002), emulsions. Formation of liposomes and emulsions still have a problem of reproducibility (Al-Kassas, 2017). Formulating a solid dispersion of these poorly soluble drugs has been a successful method for enhancement of aqueous solubility. There are several studies which suggest that amorphous solid dispersion (ASD) of poorly aqueous-soluble compounds can result in significant enhancement of solubility (Meng F. , 2015) (Li, 2014), (Meng F. T., 2015). For curcumin, binary amorphous solid dispersion with polymers like Eudragit®EPO was prepared and proved to be more soluble than pure curcumin (Li, 2014). In addition, curcumin formulated with Eudragit® E100 and HPMC showed significant enhancement in dissolution of curcumin for one hour and six hours, respectively (Meng F. , 2015).

As discussed above, the research approaches are needed to develop novel combination

therapies for tuberculosis to reduce the pill burden on patients. Based on the concept of combining an anti-TB agent with antioxidant, in this paper, we are trying to incorporate two agents at a time in one polymer matrix to prepare an amorphous system which will effectively enhance the aqueous solubility of both the agents simultaneously. We have combined the novel anti-TB agent North 2 with and anti-oxidant, curcumin. Both agents being in BCS class 2 and having problem with water solubility. Overall the goal of this research is to develop, formulate and evaluate the novel amorphous ternary solid dispersions containing North-2 and Curcumin with a different hydrophilic polymer to simultaneously enhance their aqueous solubility and solid-state stability.

2.1 Materials and Methods:

2.1.1 Materials:

Indole-2-carboxamide derivative, North 2 was synthesized in the laboratory, synthesis protocol and characterization are mentioned in the appendix of this paper. Curcumin was from Sigma (Sigma-Aldrich Co., St. Louis, MO). Polymers used in this study were obtained from, Eudragit®EPO from Degussa (Parsippany, New Jersey), PVP K90 from Spectrum (New Brunswick, New Jersey) and HPMC E5 from Dow Company (Midland, Michigan). Solvents used in the preparation of SDs and analytical method were methanol, acetone, Formic acid (HPLC grade), Acetonitrile (HPLC grade) were purchased from Fischer Scientific (Fair Lawn, New Jersey).

2.1.2 Precipitation studies- Solution:

Weighed amount of North-2, Curcumin, and polymers were dissolved in organic solvent

(acetone) separately and curcumin, North 2 and polymer solution were added in the ratio 1:1w/w (drug:polymer) for binary solutions and in the ratio of 1:1:2w/w/w (drug1:drug2:polymer) for ternary solutions. The compatibility of two compounds with polymers were evaluated based on precipitation after mixing. After obtaining clear solutions, they were dried overnight using nitrogen purging at room temperature.

2.1.3 Miscibility studies: Modified Differential Scanning Calorimetry (DSC):

Differential scanning calorimetry (MDSC) was performed by DSC Q2000 (TA instruments, New Castle, DE) equipped with a refrigerated cooling accessory (RCS90) and a data analyzer (Universal Analysis 2000, TA instruments). The equipment was calibrated with Indium. Inert atmosphere was maintained by nitrogen at a flow rate of 20 mL/min. An empty aluminum pan was used as reference. The thermal behavior of pure North 2 and PMs were characterized by a series of heating–cooling–heating cycles. Samples were heated at 10⁰C/min from 30 to 250⁰C (Cycle 1), cooled at a rate of 20⁰C/min to 30⁰C (Cycle 2), and then reheated at 10⁰C/min to 250⁰C (Cycle 3). Pure curcumin and its PMs were characterized in similar manner where Cycle 1 and Cycle 3 were from 30⁰C to 195⁰C. The final temperature is kept at around 15 C above the melting point of North 2 and Curcumin i.e., 250⁰C and 195⁰C respectively.

2.1.4 Preparation of binary and ternary solid dispersions:

North 2/Curcumin and polymer were dissolved in the appropriate solvent (methanol or acetone) at the ratio of 1:1 and 1:4w/w. Clear drug-polymer solution was then dried by using rotavapor for about 15 minutes. In case of ternary dispersions: Two compounds North 2 and Curcumin and along with the polymer were dissolved in solvent in the ratio of 1:1:2w/w/w and 2:2:1w/w/w. After obtaining clear mixtures, solutions were dried

using rotavapor for about 15 minutes. Physical mixtures (PM) of binary and ternary systems of each combination of similar ratios were prepared by physical mixing for comparison. All solid dispersions and PM were characterized by X-Ray Diffraction (XRD), Differential scanning calorimeter (DSC), and Infrared Spectroscopy (IR).

2.1.5 X-Ray Diffraction (XRD):

XRD diffraction data of PM and solid dispersions in the 2theta, ranging between 5–60 degrees were collected in focusing geometry using PANalytical Empyrean Diffractometer, operated with Cu K α radiation at 40 kV and 45 mA. A mask of 20 mm and a divergence slit of 1/4 degree was used on the incident beam path. Thin layer of powder sample was placed on a zero background Si plate and the sample holder was continuously spun at the rate of 90 deg/s during the measurement. Solid state PIXcel^{3D} detector was scanned at a rate of 0.135 deg/s to collect data and a diffracted beam monochromator for the Pixcel detector was utilized to improve signal to noise ratio.

2.1.6 Differential Scanning Calorimetry:

Three solid dispersions were tested for melting of pure compounds and glass transition temperature (T_g) using DSC and were compared with pure compounds. Weighed amount (5-8mg) of solid dispersions were tested in the hermetically sealed aluminum pans. To test the amorphous nature of ternary solid dispersions, the heating cycle from 30⁰C to 300⁰C was run at the heating rate of 10⁰C/min.

2.1.7 Fourier Transform Infrared Spectroscopy (FTIR):

Spectra Absorbance was obtained using the Nicolet iS 50 Fourier Transform Infrared Spectrometer (FT-IR) with a ZnSe ATR crystal. The spectra were signal averaged from 25 scans at 4 cm⁻¹ resolution with a dry-air purge at ambient temperature. A background was

collected to account for any interference that the environment might provide. Atmospheric compensation (to eliminate H₂O and CO₂ interference in the beam path) was used in all measurements. Background correction was carried out for every sample. Data processing for all infrared data was done using OMNIC (tm) FTIR Software.

2.1.8 Nuclear Magnetic resonance (NMR):

Binary and tertiary formulations were evaluated for their binding with the help of a proton NMR. Bruker's 400 MHz NMR instrument was used. The samples were prepared by dissolving them in deuterated solvents namely deuterated chloroform. The shift in the peaks corresponding to specific protons was observed. The solvent used to observe the chemical shifts in North 2 peaks was deuterated chloroform and that to observe the curcumin peak shifts was DMSA (deuterated Dimethyl Sulfoxide).

2.1.9 Molecular Modeling:

North 2 and curcumin were all built in MOE (Molecular Operating Environment, Chemical Computing Group, Montreal, Canada) and partial charges were assigned using the MMFF94x forcefield. EPO, HPMC and PVP were all built with at least 12 subunits in MOE and charge states (partial and full) were assigned with the AMBER10 forcefield. Molecular dynamic simulations were performed using the AMBER10 forcefield following Nosé-Poincaré-Andersen equations of motion. Molecular dynamic simulations were equilibrated at 300 K for 100 ps and produced at 300 K for 500 ps. The molecular dynamic end point pose was then energy minimized to a RMS gradient of 0.00001 kcal/(mol Å).

2.1.10 *In-vitro* dissolution:

USP apparatus II containing paddles was used for the dissolution experiment at 100rpm.

Formulated solid dispersion equivalent to 17.5mg of each drug is added to a well containing 90 mL of the medium and the physical mixture of the same ratio was added to another well (n=3). The temperature of the apparatus was maintained at 37⁰ C. The experiment was set for 12 hours. In case of ternary solid dispersion with EPO, initially, for 30 minutes, 20mL of hydrochloric acid buffer of pH 5 was added (to dissolve the EPO) and the quantity of medium was increased up to 90mL with the phosphate buffer of pH 7.4 to make the medium neutral after first 30 minutes. This condition was simulated to the human stomach and intestine pH. In the dissolution experiment of ternary solid dispersion of HPMC and PVP, medium used was phosphate buffer of pH 7.4 throughout the experiment of 12 hours. The aliquots were taken at 1, 15, 30, 60, 180, 360, and 720 minutes. Warfarin and resveratrol were added as the internal standard for North 2 and curcumin respectively. The samples were tested with LCMS.

2.1.11 UV-visible spectroscopy:

Method development: Stock solutions of curcumin and North 2 were prepared separately in methanol (20µg/ml). The solutions and blank were scanned in triplicates for their wavelength of maximum UV-Vis absorbance, in range of 250-700 nm (n=3). UV-Vis spectrophotometer with plate reader arrangement was used. The wavelength of the maximum absorbance was found for both the drugs.

Simultaneous Equations: Using standard curve equations, simultaneous equations were developed. Simultaneous equation method was then used to develop equations for concurrent quantification of curcumin and North 2. The equations were tested for standard known concentrations (5 µg/mL each) of curcumin and North 2 mixture in triplicates (n=3).

2.1.12 LCMS Method:

An Exion HPLC system (Applied Biosystems, Foster City, CA) coupled with API 5500 Q Trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) was used. The mass spectrometer was operated in electrospray ionization (ESI) positive mode with m/z 295.2(M+H) \rightarrow 135.2 for North-2 and 369.1(M+H) \rightarrow 177.0 for curcumin were selected for quantification. Chromatographic separation was performed on Phenomenex Kinetex EVO C18 column (50 \times 3.0 mm, 5 μ m) with an isocratic mobile phase containing 30:70 v/v 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a flow rate of 0.250 mL/min. The liquid chromatography-tandem mass spectrometer was controlled by Analyst 1.6.3 software. The retention times were 2.08 and 1.22 min for North-2 and curcumin respectively. The total run time for each sample was 3 min. Warfarin was used as internal standards for curcumin and north-2.

2.1.13 Stability studies:

The stability studies were done on every ternary formulation of north 2 and curcumin which found to be amorphous. The formulations were tested for any change in amorphous nature by using XRD analysis. The formulations were stored in air tight vials at room temperature for 90 days for stability testing.

2.2 Results and Discussion:

2.2.1 Drug and polymer considerations based on physicochemical properties and precipitation studies:

The system consists of two poorly soluble compounds, therefore we selected FDA

approved and commonly used hydrophilic polymers namely PVP, HPMC and EPO, which are compatible with both the compounds and do not form precipitate during mixing in presence of each other in acetone/methanol being the solvent. No precipitate of North 2 and curcumin in presence of polymers were observed except for curcumin precipitation with the methanolic solution of PVP. Another consideration for selecting polymer was their tendency to form molecular interactions with compounds. Structural analysis and previous studies were analyzed to identify the most potential groups to interact in polymers and North 2-Curcumin used in the study. Amine, carbonyl and hydroxyl groups in the polymers used, EPO, PVP and HPMC are functional groups having the potential to interact and form the hydrogen bonds in the structure (Li, 2014) (Meng F. , 2015)(Jeganathan, B.,2015),(Paradkar, A.).No studies were published for North 2 but previous studies for curcumin showed phenolic OH and carbonyl are the most potential functional group which can be involved in the interactions (Boztas, A. O.,2013). Structural studies for North-2 suggested that its carbonyl group, indole NH and amide NH can be considered as most potential functional groups. Along with interactions, crystallization tendency of compounds and their miscibility with polymers can play important role in the efficiency of polymer (Konno, H., 2006).These two compounds have different crystallization tendency, North 2 has high crystallization tendency whereas curcumin has low crystallization tendency (discussed in the under the DSC section further). The change in crystallization tendency was tested in the presence of different polymers using DSC, based on which the polymer efficiency was determined. These DSC studies are discussed further in the next section in the paper. The drug loading was another important criterion to understand the effectiveness of polymers, the prepared dispersions contained, 50% of drug loading in the solid

dispersions (1:1 w/w in binary and 1:1:2 w/w/w in ternary solid dispersions).

2.2.2 Differential scanning calorimetric analysis

2.2.2.1 Crystallization tendency of pure compounds:

The endothermic and exothermic transition in DSC heat-cool-heat cycles of pure compounds were compared with the cycles of physical mixtures (PMs) of the compounds and the polymer to determine their miscibility and effect of the polymers on the crystallization tendency of pure compounds. Three cycles were run for each sample and are like, Cycle 1: Heating cycle at 10⁰C/min; Cycle 2: Cooling cycle at 20⁰C/min; Cycle 3: Heating cycle at 10⁰C/min. Both the heating cycles were up to 250⁰C for North 2 and 195⁰C for curcumin as both have a melting point of around 231⁰C and 177⁰C respectively. The pure compounds showed sharp melting point (North 2: 231⁰C and curcumin: 177⁰C) indicating the crystallinity of the two compounds (**Figure 2.2 A**), while the physical mixture (PM) of the compounds with the polymer showed the depression in melting endotherm. North 2 in the cycle 1 showed the sharp melting point followed by the crystallization in the cycle 2 and this crystalline phase again showed the melting in the cycle 3 at around similar temperature (**Figure 2.2 A**). This suggests that North 2 has the high crystallization tendency. Similar studies with pure curcumin showed no crystallization followed by a melting in cycle 2 and cycle 3, suggesting that curcumin is low crystallization tendency compound.

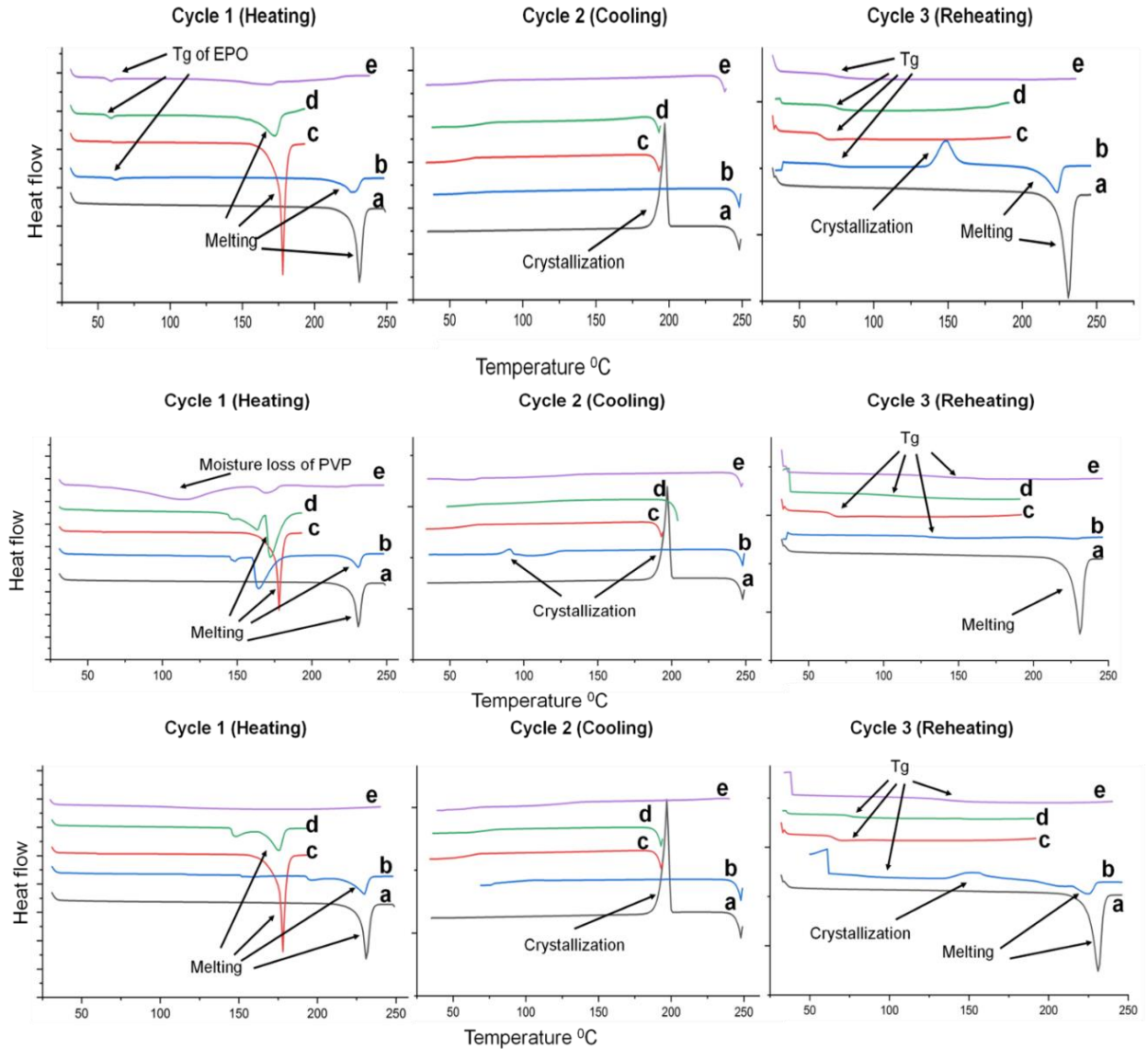


Figure 2.2: (A) DSC data of 3 cycles of the North2: Cur:EPO. a: Pure North 2; b: PM North2: EPO (1:1); c: Pure Curcumin; d: PM Curcumin:EPO (1:1); e: PM North2:Curcumin:EPO (1:1:2). (B) DSC data of 3 cycles of the North2: Cur:PVP. a: Pure North 2; b: PM North2: PVP (1:1); c: Pure Curcumin; d: PM Curcumin:PVP (1:1); e: PM North2:Curcumin:PVP (1:1:2). (C) DSC data of 3 cycles of the North2: Cur:HPMC. a: Pure North 2; b: PM North2: HPMC (1:1); c: Pure Curcumin; d: PM Curcumin:HPMC (1:1); e: PM North2:Curcumin:HPMC (1:1:2).

2.2.2.2 Miscibility between compounds and polymers:

When there is a change in endothermic event such as melting of crystalline compounds due

to presence of polymer, then it can be stated that the compound is miscible with the polymer and polymers can hold the crystalline compound in amorphous state (Chauhan H, 2015), (Meng F. T., 2015), (Qian, 2010). In this study, different selected polymers were tested for their miscibility with two compounds qualitatively. During the first and third heating cycle of pure North 2, onset of melting point was 225.44⁰C with no Tg, but that of PM of North 2 and EPO was at 218⁰C in first cycle and at 212⁰C in third cycle. Third cycle in PM North 2-EPO also showed Tg at 69⁰C and onset of crystallization at 135⁰C, this indicated the miscibility of EPO with North 2 to a considerable extent and North 2 was present in amorphous form with EPO (**Figure 2.2**). Similarly, pure curcumin showed onset of melting at 176⁰C and PM Curcumin-EPO showed at 159⁰C. This indicated the miscibility of EPO with curcumin too. Tg in the third cycle of PM Curcumin-EPO showed that curcumin was present in amorphous form with EPO. When both, North 2 and Curcumin were mixed with EPO, first cycle did not show any melting, indicated miscibility of EPO with both compounds. With PVP, North 2's onset of melting was at 224⁰C which was little less than pure North 2 (225⁰C), indicated less miscibility with North 2 as compared to EPO. Curcumin showed onset of melting with PVP at 169⁰C which is less as compared to EPO's effect, indicating less miscibility of curcumin with PVP (**Figure 2.2**). Two compounds together with PVP showed small endothermic event in first cycle, this indicated less miscibility with two compounds with PVP. HPMC behaved in the similar manner with two compounds as of EPO in all the DSC cycles. Onset of melting of North 2 in first and third cycle in PM North 2-HPMC was 220⁰C. Third cycle of North 2-HPMC showed Tg (around 81⁰C) and crystallization at 134⁰C, indicating similar behavior of HPMC with North 2 as that of EPO, but little less miscibility than EPO. Curcumin stated

melting at 166⁰C, indicating less miscibility than EPO. North 2 and Curcumin, together with HPMC showed no melting suggesting good miscibility (**Figure 2.2**). Hence, based on this DSC study, rank of polymers in terms of miscibility with two compounds is EPO>HPMC>PVP.

Table 2.1: Exothermic and endothermic events in the DSC heat-cool-heat cycles

	Cycle 1: Heating 25-240 C			Exothermic crystallization (Cycle 2)			Endothermic melting (Cycle 3)		
	Melting Temp	Melting Enthalpy	Tg ⁰ C	Temp		Tg ⁰ C	Temp	Melting Enthalpy	Tg ⁰ C
Pure North 2	231.15	55.21		197.26	66.77		231	54.12	
Pure curcumin	177.92	99.56					223	23	61
PM North2:EPO (1:1)	225	25		148	25.05		224		71
PM Cur:EPO (1:1)	172.99	39.74							68
PM North2:HPMC (1:1)	229	27.57	81	149	8.53		225	10.97	82
PM Cur:HPMC (1:1)	175	34.82							71
PM North2:Cur:EPO (1:1:2)	169.27	7.86							68
PM North2:Cur:HPMC (1:1:2)			107						68,81

2.2.2.3 Effect of polymers on crystallization tendency of North-2 and curcumin:

When PM of North 2 and EPO were run for three cycles, compound showed melting in cycle 1 and no crystallization peak was observed in the cycle 2 but in cycle 3 there was a crystalline peak followed by the melting peak (**Figure 2.2A**), indicating that the crystallization tendency of North 2 reduced from high to moderate level in the presence of EPO. Similar studies were done with PVP and HPMC with North 2 and it was observed that crystallization tendency of North 2 reduced to moderate in presence of polymers. When similar set of cycles were run for curcumin, there was no change in its crystallization tendency. Pure curcumin and PM of curcumin with polymers were having low crystallization tendency (**Figure 2.2B**). Ternary PM that is two compounds and one polymer showed no melting peaks and crystallization peaks in any of DSC cycles. This indicates that the polymers are miscible and having the effect of the crystallization tendency. The melting enthalpy of the pure compounds in the cycle 1 was found to be reduced in presence of the polymers (**Table 2.1**) and the peaks were widely spread as compared to that of the pure compounds, this indicates that the presence of the polymer and interaction between the polymer and compounds decreases the crystallinity of the compounds. Binary and ternary PMs with EPO showed the glass transition temperature of EPO at about 60⁰C (**Figure 2.2A**, Heating cycle). In the PM of two compounds and a polymer in each of the three formulations, it was seen that there was a significant depression in the melting peak. The glass transition temperature (T_g) of the pure compounds in the cycle 3 were increased in presence of the polymer (**Figure 2.2A**, **Table 2.1**) and in ternary PM like North 2:Cur:HPMC, there were two T_g observed. These T_g maybe observed because of the two compounds in the same polymer at the same time. In

the case of PVP (**Figure 2.2B, Table 2.1**) a broad peak is observed initially from 50⁰C to 150⁰C indicating the presence of the moisture since the PVP is hygroscopic polymer. This was confirmed from the TGA plot of PVP and PM of the North 2, curcumin and PVP. This study of PMs showed that three polymers reduced the crystallization tendency of North 2 and had no effect on curcumin crystallization tendency which may result in additional stability of amorphous ternary systems after storage. Based on this discussion, rank order of capacity of amorphisation of polymers for two crystalline compounds, North 2 and curcumin is EPO>HPMC>PVP.

2.2.2.4 X-Ray Diffraction (XRD):

The pure curcumin showed the sharp diffraction peaks at 2θ between 12 to 29 (Meng F. T., 2015), (Wegiel, L. A., 2014) , specifically at 9, 13.3, 15.8, 19.7, and 21.6, and north 2 showed the diffraction peaks between 5 to 30 of 2θ , specifically at 7, 14.4, 16.4, 19.3, 27.5, and 29.9 (**Figure 2.3**). XRD data of binary and ternary dispersions was plotted and sharp peaks showed that the binary solid dispersion of North 2:EPO (1:1) of North 2:HPMC were crystalline whereas, the ternary formulation of North 2:Cur:EPO (1:1:2) and North 2:Cur:HPMC were found to be an amorphous system (**Figure 2.3A, B**). In case of PVP, both binary (North 2:PVP) and ternary (North 2:Cur:PVP) formulations were found to be amorphous (**Figure 2.3D**). All the PMs were crystalline. In binary dispersion, North 2:EPO, though the crystallization peaks corresponding to pure North 2 at 7, 14.4, 16.4, and 19.3 are present, but the intensity of peaks is 2-3 times less than that of pure North 2. Similarly with North2:HPMC, intensity of peaks at 7, 14.4, and 19.3 is very low. Hence it can be concluded that EPO and HPMC were able to interact with some amount of pure compounds efficiently but could not disperse all the North 2 molecules present.

This suggests that reduction in North 2 loading in binary dispersions might lead to amorphous dispersions.

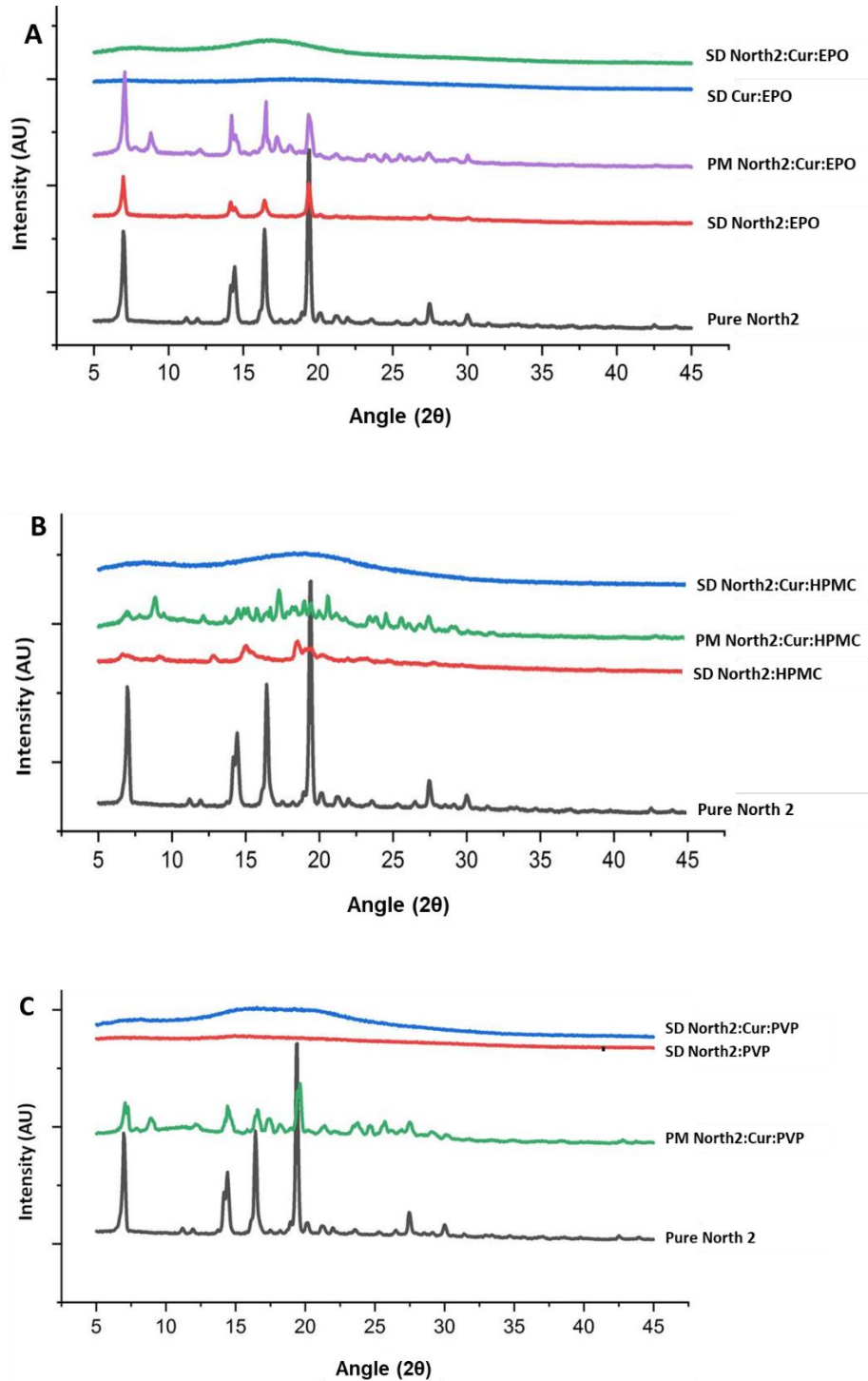


Figure 2.3: (A) XRD plot of Eudragit EPO formulations with physical mixture and binary formulations; a: SD North 2:Cur:EPO; b: SD Cur:EPO; c: PM North 2:Cur:EPO; d: SD North 2:EPO; e: Pure North 2;

(B) XRD plot of PVP formulations with physical mixture and binary formulations; a: SD North 2:Cur:P; b: SD North 2:PVP; c: PM North 2:Cur:PVP; d: Pure North 2;

(C) XRD plot of HPMC formulations with physical mixture and binary formulations; a: SD North 2:Cur:HPMC; b: PM North 2:Cur:HPMC; c: SD North 2:HPMC; d: Pure North 2

Note: Pure curcumin is not added in the plots and its combinations with the polymers are explained in the discussion section with the reference

2.2.2.5 Differential Scanning Calorimetry (SDs):

Ternary dispersions North 2:Cur:EPO, North 2:Cur:PVP and North 2:Cur:HPMC (1:1:2) showed no melting associated with curcumin and North-2 (**Figure 2.4**) confirming the formation of amorphous dispersions Ternary dispersions containing EPO ($T_g \sim 45^\circ\text{C}$) and PVP ($T_g \sim 154^\circ\text{C}$) showed degradations after 200°C . Thus, the study could not investigate the melting of North 2 in North 2:Cur:EPO and North 2:Cur:HPMC because the solid dispersions started degrading at the temperature below melting point of North 2. But the appearance of glass transition temperature (T_g) in all the three solid dispersions confirmed the formation of amorphous dispersions. T_g for North 2:Cur:EPO, North 2:Cur:PVP and North 2:Cur:HPMC were 136°C , 193.2°C and 176°C (**Figure 2.4**). The presence of glass transition event near 50°C in North 2:Cur:EPO and near 126°C in North 2:Cur:PVP are associated with the T_g of respective polymers (**Figure 2.4**). The wide endothermic peak below 100°C in North 2:Cur:PVP can be associated with the moisture loss, PVP being a

highly hygroscopic polymer (Meng F. T., 2015).

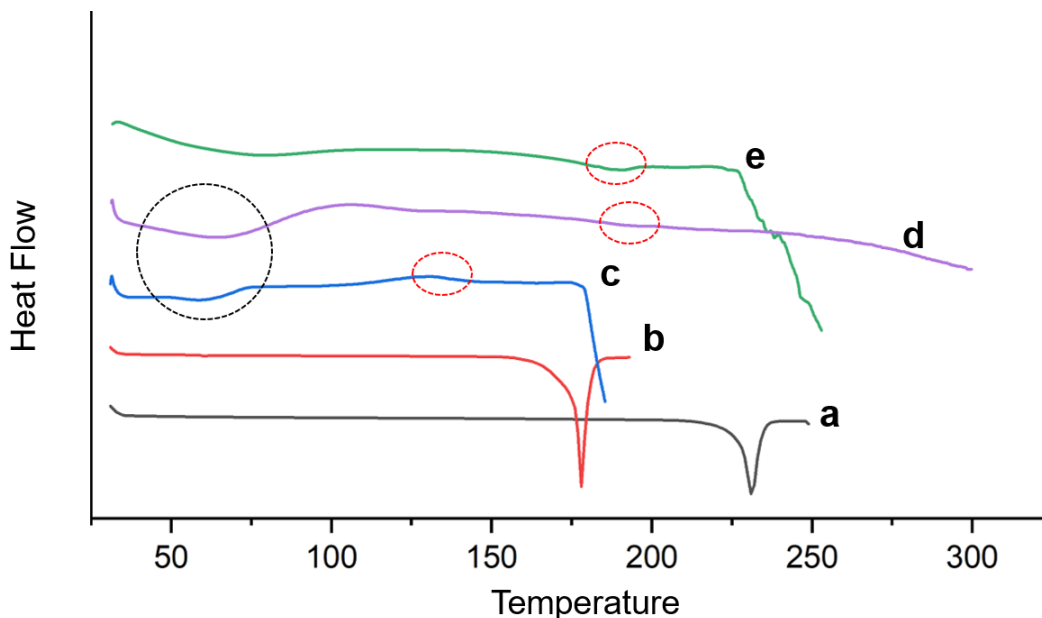


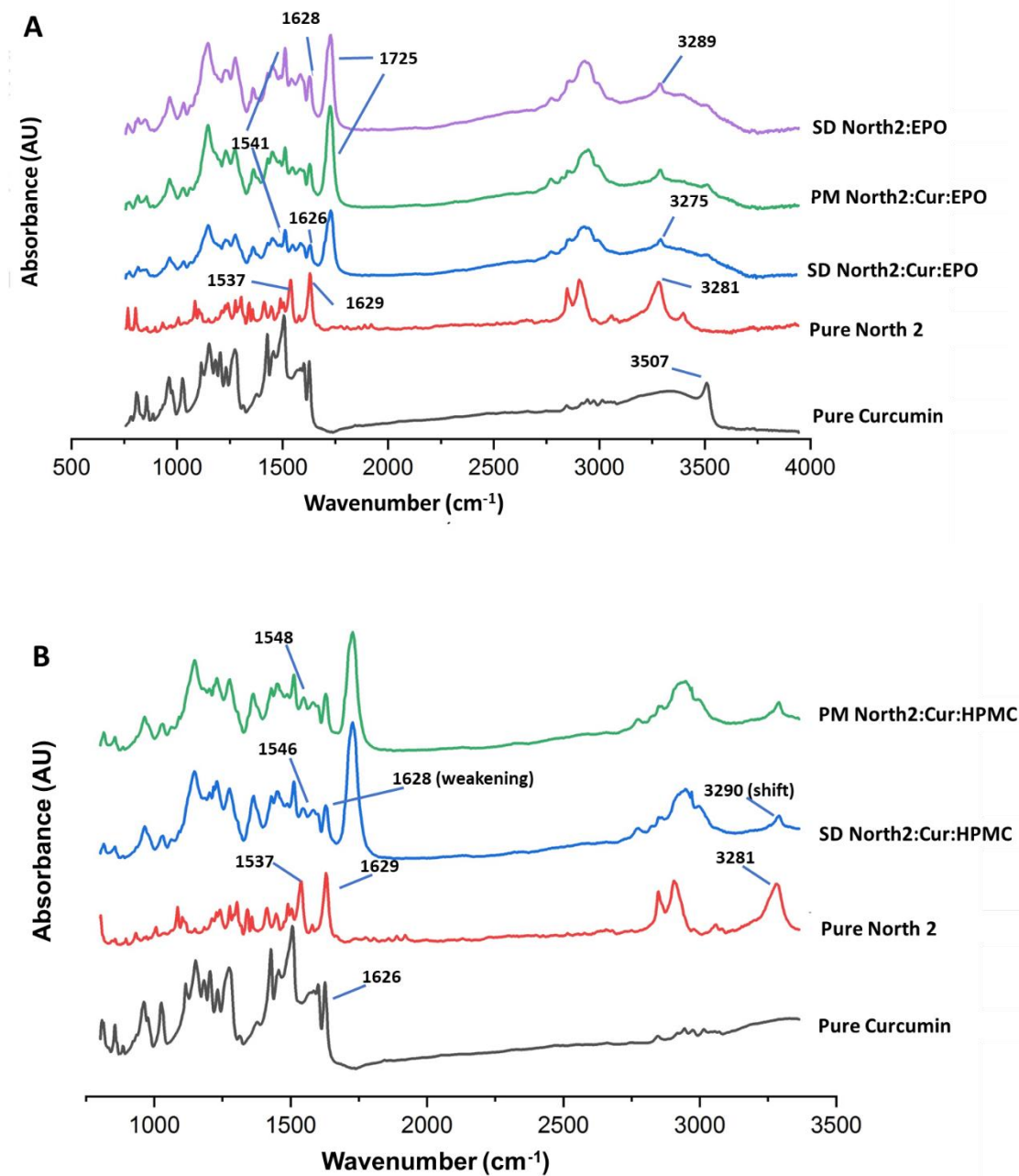
Figure 2.4: DSC plot for ternary SDs; a: Pure North 2; b: Pure curcumin; c: SD North 2:Cur:EPO; d: SD North 2:Cur:PVP; e: SD North 2:Cur:HPMC; (—) - moisture loss; (---) - Tgs

2.2.2.6 Fourier Transform Infrared Spectroscopy (FTIR):

IR analysis of pure curcumin and PMs showed characteristic absorption peaks at 3507 cm^{-1} and 1626 cm^{-1} for the phenolic hydroxyl and carbonyl groups, respectively (**Figure 2.5A**). It has been reported that curcumin exists in a predominant enol-keto form rather than diketone form (Meng F. T., 2015), this is due to the increased conjugation from the α,β -unsaturated ketone and resulting intramolecular hydrogen bond. This results in a bathochromic shift of curcumin carbonyl group, which supports a low wave number (1626 cm^{-1}). The peaks at 3281 cm^{-1} (indole N-H), 1629 cm^{-1} (carbonyl group, C=O), 1504 cm^{-1} , 1490 cm^{-1} , 1446 cm^{-1} (strong to weak absorption peaks representing aromatic ring of indole), and the peak at 1537 cm^{-1} (N-H bending strongly coupled with C-N stretching, amide) are attributed to the North 2 structure (**Figure 2.5A**). These peaks are

present in the physical mixtures representing that there is no interaction in physical mixtures. In the case of North 2:Cur:EPO (**Figure 2.5A**), the shift of indole N-H peak from 3281 cm^{-1} (in pure drug and physical mixture) to 3289 cm^{-1} in solid dispersion of North 2 and EPO and 3275 cm^{-1} in the ternary solid dispersion suggesting hydrogen bonding interactions between indole N-H of North 2 and EPO. Also, the hypochromic shift of the N-H bond of the amide from 1537 cm^{-1} (in pure North 2 and PM) to 1541 cm^{-1} in binary and ternary solid dispersions showed the involvement of the N-H (amide) in hydrogen bonding. And shift of the carbonyl peak from 1629 cm^{-1} to 1628 cm^{-1} in binary solid dispersion and 1626 cm^{-1} in ternary solid dispersion suggested that this oxygen is involved in hydrogen bonding as an acceptor. For curcumin, there is a weakening (lower absorption) of the absorption peak of O-H bond (3507 cm^{-1}) in the ternary solid dispersion, suggesting O-H hydrogen bonding with the polymer. This interaction is also shown in the molecular modeling studies performed for the same combinations which is discussed further in the article. In the formulation containing PVP (**Figure 2.5B**), the shift of the peak from 3281 cm^{-1} (in pure North 2 and physical mixture) to 3283 cm^{-1} and its broadening seems to suggest that indole N-H is involved in molecular interaction with polymer. Similarly, the bathochromic shift of the carbonyl peak to 1628 cm^{-1} from 1629 cm^{-1} indicates the involvement of carbonyl oxygen in molecular interaction. There might be a contribution of O-H bond present in curcumin, since there is a weakening (lower absorption) of the O-H bond peak at 3507 cm^{-1} . In the formulation containing HPMC, (**Figure 5C**) the shift of indole N-H from 3281 cm^{-1} to 3290 cm^{-1} and weakening of amide N-H bond at 1546 cm^{-1} and shifting from 1537 cm^{-1} (which represents amide N-H in pure North 2) suggested the involvement of hydrogen bonding between North 2 and polymer. However, the data was

not able to conclude that this carbonyl was the functional group from either North 2 or curcumin since both of the structures have carbonyl group and in the ternary complex only one carbonyl peak was observed (**Figure 2.5A, 2.4B, 2.4C**).



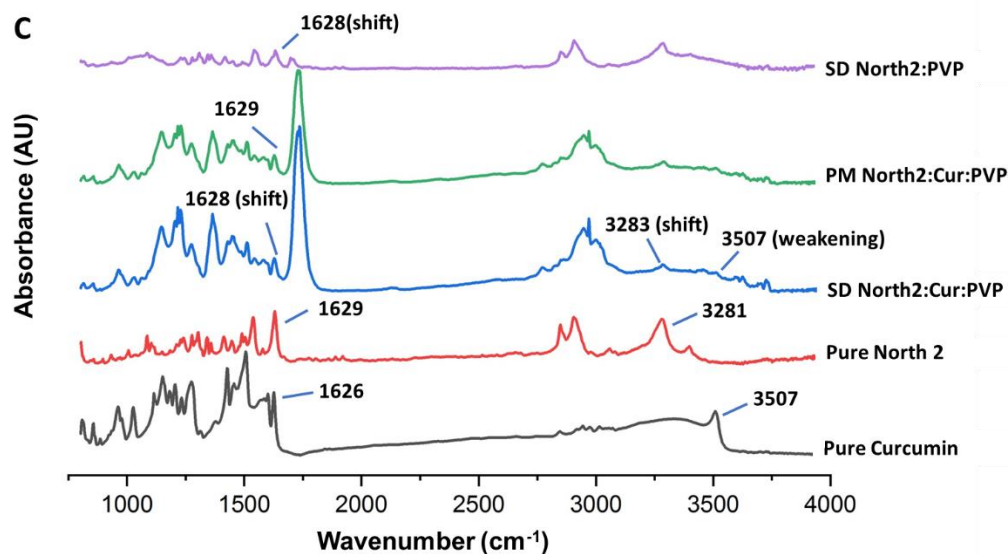


Figure 2.5: FTIR plots of solid dispersions (SD) and Physical Mixtures (PM) with (A) EPO; (B) HPMC; and (C) PVP

2.2.2.7 Nuclear Magnetic Resonance (NMR):

FTIR data showed the interactions between the compounds and polymer, these interactions were hydrogen bonding. In addition to this, this study has also investigated the solution phase interaction using NMR analysis, as these interactions were confirmed by comparing NMR data of pure compounds with the binary and ternary solid dispersions. For this study, to confirm the functional group of North 2 that is involved in the interaction, pure North 2, its binary SD with all three polymers and ternary SD with curcumin and other three polymers were studied after dissolving the formulations in deuterated chloroform. The chemical shift represented by the delta value in the **Table 2.2-Table 2.4** was taken into consideration to evaluate the involvement of the particular functional group in the interaction. The upfield shift (positive delta) supports the notion of having more resonance and denser electron environment around a particular proton, meaning the proton may be

involved in the interactions resulting in the upfield shift of the peak to the shielded region in the NMR (Pham, T. N.,2010). In this section, shifts for North 2 and curcumin are mentioned separately. **North 2:** In North 2, the functional groups that have the potential to form non-covalent interactions are the indole and amide N-H groups. Pure North 2 showed the indole N-H peak at 9.726 ppm and amide N-H peak at 5.902 ppm. There were upfield chemical shifts (having positive delta) in the peak of indole N-H and amide N-H which indicated that these protons are involved in the interactions (**Table 2.2-Table 2.4**). The minimum delta value for the indole N-H was approximately 0.4583 ppm and the highest delta value for the same function group was found to be 0.6742 ppm. There were no peak shifts observed in the aliphatic and aromatic regions of the compounds with any of the three polymers used, suggesting that these protons are not involved in the bonding interactions. The largest delta was observed for the indole N-H bond in every formulation. In case of EPO formulation (North 2:Cur:EPO), the delta values (upfield shift) in the indole N-H was 0.6251 ppm and 0.6749 ppm in the binary complex of North 2:EPO and ternary complex of North 2:Cur:EPO respectively (**Table 2.2**). Similarly, in the case of North 2:Cur:PVP it was 0.4583 ppm and 0.6324 ppm in the binary and ternary complex respectively (**Table 2.3**). With North 2:Cur:HPMC, it was approximately 0.6621 ppm and 0.4819 ppm in binary and ternary complex (**Table 2.4**). In case of amide N-H there was minimum delta value of 0.0099 ppm and highest delta value for amide N-H was found to be 0.05891 ppm in the peak in any of the three formulations (**Table 2.2-Table 2.4**) suggesting that the amide N-H may not be involved in the interactions but might be surrounded with the polymer or the other drug moiety (curcumin) as the minor shift observed was the upfield shift. **Curcumin:** To observe the shift in the curcumin functional

group peaks the NMR were run with the deuterated DMSO, as the proton in the functional group having the potential of interaction in the curcumin structure (phenolic OH) gets exchanged with deuterium of chloroform. The functional groups that may interact are the phenolic OH groups that are present on the two rings and carbonyl group in the middle of the structure, however we cannot observe the carbonyl group in proton NMR. There was no observed chemical shifts for any curcumin proton in binary or ternary complexes, suggesting that curcumin is quickly released from polymer in DMSO. Thus, the data obtained from the NMR is a tool to confirm the presence of binding interactions, however, However, we cannot conclude that these interactions are between the two compounds or between the drug and polymer and the exact nature of the binding interaction may not be affirmatively stated.

Table 2.2: NMR peaks of pure North 2 and its complexes with EPO (in ppm)

North 2 peaks	Pure North 2	North 2:EPO	Δ shift	North 2:Cur:EPO	Δ shift
A (indole N-H)	9.7261	9.101	0.6251	9.0512	0.6749
B (aromatic proton)	7.637-7.4953	7.637-7.4954	≈ 0	7.637-7.4957	≈ 0
C (aromatic proton)	7.4933-7.4726	7.4933-7.4727	≈ 0	7.4933-7.4730	≈ 0
D (aromatic proton)	7.1410-7.1010	7.1410-7.1011	≈ 0	7.1410-7.1014	≈ 0
E (aromatic proton)	6.7582-6.7511	6.7582-6.7512	≈ 0	6.7582-6.7515	≈ 0

F (amide N-H)	5.9023	5.852	0.0503	5.8434	0.0589
G (aliphatic protons)	2.1668	2.1668	≈0	2.1668	≈0
H (aliphatic protons)	1.7844-1.7121	1.7844-1.7122	≈0	1.7844-1.7125	≈0

Table 2.3: NMR peaks of pure North 2 and its binary and ternary complexes with PVP (in ppm)

North 2 peaks	Pure North 2	North 2:PVP	Δ shift	North2:Cur:PVP	Δ shift
A (indole N-H)	9.7261	9.2678	0.4583	9.0937	0.6324
B (aromatic proton)	7.637-7.4953	7.637-7.4955	≈0	7.637-7.4958	≈0
C (aromatic proton)	7.4933-7.4726	7.4933-7.4728	≈0	7.4933-7.4731	≈0
D (aromatic proton)	7.1410-7.1010	7.1410-7.1012	≈0	7.1410-7.1015	≈0
E (aromatic proton)	6.7582-6.7511	6.7582-6.7513	≈0	6.7582-6.7516	≈0
F (amide N-H)	5.9023	5.8978	0.0045	5.8474	0.0549
G (aliphatic protons)	2.1668	2.1668	≈0	2.1668	≈0

H (aliphatic protons)	1.7844-1.7121	1.7844-1.7123	≈ 0	1.7844-1.7126	≈ 0
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Table 2.4: NMR peaks of pure North 2 and its binary and ternary complexes with HPMC (in ppm)

North 2 peaks	Pure North 2	North 2:HPMC	Δ shift	North2:Cur:HPMC	Δ shift
A (indole N-H)	9.7261	9.064	0.6621	9.2442	0.4819
B (aromatic proton)	7.637-7.4953	7.637-7.4956	≈ 0	7.637-7.4959	≈ 0
C (aromatic proton)	7.4933-7.4726	7.4933-7.4729	≈ 0	7.4933-7.4732	≈ 0
D (aromatic proton)	7.1410-7.1010	7.1410-7.1013	≈ 0	7.1410-7.1016	≈ 0
E (aromatic proton)	6.7582-6.7511	6.7582-6.7514	≈ 0	6.7582-6.7517	≈ 0
F (amide N-H)	5.9023	5.8518	0.0505	5.8924	0.0099
G (aliphatic protons)	2.1668	2.1668	≈ 0	2.1668	≈ 0

H (aliphatic protons)	1.7844-1.7121	1.7844-1.7124	≈ 0	1.7844-1.7127	≈ 0
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2.2.2.8 Molecular modeling:

Molecular modeling is very common tool to evaluate the intermolecular interaction in amorphous solid dispersions. This is generally used as a supplemental study for interaction studies because it gives us the information about the potential interaction functional groups, and the *in-silico* interactions between the compounds and polymers (Baghel, S.,2016). In many solid dispersion studies, molecular modeling is used to predict or confirm the interactions based on minimum energy levels of complexes(Mura, P., 1996). Molecular dynamics studies were performed to rationalize binary and ternary interactions determined with North 2, curcumin and polymer as described above. Binary systems between North 2 and each polymer (EPO, HPMC and PVP) are shown in **Figure 2.6**, panels A, B and C. Hydrogen bonding (H-bonding) can be seen between the North 2 amide hydrogen and EPO and PVP. In addition, H-bonding was also modeled between the North 2-indole hydrogen and PVP. Hydrophobic interactions between the North 2-indole ring and EPO and HPMC were shown. These data are consistent with ^1H NMR data described above. This hydrophobic interaction could influence the indole-hydrogen proton environment, thus rationalizing the ^1H NMR shift seen with North 2 containing binary systems. There was no interaction seen with the North 2-amide hydrogen and HPMC (**Figure 2.6**, panel B). Ternary systems with North 2, curcumin and each polymer (EPO, HPMC and PVP) were

also subjected to molecular dynamics simulations (**Figure 2.6**, panels D, E and F). H-bonding between the North 2-amide hydrogen and EPO, HPMC and PVP were seen in the simulations. Interesting, the ternary system with HPMC was able to show a hydrogen bond with the North 2-indole hydrogen and HPMC. Similar to the North 2 binary systems, hydrophobic interactions between the North 2-indole ring and EPO and HPMC as well as PVP. These data help rationalize chemical shift change of both the North 2-amide and -indole hydrogen atoms seen in the ^1H NMR experiments. North 2 and curcumin were seen to interact with each other through slipped parallel Van der Waals interactions between the phenyl ring of curcumin and indole ring on North 2 in the ternary systems with EPO and HPMC (**Figure 2.6**, panels D and E). No North 2 and curcumin interactions were seen in the ternary complex with PVP.

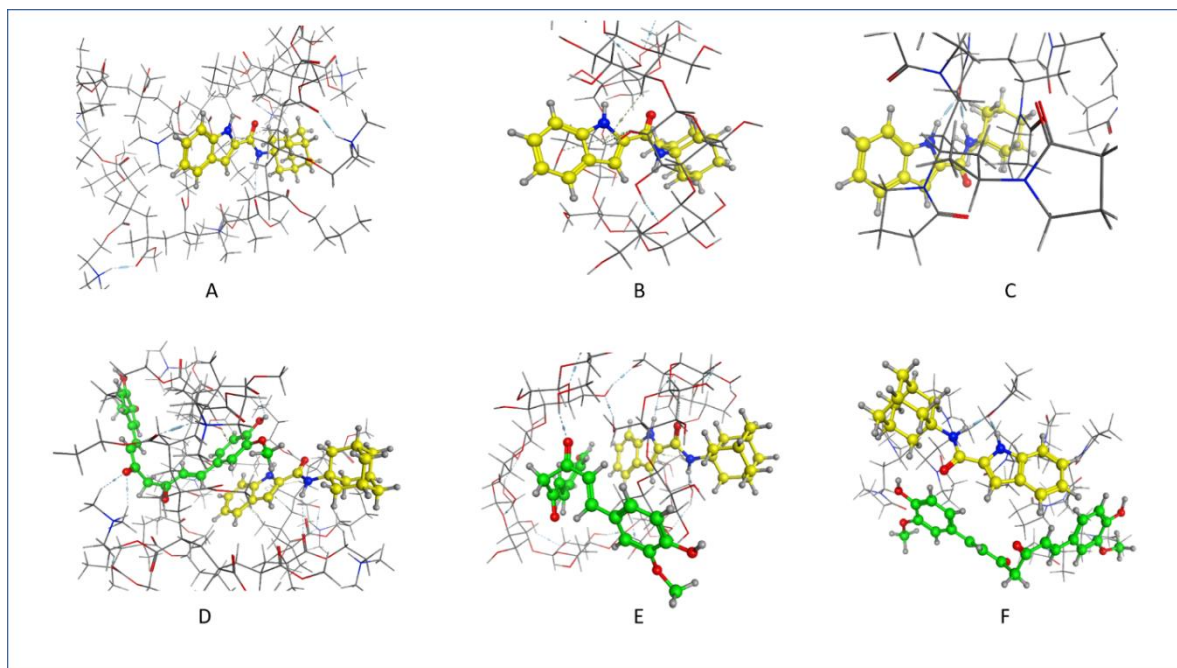


Figure 2.6: Molecular dynamic simulations showing N2-EPO (panel A), N2-HPMC (panel B), and N2-PVP (panel C) binary systems and N2-Cur-EPO (panel D), N2-Cur-HPMC (panel E), and N2-Cur-PVP (panel F) ternary systems. In each panel, hydrogen atoms are light grey, oxygen atoms are red, nitrogen atoms are blue, and carbon atoms are yellow for N2, green for curcumin, and grey for polymers. Blue dotted lines between drugs and

polymers denote hydrogen bonding seen in A, C, D, E, and F. Grey dotted lines between drugs and polymers denote hydrophobic interactions seen in panels A, B, and D.

2.2.2.9 UV-Visible Spectroscopy:

The wavelengths of the maximum absorbance of Curcumin and North 2 were found to be 424nm and 298nm respectively. The standard curve of the serial dilutions of the two drugs followed the Lambert's law of linearity from 20 μ g/mL to 0.01 μ g/mL.

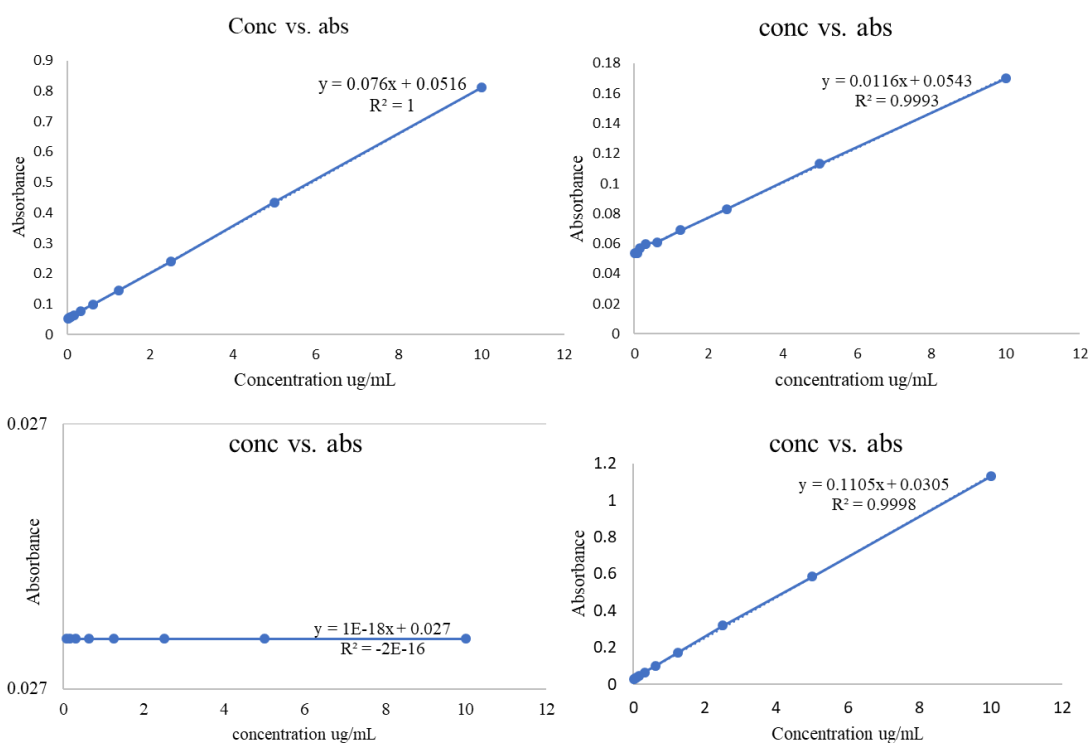


Figure 2.7: Standard curves: a) North 2 at 298nm b) Curcumin at 298nm c) North 2 at 424nm d) Curcumin at 424nm

The curcumin showed absorbance at 298nm and 424nm with the R^2 value more than 0.99 and North 2 showed absorbance at only 298nm and followed the linearity with R^2 value more than 0.99. The simultaneous method was developed according to following:

Equations derived from the standard curves:

Equation 1 Curcumin₂₉₈: $y = 0.0116x + 0.0543$

Equation 2 North 2₂₉₈: $y = 0.076x + 0.0516$

Equation 3 Curcumin₄₂₄: $y = 0.1105x + 0.0305$

Equation 4 North 2₄₂₄: $y = 1E-18x + 0.027$

For a mixture of Curcumin and North 2

At wavelength of 298nm:

Equation 5 $\text{Absorbance}_{298} = (K_{298\text{cur}} * C_{\text{cur}}) + (K_{298\text{north2}} * C_{\text{north2}})$

At wavelength of 424nm:

Equation 6 $\text{Absorbance}_{424} = (K_{424\text{cur}} * C_{\text{cur}}) + (K_{424\text{north2}} * C_{\text{north2}})$

Thus,

Equation 7 $C_{\text{cur}} = \text{Absorbance}_{424} / K_{424\text{cur}}$

Equation 8 $C_{\text{north2}} = [\text{Absorbance}_{298} - (K_{298\text{cur}} * C_{\text{cur}})] / K_{298\text{north2}}$

This method was developed but was not used for the analytical quantitation of two compounds because the results from this method could detect concentrations in the range of $\mu\text{g/mL}$ which was a limitation for the detection and quantification of compounds after dissolution experiments. For better detection and quantification LC-MS/MS method was developed and validated.

2.2.2.10 In-vitro dissolution studies:

Prasad et al., have designed ternary solid dispersions using two polymers and achieved dissolution enhancement of indomethacin (D Prasad, Amorphous stabilization and dissolution enhancement of amorphous ternary solid dispersions: combination of polymers

showing drug-polymer interaction for synergistic effects., 2014) There have been studies which focus on the designing multidrug as solid dispersions (Arca H. C.-G., 2017) (Alhalaweh, 2016). Riekes et al., have used this approach to develop the amorphous ternary solid dispersions of fixed dose combinations. They have studied ternary solid dispersion of hypolipidemic compounds, Lovastatin and Ezetimibe with 25% of drug loading in 75% of polymer. They have achieved around 50% release for both the compounds from 25% drug loading (Riekes M. K., 2016). There has been a development of solid dispersion for anti-TB treatment. Arca et al. performed solid dispersion of rifampin at different pH, because this drug is not stable at all pH. At gastric pH, the release of rifampicin was found to be 90% where in intestinal pH this concentration rapidly reduced (Arca H. Ç.-G., 2018). Current research study is focused on enhancement of dissolution of both North 2 and curcumin for combination treatment of tuberculosis. Having confirmed that all the ternary solid dispersions were amorphous, the dissolution experiments were designed to understand the in-vitro drug release behavior of curcumin and North-2. Eudragit®EPO is soluble in aqueous medium at pH 5-5.5 (Rowe, 2006), hence in case North 2:Cur:EPO, the medium contains the hydrochloric acid buffer of pH 5.5 for initial 30 minutes of dissolution and the phosphate buffer of pH 7.4 for rest of the time period of dissolution, this mimics the gastric and intestinal conditions of body (Arca H. Ç.-G., 2018). For North 2:Cur:PVP and North 2:Cur:HPMC ternary solid dispersions, the medium was phosphate buffer throughout the dissolution experiment. The dissolution studies showed that the concentration of the compounds released from the ternary amorphous complexes with HPMC, EPO and PVP were significantly higher than their physical mixtures. North 2 and curcumin followed different release pattern from North 2:Cur:EPO (1:1:2). For both the

compounds, initial dose added in the dissolution was 17.5 mg in each ternary solid dispersion. From North 2:Cur:EPO, concentration of North 2 was $55.16 \pm 4.10 \mu\text{g/mL}$ at 12 hrs., which is around 29% of initial concentration. Similarly, concentration of curcumin was $167.33 \pm 11.24 \mu\text{g/mL}$ at 6 hrs. which is around 89% of initial concentration. The release of North 2 and curcumin from equivalent PM was 0.52% and 1.49% respectively. Compared to PM, the release for North 2 and curcumin was increased by 55 folds and 59 folds respectively. (**Table 2.5, Figure 2.7**). The concentration of the North 2 released from North 2:Cur:PVP (1:1:2) was $20.44 \pm 1.48 \mu\text{g/mL}$ at 12 hr, which was about 10% of the initial drug concentration. Curcumin concentration released from North 2:Cur:PVp was $13.60 \pm 1.24 \mu\text{g/mL}$ which was around 7% of the initial concentration. This was found to be a 5 folds and 5 folds increase, as compared to physical mixtures, in the release of North 2 and curcumin respectively (**Figure 2.7**). The concentration of the North 2 and curcumin released from North 2:Cur:HPMC (1:1:2) was $8.26 \pm 0.43 \mu\text{g/mL}$ and $11.73 \pm 1.52 \mu\text{g/mL}$ respectively. This was around 4% and 6% release from initial concentration respectively. And this was found to be 4- and 6-fold increase in release of North 2 and curcumin respectively as compared to PM. (**Figure 2.7**).

An area under the curve (AUC), which is determined by dividing the difference between consecutive concentrations by the difference in respective time, showed that AUC for all the solid dispersions were very higher than respective PMs. All the AUC values mentioned in this section are at 12 hours of the dissolution study and are shown in **Table 2.5**. In North 2:Cur:EPO, AUC for North 2 and curcumin were found to be $3.19 \pm 0.19 \mu\text{g/mL/min}$ and $25.21 \pm 1.95 \mu\text{g/mL/min}$ respectively, whereas AUC of North 2 and curcumin from physical mixture were $0.06 \pm 0.004 \mu\text{g/mL/min}$ $0.33 \pm 0.08 \mu\text{g/mL/min}$ respectively. This

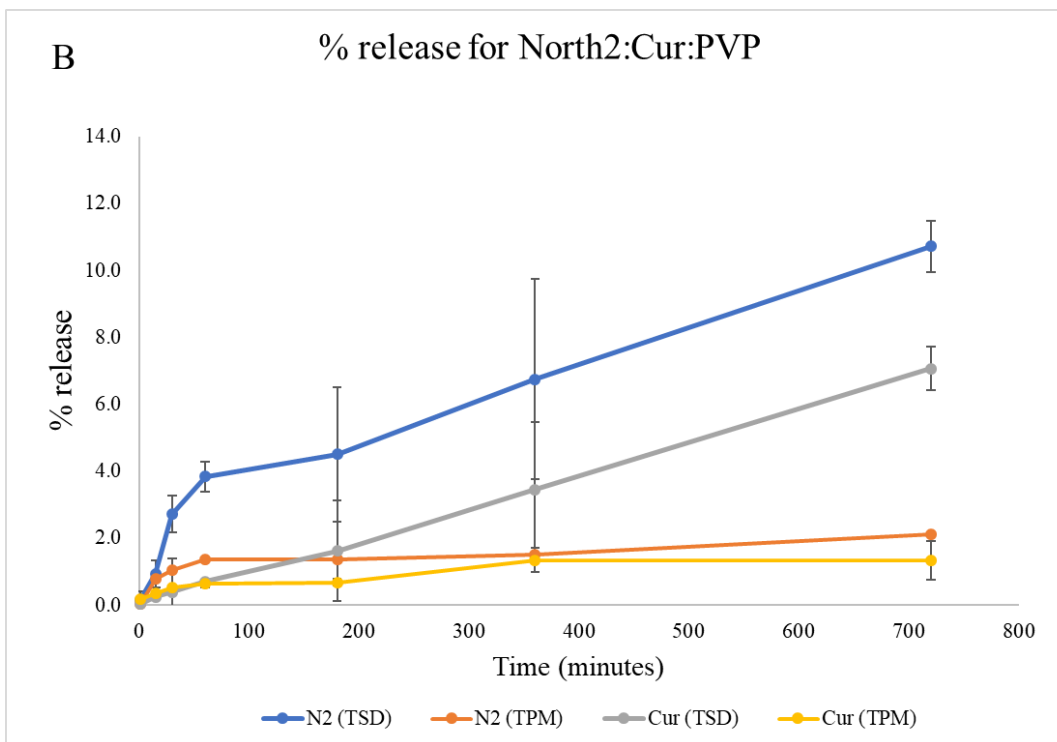
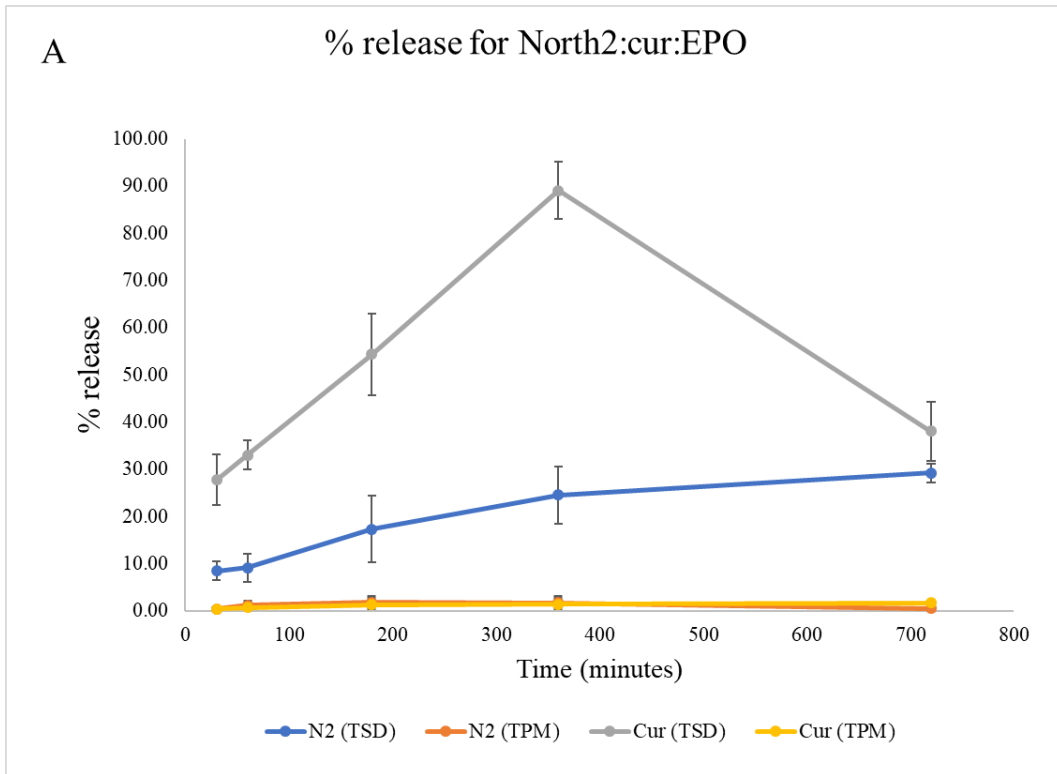
was around 53- and 73-folds increase in AUC compared to PM. This suggests that bioavailability of compounds is much higher when released from SDs as compared to PMs. In case of North 2:Cur:PVP, SD gave the AUC of North 2 and curcumin were 0.23 ± 0.04 $\mu\text{g/mL/min}$ and 0.047 ± 0.002 $\mu\text{g/mL/min}$ respectively. And in PM the AUC of North 2 and curcumin after the dissolution was 0.08 ± 0.001 $\mu\text{g/mL/min}$ and 0.02 ± 0.001 $\mu\text{g/mL/min}$ respectively. Though this increase in AUC was not as high as North 2:Cur:EPO, solid dispersion gave an enhancement as compared to PM. Similarly for North 2:Cur:HPMC, AUC of North 2 and curcumin from ternary solid dispersion was 0.43 ± 0.006 $\mu\text{g/mL/min}$ and 0.15 ± 0.02 $\mu\text{g/mL/min}$ respectively. For equivalent PM it was 0.047 ± 0.002 $\mu\text{g/mL/min}$ and 0.0049 ± 0.003 $\mu\text{g/mL/min}$ for North 2 and curcumin respectively. This data indicates that the ternary solid dispersions were able to release the compounds to the greater extent as compared to PMs. More amount of both the compounds was available for longer time in dissolution medium. On the other hand, AUC for North 2 of the amorphous dispersion with PVP was found to be 0.13 ug/mL/min which was 3.2 times more than the AUC of physical mixture which was 0.042 ug/mL/min . Similarly, AUC for curcumin of the amorphous dispersion with PVP was found to be 0.031 ug/mL/min which was 1.55 times more than the AUC of physical mixture which was 0.02 ug/mL/min . An area under the curve (AUC) for North 2 of the amorphous dispersion with HPMC was found to be 0.099 ug/mL/min which was 4.95 times more than the AUC of physical mixture which was 0.02 ug/mL/min . Similarly, AUC for curcumin of the amorphous dispersion with HPMC was found to be 0.044 ug/mL/min which was 4.88 times more than the AUC of physical mixture which was 0.009 ug/mL/min .

Table 2.5: The C_{max} , T_{max} and AUC of North 2 and curcumin in prepared ATSD

	North2:Cur:EPO				North2:Cur:PVP				North2:Cur:HPMC			
	North 2		Curcumin		North 2		Curcumin		North 2		Curcumin	
	PM	SD	PM	SD	PM	SD	PM	SD	PM	SD	PM	SD
C _{max}	1.67	55.1	3.2±	167.3	2.10	20.4	13.6	1.33	1.0	8.26	1.46	11.7
(ug/m	±1.2	6±4.	0.36	3±11.	±0.0	4±1.	0±1.	±0.5	±0.4	±0.2	±0.5	3±1.
L)		10		24	2	48	24	8	0	3	0	52
T _{max}	720	720	360	360	720	720	720	720	360	360	720	360
(Minu												
tes)												
AUC	3.07	0.06	0.33	25.07	0.08	0.25	0.02	0.05	0.04	0.43	0.00	0.15
(ug/m	±0.3	±0.0	±0.0	±1.90	±0.0	±0.0	±0.0	±0.0	±0.0	±0.0	6±0.	±0.0
L/min	6	06	8		0	5	1	1	0	1	00	2
)												

Dissolution data was plotted using various mathematical models to understand the release mechanism of curcumin and North-2. The dissolution data was divided in two different phases namely, release and precipitation as the dissolution data showed the bell shaped graph meaning there is the precipitation of the drug after certain time in aqueous medium since the compounds are poorly water soluble. The dissolution phase was plotted models like first order, zero order, Higuchi model, Hixson Crowell model and Korsmeyer peppas model. The release of North 2 from North 2:Cur:EPO was found to be following the Hixson-Crowell model for the release data (cube root of drug remaining v/s time) of drug dissolution with the regression of 0.9928. According to Hixson-Crowell model, larger the

area faster is the dissolution and it describes the drug release with changes in surface area of particles (Dash, S., 2010),(Shoaib, M. H., 2006). This tells us that North 2 particles in polymer matrix were reached to very small size leading to the large surface area. This is because the linearity in Hixson-Crowell model suggests that the geometrical shape of the dosage form diminishes proportionally over the time (Hixson, 1931),(Singhvi, G., 2011).And as there was not precipitation started for North 2 after 12 hrs. of dissolution, there might be the further increase of North 2 release. Whereas the release data for the curcumin from North 2:Cur:EPO was found to be best fitted in Higuchi model (Square root of time v/s % release of the drug) with the regression of 0.9721. Since release of curcumin follows Higuchi model, it can be said that there is a slow release of curcumin from EPO matrix. The concentration of curcumin remains constant for certain period of time, i.e., even though there is decline in drug loading in the matrix during the process, the thermodynamic activity remains at unity for most of the delivery time as the initial drug loading is much higher than the solubility of the drug(Alves, T. F. R., 2018),(Paul, D. R., 2011).Similarly, studies done with North 2:Cur:PVP showed that North 2 in this case follows Higuchi model of dissolution with regression of 0.9608. As there was no precipitation observed of North 2 in North 2:Cur:PVP, all the release data were analyzed as a drug release without dividing it into two phases. This tells us that the super-saturation for North 2 has not yet achieved and hence there might be further increase in the release of North 2 if the study is continued. Whereas curcumin in the ternary formulation with PVP followed zero order release model of the drug dissolution profile for initial 12 hours with regression of 0.999.



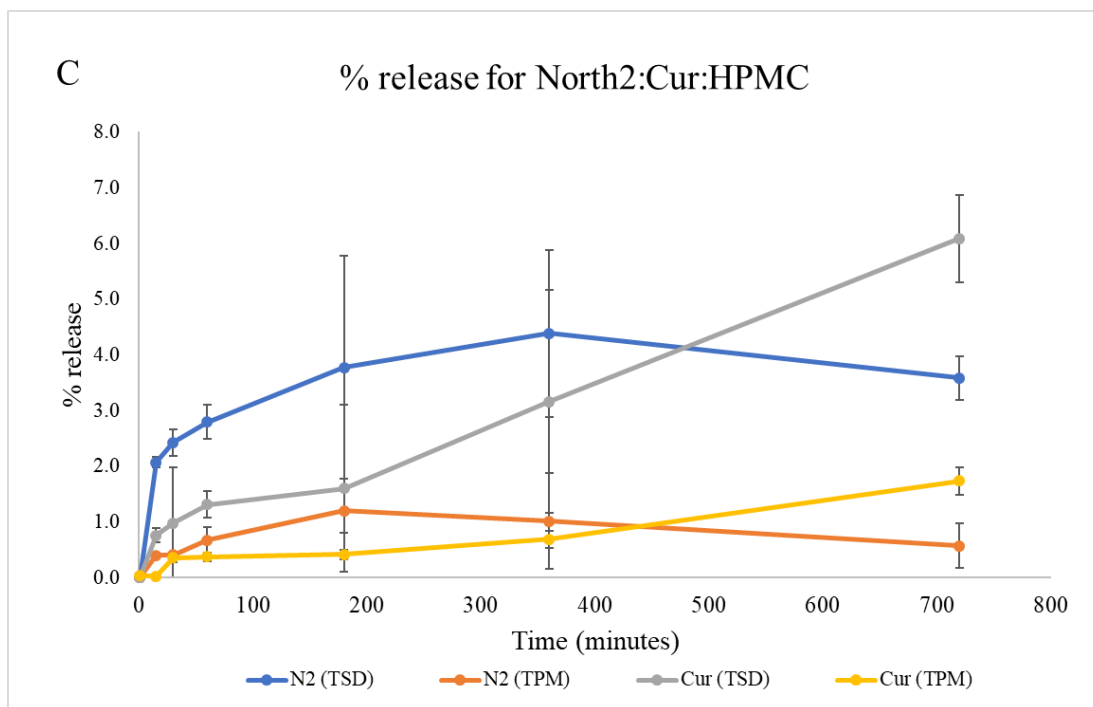


Figure 2.8: Dissolution release profiles of North 2 and curcumin in their physical mixtures and solid dispersions. (7A): Solid dispersion of North2 and Cur with EPO formulation; (7B): Solid dispersion of North2 and Cur with PVP formulation; (7C): Solid dispersion of North2 and Cur with HPMC formulation;

(TPM: Ternary Physical Mixture; TSD: Ternary Solid Dispersion)

2.2.2.11 Stability studies:

XRD is often used as one of the important analytical technique to test the long-term solid-state stability of amorphous solid dispersions (Tonnesen, 2002),(Meng F, 2015).This tells about the amorphicity of the dispersions in physical state. There have been studies where

amorphous dispersions were subjected to different conditions and chemical stability is tested using XRD(Li, B., 2013). In this paper, all the amorphous ternary solid dispersions were stored at room temperature for 3 months and tested for stability. No crystallinity in the three amorphous ternary solid dispersions, North 2:Cur:EPO, North 2:Cur:PVP and North 2:Cur:HPMC (1:1:2) was observed for three months (Figure 2.8). Overall, this concludes that the ternary amorphous dispersions, North 2:Cur:EPO, North 2:Cur:PVP and North 2:Cur:HPMC (1:1:2) stayed stable over the period of 90 days in terms of amorphicity.

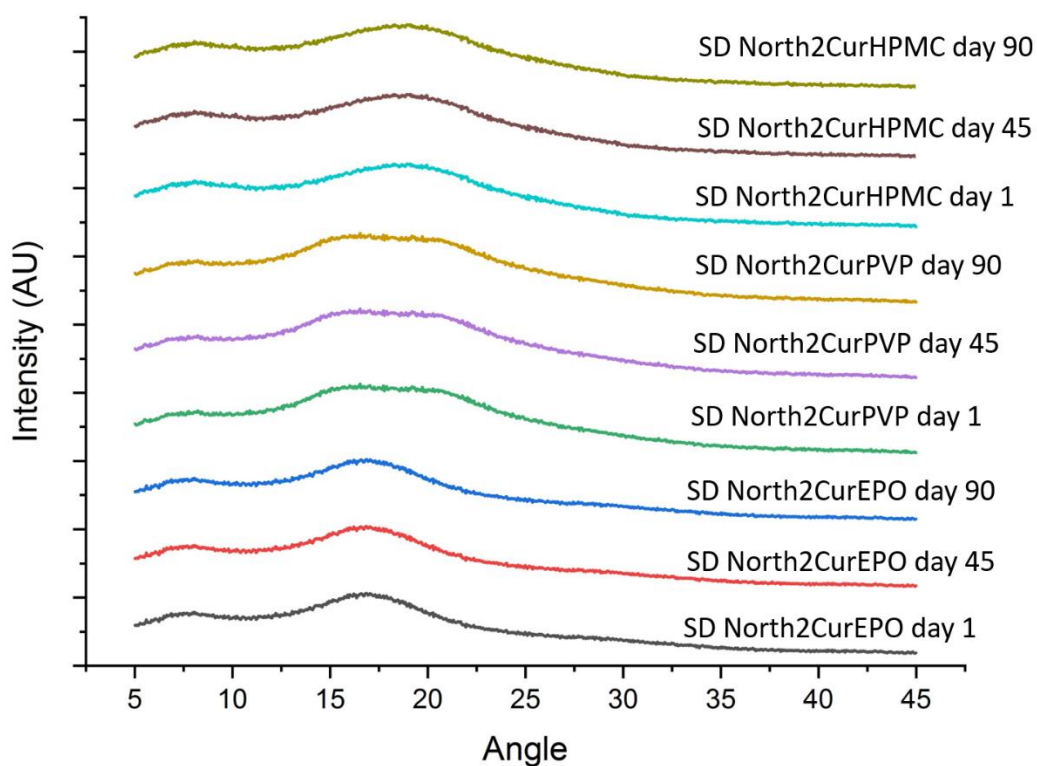


Figure 2.9: XRD plots of amorphous ternary solid dispersions of North2 and Curcumin with EPO, PVP, and HPMC stored at room temperature for 1, 45, and 90 days.

2.3 Conclusion:

The novel amorphous ternary solid dispersion of novel, highly potent, anti-tubercular agent, indole-2-carboxamide derivative, North 2 with enhanced aqueous solubility of both North 2 and curcumin were successfully prepared and evaluated. The ternary dispersions having the high drug loading of 50%, i.e. 1:1:2 (North 2:Curcumin:Polymer) showed the increase in dissolution of both compounds as compared to their physical mixtures. Among polymers, EPO was found to be working best with the combination of two compounds by increasing the compounds' release in dissolution by around 55 and 59 folds for North 2 and curcumin respectively. Possible reason for enhanced solubility was investigated and confirmed as hydrogen bonding by studying interactions of the compounds with the polymers by FTIR, NMR, and molecular modeling. These results concluded that interactions between two compounds and polymer in ternary dispersions can be used for solubility enhancement of two poorly soluble compounds. In future, different fixed dose combination of North 2 or other potent indole-2-carboxamide derivatives can be formulated as ternary amorphous solid dispersions. This will be effective approach for the formulation of different fixed dose combinations involving poorly soluble compounds for the treatment of challenging diseases such as cancer, diabetes, AIDS, and cardiac disorders.

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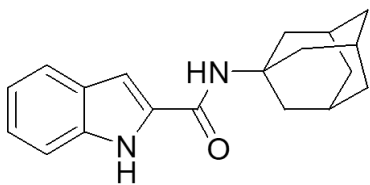
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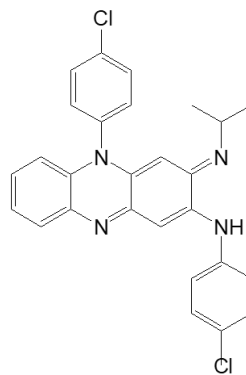
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3 Combination of North 2, anti-TB compound and clofazimine, established anti-TB agent

Clofazimine is the prototype riminophenazine antibiotic (**Figure 3.1**). It was originally described in 1957 (BARRY, et al., 1957). This molecule is very active against *Mycobacterium tuberculosis*, including multidrug-resistant mycobacterial strains (Reddy VM, 1999). Clofazimine has not been reported as active against other bacteria hence it is considered as narrow spectrum an anti-tubercular agent (I. Chopra, 1998). Clofazimine is active against gram positive as well as gram negative multi drug resistant mycobacteria, in case of gram positive bacteria MIC is 0.5-2mg/L (Moloko C. Cholo, 2012). Clofazimine is active against resistant species, Isoniazid resistant *M. tuberculosis* MIC= 0.39 µg/mL, Multi-drug resistant strains MIC =0.12-2 µg/mL, Pyrazinamide resistant *M. tuberculosis* MIC= 0.25-0.5 µg/mL, Pyrazinamide resistant *M. tuberculosis* strains MIC= 6.25 µg/mL (Reddy VM, 1999) (De Logu A, 2002). Despite being impressively active against mycobacteria, low water solubility of clofazimine is the rate limiting step for its absorption. There have been number of strategies to improve the solubility, mainly by formulation of solid dispersions. Polymers like PVP, cyclodextrins, polyethylene glycol were used to improve the dissolution by formulating solid dispersions (Kailasam S, 1994) (Krishnan TR, 1991).



A) North 2



B) Clofazimine

Figure 3.1: Chemical Structure of A) North 2, B) Clofazimine

In this chapter clofazimine is formulated with anti-tubercular agent, North 2 for the combination therapy. Wei Li, et. al. have studied indole-2-carboxamide derivatives with different anti-TB drugs and tested them for the synergistic effects. Clofazimine was found to give FIC (Functional Inhibitory Concentration) value of 0.5 with the series of indole-2-carboxamide derivatives, indicating that clofazimine has synergistic effects with North 2 (Wei Li, 2017). Hence in this study, two anti-TB agents are used in the combination with hydrophilic polymers to improve the solubility of both the compounds, North 2 and clofazimine simultaneously. Based on the screening of the polymers done in the last chapter, EPO, PVP and HPMC were screened for the formation of ternary solid dispersions and ternary solid dispersions were tested for XRD, FTIR, and DSC studies. Crystallinity of both the compounds was tested in presence of polymers. Amorphous ternary solid dispersion was tested for dissolution of North 2 and clofazimine and solubility of both the compounds was improved as compared to physical mixtures.

3.1 Methods:

3.1.1 Miscibility studies: Modified Differential Scanning Calorimetry (DSC):

This study was performed similar to the combination of North 2 and clofazimine. Differential scanning calorimetry (MDSC) was performed by DSC Q2000 (TA instruments, New Castle, DE) equipped with a refrigerated cooling accessory (RCS90) and a data analyzer (Universal Analysis 2000, TA instruments). The equipment was calibrated with Indium. Inert atmosphere was maintained by nitrogen at a flow rate of 20 mL/min. An empty aluminum pan was used as reference. The thermal behavior of pure North 2 and PMs were characterized by a series of heating–cooling–heating cycles. Samples were heated at 10⁰C/min from 30 to 250⁰C (Cycle 1), cooled at a rate of 20⁰C /min to 30⁰C (Cycle 2), and then reheated at 10⁰C /min to 250⁰C (Cycle 3). Pure clofazimine and its PMs were characterized in similar manner where Cycle 1 and Cycle 3 were from 30⁰C to 250⁰C. The final temperature is kept at around 15⁰C above the melting point of North 2 and clofazimine i.e. 250⁰C.

3.1.2 X-ray Diffraction:

XRD diffraction data of PM and solid dispersions in the 2theta, ranging between 5–60 degrees were collected in focusing geometry using PANalytical Empyrean Diffractometer, operated with Cu K α radiation at 40 kV and 45 mA. A mask of 20 mm and a divergence slit of 1/4 degree was used on the incident beam path. Thin layer of powder sample was placed on a zero background Si plate and the sample holder was continuously spun at the rate of 90 deg/s during the measurement. Solid state PIXcel^{3D} detector was scanned at a rate of 0.135 deg/s to collect data and a diffracted beam monochromator for the Pixcel

detector was utilized to improve signal to noise ratio. This XRD method was used for crystallization studies and evaluation of ternary solid dispersions

3.1.3 Crystallization Studies:

Initially, concentrated solutions of North2 (10 mg/mL), clofazimine (10 mg/mL) and polymers (20 mg/mL) were prepared in acetone. 100 μ L of each sample was placed on the glass sample holder and allowed to dry for 5 minutes. The thin layer developed was tested according to the XRD procedure. The sample was tested at different time points starting from 5 min until 45 min. Samples are described in the table.

Table 3.1: Combination of compounds and polymers and their ratios used in crystallization study

Sample	Ratio
North 2	
CFZ	
North 2:EPO	1:1
CFZ:EPO	1:1
CFZ:EPO	1:2
CFZ:EPO	1:3
North2:CFZ:EPO	1:1:2
North2:CFZ:EPO	1:1:1
CFZ:PVP	1:1
CFZ:PVP	1:3
North2:CFZ:PVP	1:1:1

3.1.4 Fourier Transform Infrared Spectroscopy (FTIR):

Spectra Absorbance was obtained using the Nicolet iS 50 Fourier Transform Infrared Spectrometer (FT-IR) with a ZnSe ATR crystal. The spectra were signal averaged from 25 scans at 4 cm^{-1} resolution with a dry-air purge at ambient temperature. A background was collected to account for any interference that the environment might provide. Atmospheric compensation (to eliminate H₂O and CO₂ interference in the beam path) was used in all measurements. Background correction was carried out for every sample. Data processing for all infrared data was done using OMNIC(tm) FTIR Software.

3.1.5 Solubility Studies:

Aqueous solubility of North2:CFZ:EPO was performed in acetate buffer (0.1M, 5.2 pH) and phosphate buffer (0.01M, 7.4 pH). 5mg (Equivalent to 1.25mg of pure compounds) of sample was tested for the solubility in 10mL of both the buffer. Temperature was maintained at 37⁰C. Aliquots were taken at certain time points: 5min, 1hr, 3hr,6hr. Magnetic stirrers at 100rpm were used to rotate the aqueous solution in vials. In case of pure compounds, 1.25mg of North 2 and clofazimine in 10mL of buffer was used for the comparison. For North2:CFZ:HPMC and North2:CFZ:PVP, aqueous solubility were tested with phosphate buffer because of good solubility of HPMC and PVP at pH 7.4. The samples were tested with LCMS method.

3.1.6 *In vitro* dissolution:

USP apparatus II containing paddles was used for the dissolution experiment at 100rpm. Formulated solid dispersion equivalent to 15mg of each drug is added to a well containing 90 mL of the medium and the physical mixture of the same ratio was added to another well (n=3). The temperature of the apparatus was maintained at 37⁰ C. The experiment was set

for 12 hours. In case of ternary solid dispersion with EPO, initially, for 30 minutes, 20mL of acetate buffer of pH 5 was added (to dissolve the EPO) and the quantity of medium was increased up to 90mL with the phosphate buffer of pH 7.4 to make the medium neutral after first 30 minutes. This condition was simulated to the human stomach and intestine pH. The aliquots were taken at 1, 15, 30, 60, 180, 360, and 720 minutes. Warfarin was added as the internal standard for North 2 and clofazimine respectively. The samples were tested with LCMS.

3.1.7 LCMS Method:

An Exion HPLC system (Applied Biosystems, Foster City, CA) coupled with API 5500 QTrap mass spectrometer (Applied Biosystems, Foster City, CA, USA) was used. The mass spectrometer was operated in electrospray ionization (ESI) positive mode with m/z 295.2 $[M+H]^+ \rightarrow 135.2$ for North-2 and 473 $[M+H]^+ \rightarrow 431.0$ for clofazimine were selected for quantification. Chromatographic separation was performed on Phenomenex Kinetex EVO C18 column (50×3.0 mm, $5 \mu\text{m}$) with an isocratic mobile phase containing 20:80 v/v 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a flow rate of 0.250 mL/min. The liquid chromatography-tandem mass spectrometer was controlled by Analyst 1.6.3 software. The retention times were 1.6 and 0.82 min for North 2 and clofazimine respectively. The total run time for each sample was 3 min. Warfarin was used as internal standards for clofazimine and North 2.

3.1.8 Scanning Electron Microscopy:

An aluminum mount was coated with an adhesive using an adhesive Tab. The solid dispersion was placed on the adhesive surface, excess sample that did not adhere to the

surface was gently blown away leaving a single layer of particles on the surface of the mount. The sample was then placed in an EMS/Quorum ES R sputter coater and coated with gold/palladium to render it conductive. Sample was viewed in a 57 scanning electron microscope at 15 kilovolts voltage. Images were captured as TIFF's using a Quartz PCI system. The dried ATSD and the filtered and dried samples after the dissolution studies were tested for the SEM.

3.2 Results and discussion:

3.2.1 Differential scanning calorimetric analysis):

3.2.1.1 Crystallization tendency of pure compounds:

This study has been designed according the experiments done for the combination of clofazimine and North 2 in Chapter 2. Three cycles in the DSC system were studied. Endothermic and exothermic transition in DSC heat-cool-heat cycles of pure compounds were compared with the cycles of physical mixtures (PMs) of the compounds and the polymer to determine their miscibility and effect of the polymers on the crystallization tendency of pure compounds. Three cycles were run for each sample and are like, Cycle 1: Heating cycle at 10⁰C/min; Cycle 2: Cooling cycle at 20⁰C/min; Cycle 3: Heating cycle at 10⁰C/min. Both the heating cycles were up to 250⁰C as North 2 and clofazimine have a melting point of around 231⁰C and 224⁰C respectively. The pure compounds, North 2 and clofazimine showed sharp melting point at 231⁰C and 224⁰C indicating the crystallinity of the two compounds (**Figure 3.2**), while the physical mixture (PM) of the compounds with the polymer showed the depression in melting endotherm. For North 2, the DSC data from

the chapter 2 was used to compare with clofazimine and ternary PMs. Similar studies with pure clofazimine showed no crystallization followed by a melting in cycle 2 and cycle 3, this suggested that clofazimine is a low crystallization tendency compound.

3.2.1.2 Miscibility between compounds and polymers:

When there is a change in endothermic event such as melting of crystalline compounds due to presence of polymer, then it can be stated that the compound is miscible with the polymer and polymers can hold the crystalline compound in amorphous state (Chauhan H, 2015) (Meng F. T., 2015) (Qian, 2010). Similar to the studies with North2-CFZcuimin, in this study, different selected polymers were tested for their miscibility with two compounds qualitatively. Similar results for North 2 from Chapter 2 were utilized to compare them with clofazimine and ternary PMs. Pure clofazimine showed onset of melting at 223.28⁰C and PM Clofazimine-EPO showed at 208.85⁰C. This indicated the miscibility of EPO is miscible with clofazimine. Tg at 68⁰C in the third cycle of PM Clofazimine-EPO showed that clofazimine was present in amorphous form with EPO. Two different ratios of North2:CFZ:EPO were tested, since 1:1:2 gave a melting event in the heating cycle. When both, North 2 and Clofazimine were mixed with EPO, North2:CFZ:EPO 1:1:2, first cycle showed melting at 193⁰C. This melting was broad and started at 186⁰C, Tg was observed in third cycle indicating the amorphous nature. This indicated partial miscibility of EPO with both compounds. In North2:CFZ:EPO 1:1:3, no sharp melting was observed in cycle 1. This suggested that combination of clofazimine and North 2 needed larger amount of EPO to be in amorphous state. Clofazimine showed onset of melting with PVP at 220⁰C which is less as compared to EPO's effect, indicating less miscibility of clofazimine with PVP (**Figure 3.2**). HPMC behaved in the similar manner with two compounds as of EPO

in all the DSC cycles. Clofazimine started melting at 218⁰C, indicating less miscibility that EPO. North 2 and Clofazimine, together with HPMC showed melting suggesting a poor miscibility (**Figure 3.2**). Hence, based on this DSC study, rank of polymers in terms of miscibility with two compounds is EPO>PVP>HPMC.

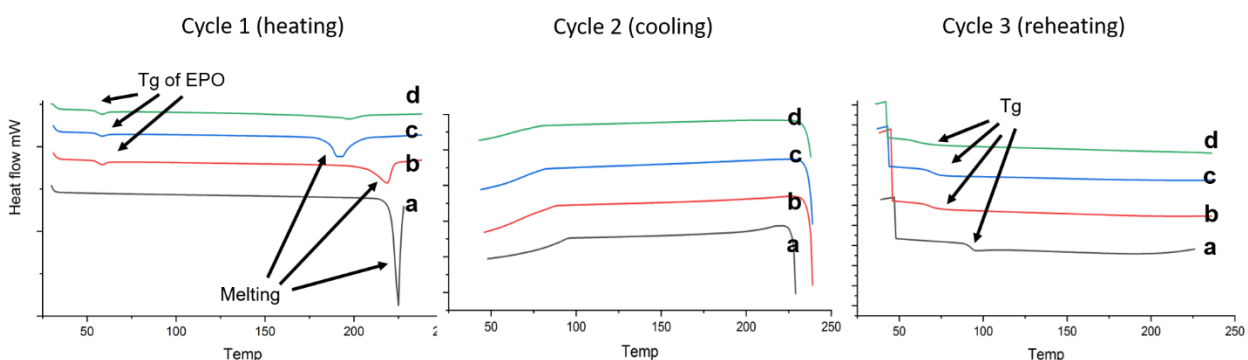


Figure 3.2: DSC plots of a: Pure CFZ; b: PM CFZ:EPO; c: PM N2:CFZ:EPO 1:1:2; d: PM N2:CFZ:EPO 1:1:3

3.2.1.3 Effect of polymers on crystallization tendency of North-2 and clofazimine:

When PM of North 2 and EPO were run for three cycles, compound showed melting in cycle 1 and no crystallization peak was observed in the cycle 2 but in cycle 3 there was a crystalline peak followed by the melting peak (**Figure 3.2**), indicating that the crystallization tendency of North 2 reduced from high to moderate level in the presence of EPO. Similar studies were done with PVP and HPMC with North 2 and it was observed that crystallization tendency of North 2 reduced to moderate in presence of polymers. When similar set of cycles were run for clofazimine, there was no change in its crystallization tendency. Pure clofazimine and PM of clofazimine with polymers were having low crystallization tendency (**Figure 3.2**). Ternary PM that is two compounds and one polymer showed no melting peaks and crystallization peaks in any of DSC cycles. This

indicates that the polymers are miscible and having the effect of the crystallization tendency. The melting enthalpy of the pure compounds in the cycle 1 was found to be reduced in presence of the polymers (**Table 3.1**) and the peaks were widely spread as compared to that of the pure compounds, this indicates that the presence of the polymer and interaction between the polymer and compounds decreases the crystallinity of the compounds. Binary and ternary PMs with EPO showed the glass transition temperature of EPO at about 60⁰C (**Figure 3.2**, Heating cycle). In the PM of two compounds and a polymer in each of the three formulations, it was seen that there was a significant depression in the melting peak. The glass transition temperature (T_g) of the pure compounds in the cycle 3 were increased in presence of the polymer (**Table 3.1**) and in ternary PM like North 2:CFZ:HPMC, there were two T_g observed. These T_g maybe observed because of the two compounds in the same polymer at the same time. In the case of PVP (**Table 3.1**) a broad peak is observed initially from 50⁰C to 150⁰C indicating the presence of the moisture since the PVP is hygroscopic polymer. This was confirmed from the TGA plot of PVP and PM of the North 2, clofazimine and PVP. This study of PMs showed that three polymers reduced the crystallization tendency of North 2 and had no effect on clofazimine crystallization tendency which may result in additional stability of amorphous ternary systems after storage. Based on this discussion, rank order of capacity of amorphisation of polymers for two crystalline compounds, North 2 and clofazimine is EPO>HPMC>PVP.

3.2.1.4 Crystallization of compounds in absence and presence of polymers using XRD:

In this study, XRD was first time utilized to evaluate the crystallization tendency of pure compounds (North 2 and clofazimine) and compounds in presence of polymer was tested. This study was designed to observe the crystallization pattern of pure compounds from acetone and if there is any difference in the pattern when compounds are in a certain ratio with the polymers. After placing 100 μ L of each sample on the sample holder, it was allowed to dry for 5 minutes and the dry thin layer developed was tested for the presence of crystals of pure compounds at 5 min, 10min, 20 min, and 45min. Crystallization of pure compounds from the acetonic solution was compared with the crystallization of compounds from Compound: Polymer solutions. In case+ of North2:CFZ:EPO, crystallization of North 2 and clofazimine was evaluated from North 2 solution, clofazimine solution, North2:EPO solution, CFZ:EPO solution, and North2:CFZ:EPO solutions. Significant crystallization peaks at 13, 18 and 20 for North 2 were observed in the sample of pure North 2 solution after 5 minutes (**Figure 3.3**).

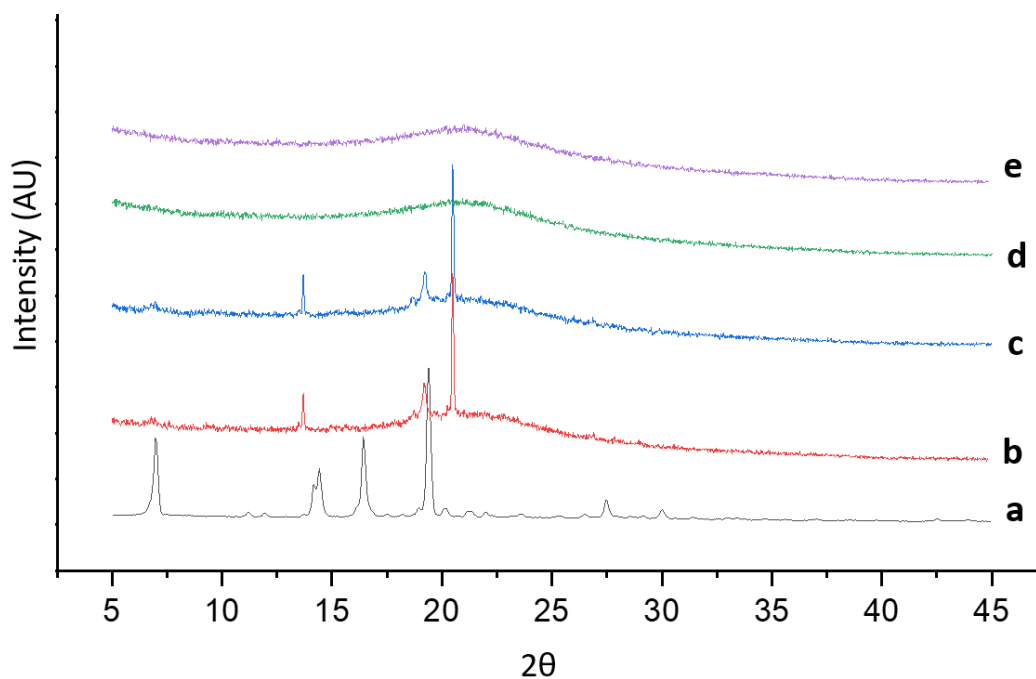


Figure 3.3: XRD plots of a: Pure North2; b: North2, 5 mins; c: North2, 20 mins; d: North2:EPO (1:1), 5 mins; e: North2:EPO (1:1), 20 mins after acetone evaporation

The intensity of these peaks was observed to be increased at 20 minutes, and the observation was stopped at 20 minutes. North2:EPO in the ratio of 1:1 was tested at 5 mins, 10 mins, 20 mins, and 45 mins and no crystallization peaks were observed in this case (**Figure 3.4**). This indicated that EPO could keep North 2 in the amorphous state and North 2 did not crystallize out even when acetone was completely dried out. Since 1:1 ratio of North 2 and EPO was found to be amorphous, no ratios with higher amount of EPO were tried. Similarly, for clofazimine, pure clofazimine solution after 5 mins showed a crystallization peak which was observed to be having higher intensity after 20 mins. The same peak was observed in CFZ:EPO 1:1 ratio at 5 mins and the intensity of the peak was increased after 20 mins, indicating that there was need of more polymer to stop the crystallization of clofazimine. Hence 1:2 ratio of CFZ:EPO was tested, and it was observed to be having the peak of lesser intensity than 1:1 ratio at 5 mins (**Figure 3.4**). This intensity increased a little at 20 mins. Lastly, 1:3, CFZ:EPO was tried and no crystallization peak was observed even after 45 mins, indicating clofazimine needed more amount of EPO to be in amorphous state than North 2. Finally, ternary solutions were tested (**Figure 3.5**). North2:CFZ:EPO in a ratio of 1:1:1 showed very small crystallization peaks but 1:1:2 ratio was completely amorphous even after 45 mins, indicating that presence of North 2 in the combination had some effect on the crystallization of clofazimine.

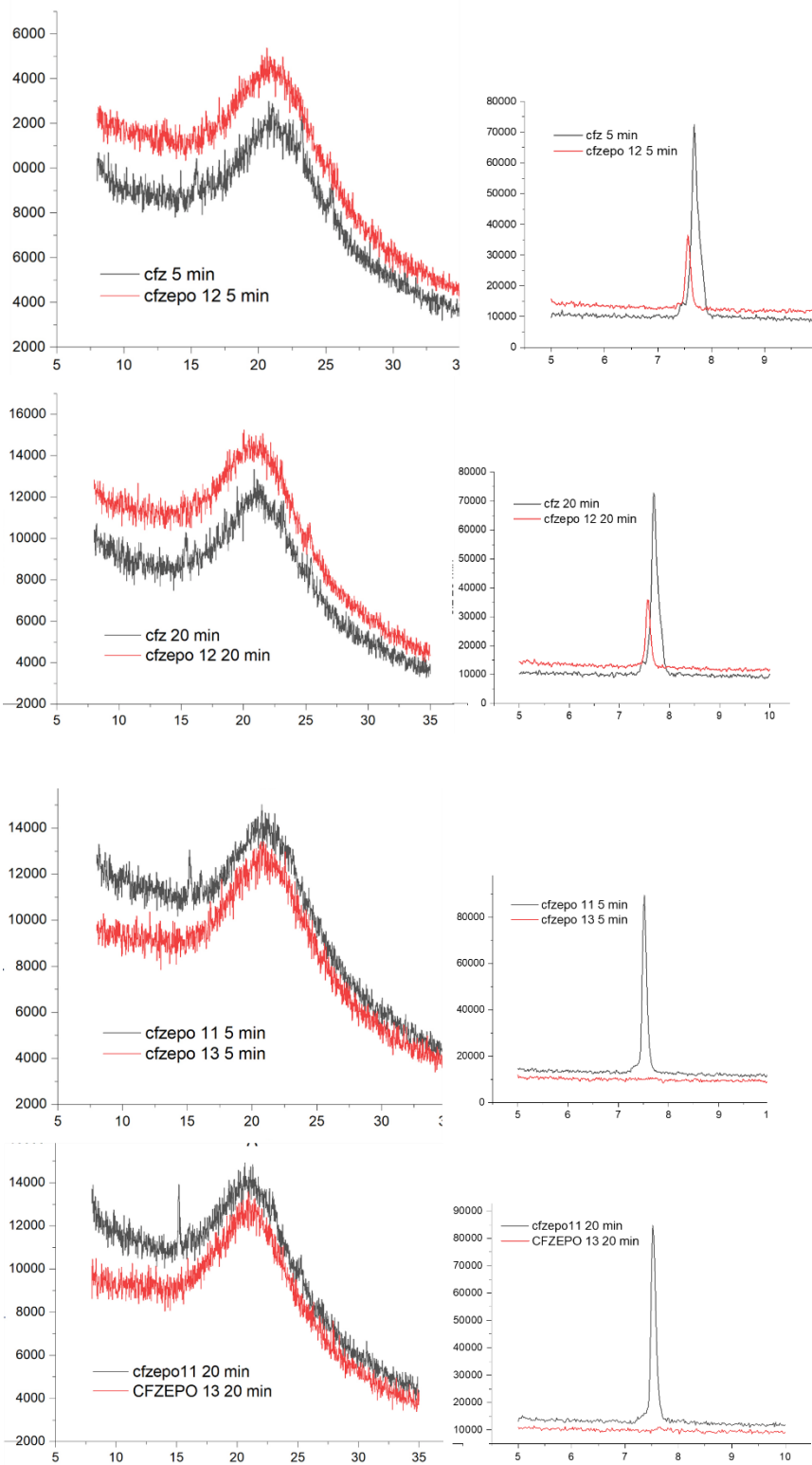


Figure 3.4: XRD plots of clofazimine after acetone evaporation in presence of EPO

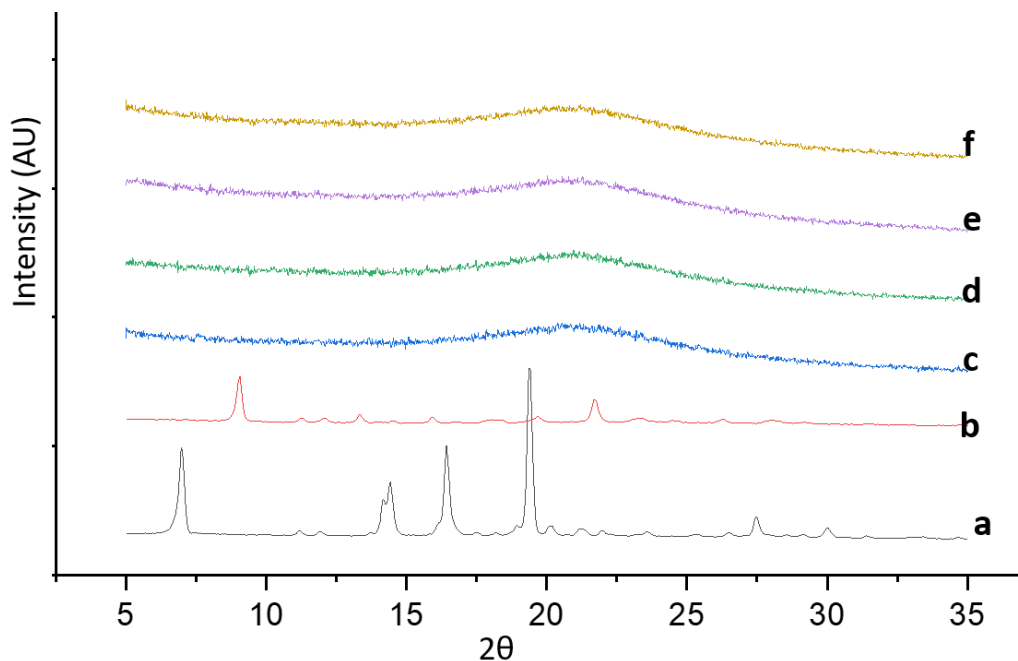


Figure 3.5: XRD plots of a: Pure North2; b: Pure CFZ; c: N2:CFZ:EPO (1:1:1), 5 min; d: N2:CFZ:EPO (1:1:1), 20 min; e: N2:CFZ:EPO (1:1:2), 5 min; f: N2:CFZ:EPO (1:1:2), 20 mins after acetone evaporation

3.2.1.5 X-Ray Diffraction:

The pure clofazimine showed the sharp diffraction peaks at 2θ between 8 to 25 (Ashlee D. Brunaugh, 2017), specifically at 9, 13, 16, and 32, and north 2 showed the diffraction peaks between 5 to 30 of 2θ , specifically at 7, 14.4, 16.4, 19.3, 27.5, and 29.9 (**Figure 3.6A**). XRD data of binary and ternary dispersions was plotted and sharp peaks showed that the binary solid dispersion of North 2:EPO (1:1) and CFZ:EPO (1:1) were crystalline whereas, the ternary formulation of North 2:CFZ:EPO (1:1:2) was found to be an amorphous system (**Figure 3.6A**). Binary CFZ:PVP and CFZ:HPMC (1:1), Ternary North 2:CFZ:PVP and North2:CFZ:HPMC (1:1:2) formulations were found to be crystalline (**Figure 3.6B**). All the PMs were crystalline. In binary dispersion, North 2:EPO, though the crystallization peaks corresponding to pure North 2 at 7, 14.4, 16.4, and 19.3 are

present, but the intensity of peaks is 2-3 times less than that of pure North 2. Similarly with North 2:HPMC, intensity of peaks at 7, 14.4, and 19.3 are low. In binary CFZ:EPO,CFZ:PVP and CFZ:HPMC (1:1) crystalline peaks at 9, 13, 16, and 32 were corresponding to pure clofazimine but similar to the case of binary North2 solid dispersions, intensity of clofazimine crystalline peaks were observed to be having lesser intensity than pure clofazimine. In ternary North 2:CFZ:PVP and North 2:CFZ:HPMC (1:1:2) intensity of crystalline peaks were less than the pure compounds. This indicates that the more amount of polymer can be utilized to get a completely amorphous ternary system. This finding related well with the crystallization studies done using XRD, where certain ratios of the combinations were studied to evaluate the amount of the polymer needed to hold the pure compounds in amorphous state.

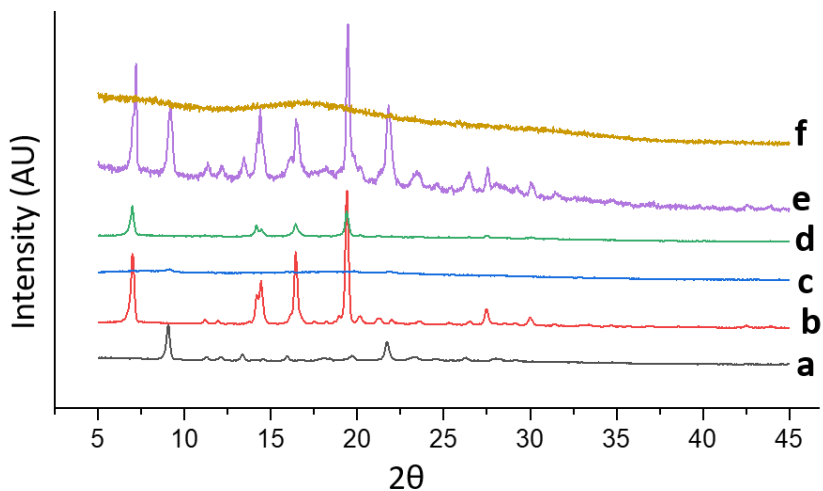


Figure 3.6 (A): XRD plots of a:CFZ; b:N2; c: SD CFZ:EPO (1:1); d: SD N2:EPO (1:1); e: PM N2:CFZ:EPO (1:1:2); f: SD N2:CFZ:EPO (1:1:2)

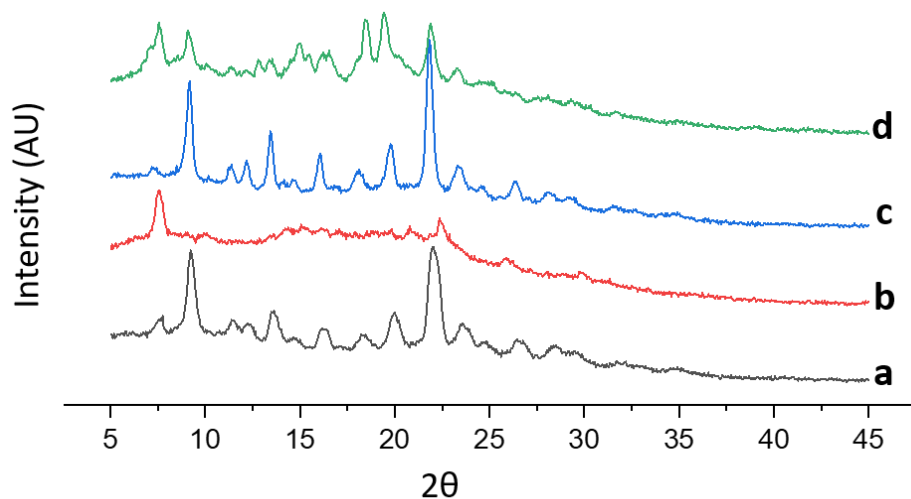


Figure 3.6(B): XRD plots of a: SD CFZ:PVP (1:1); b: SD N2:CFZ:PVP (1:1:2); c: SD CFZ:HPMC (1:1); d: SD N2:CFZ:HPMC (1:1:2)

3.2.1.6 Fourier Transform Infrared Spectroscopy:

IR analysis of pure clofazimine and PMs showed characteristic absorption peaks at 1625 cm^{-1} and 1556 cm^{-1} for CN imine and NH amide groups, respectively (**figure 3.7**) (Haichen Nie, 2019). The peaks at 3281 cm^{-1} (indole N-H), 1629 cm^{-1} (carbonyl group, C=O), 1504 cm^{-1} , 1490 cm^{-1} , 1446 cm^{-1} (strong to weak absorption peaks representing aromatic ring of indole), and the peak at 1537 cm^{-1} (N-H bending strongly coupled with C-N stretching, amide) are attributed to the North 2 structure (**Figure 3.7**). These peaks are present in the physical mixtures representing that there is no interaction in physical mixtures. In the case of North 2:CFZ:EPO (**Figure 3.7**), the shift of indole N-H peak from 3281 cm^{-1} (in pure drug and physical mixture) to 3289 cm^{-1} in solid dispersion of North 2 and EPO and 3265 cm^{-1} in the ternary solid dispersion suggesting hydrogen bonding interactions between indole N-H of North 2 and EPO. Also, the hypsochromic shift of the N-H bond of the amide from 1537 cm^{-1} (in pure North 2 and PM) to 1541 cm^{-1} in binary

SD North2:EPO was observed and ternary SD North2:CFZ:EPO showed no shift of absorption peak of N-H amide. This indicates no involvement of the N-H (amide) in hydrogen bonding in ternary North 2:CFZ:EPO. This study showed that there might be change in the vicinity of amide N-H when clofazimine was added with North 2 and EPO. Shift of the carbonyl peak of North 2 from 1629 cm^{-1} to 1628 cm^{-1} in binary SD North 2:CFZ and 1626 cm^{-1} in ternary SD North2:CFZ:EPO suggested that this oxygen is involved in hydrogen bonding as an acceptor. For clofazimine, in binary SD CFZ:EPO 1:1, there is a broadening carbonyl absorption peak 1722 cm^{-1} of EPO, indicating that carbonyl from EPO might be involved in the interactions. This broadening lead to the shift in binary SD CFZ:EPO 1:3 to 1728 cm^{-1} and also shift of amide NH of clofazimine peak from 1556 cm^{-1} to 1562 cm^{-1} was observed. This indicated that as the concentration of polymer increased, clofazimine started interacting with polymer functional groups. This interaction resulted into the amorphous form of the binary SD CFZ:EPO (this is discussed in the XRD studies of solid dispersions). In ternary SD North 2:CFZ:EPO 1:1:2, carbonyl group of EPO shifted from 1722 cm^{-1} to 1727 cm^{-1} with the shift of NH amide of clofazimine from 1556 cm^{-1} to 1563 cm^{-1} indicating the interactions between these functional groups in presence of North 2. While the shift on indole NH peak of North 2 indicated that carbonyl of EPO might be involved in the hydrogen bonding with both the active compounds. In binary SD, interaction between clofazimine and EPO was not enough to be resulted in amorphous form but in ternary SD, presence of North 2 and the interaction of functional groups of both the compounds with carbonyl of EPO resulted into better interactions and distribution of two compounds in polymer matrix. This gave the completely amorphous ternary solid dispersion.

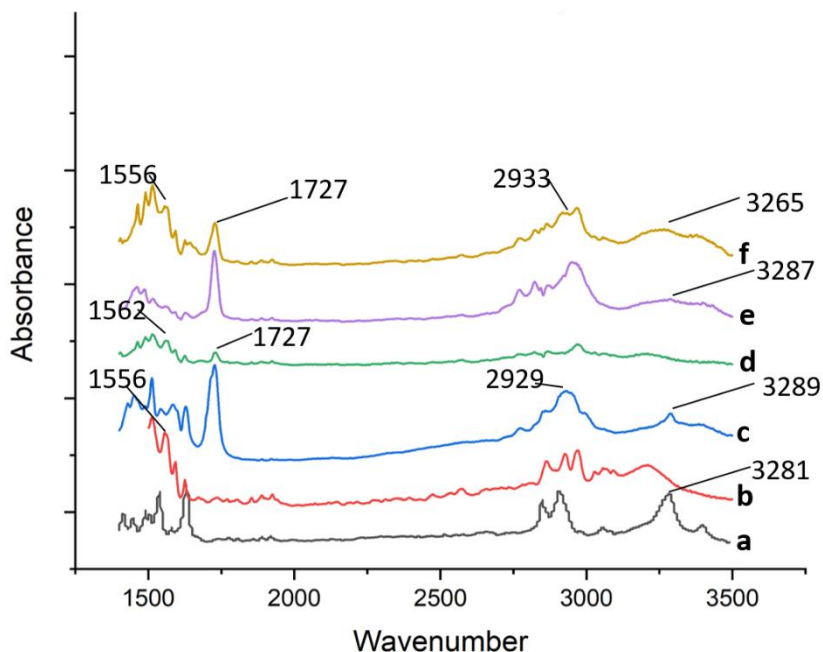


Figure 3.7: FTIR plots of a: North 2; b: Clofazimine; c: SD North2:EPO; d: SD CFZ:EPO; e: PM North2:CFZ:RPO; f: SD North2:CFZ:EPO

3.2.1.7 Aqueous solubility studies of ternary solid dispersions:

Aqueous solubility study was designed for North2: CFZ: EPO, North2: CFZ: PVP and North2: CFZ: HPMC to have a better idea for the dissolution study design. This study was performed in presence of acetate buffer and phosphate buffer to observe the release of compounds from EPO matrix at two different pH. Since PVP and HPMC are both soluble at neutral pH, North2: CFZ: PVP and North2: CFZ: HPMC were studied in phosphate buffer. It was observed that in presence of acetate buffer, the release of the compounds was more as compared to phosphate buffer. This is because EPO dissolved at slightly acidic pH at around 5.5. Once the polymer matrix is dissolved the compounds were released at faster rate. It was observed that around 3.5 ng/mL of North 2 and 89.26 ng/mL of clofazimine was released from 5 mg of SD North2: CFZ: EPO, equivalent to 1.25 mg of pure compound

whereas concentration released in presence of phosphate buffer was 1 ng/mL and 14 ng/mL for North 2 and clofazimine respectively (**Figure 3.8A, 3.8B**). The release of North 2 in presence of acetate buffer was approximately 3-4 times more than that of phosphate buffer and that of clofazimine was 6 times more than in phosphate buffer, hence acetate buffer was used for the further dissolution studies for initial 30 minutes of the experiment. This study was also utilized to compare the release of the compounds from solid dispersions as compared to PMs and pure compounds. The release of North 2 and clofazimine from PM was 0.41 ng/mL and 21 ng/mL which was around 8 times and 4 times less as compared to the release from SD (**Figure 3.8A, 3.8B**). The release of North 2 and clofazimine from North 2:CFZ:HPMC was 1.30 ng/mL and 1.9 ng mL and that from North 2:CFZ:PVP was 1 ng/mL and 8.28 ng/mL. This release from SD North 2:CFZ:PVP and SD North2:CFZ:HPMC was significantly less as compared to SD North2:CFZ:EPO. Based on this data, it was proved that only North 2:CFZ:EPO was able to increase the solubility of both the compounds in presence of acetate buffer. Hence for dissolution studies only North 2:CFZ:EPO was selected and the study was designed in such way that acidic pH was maintained for initial 30 minutes and neutral pH was maintained for the rest of the study simulating the stomach and intestinal conditions.

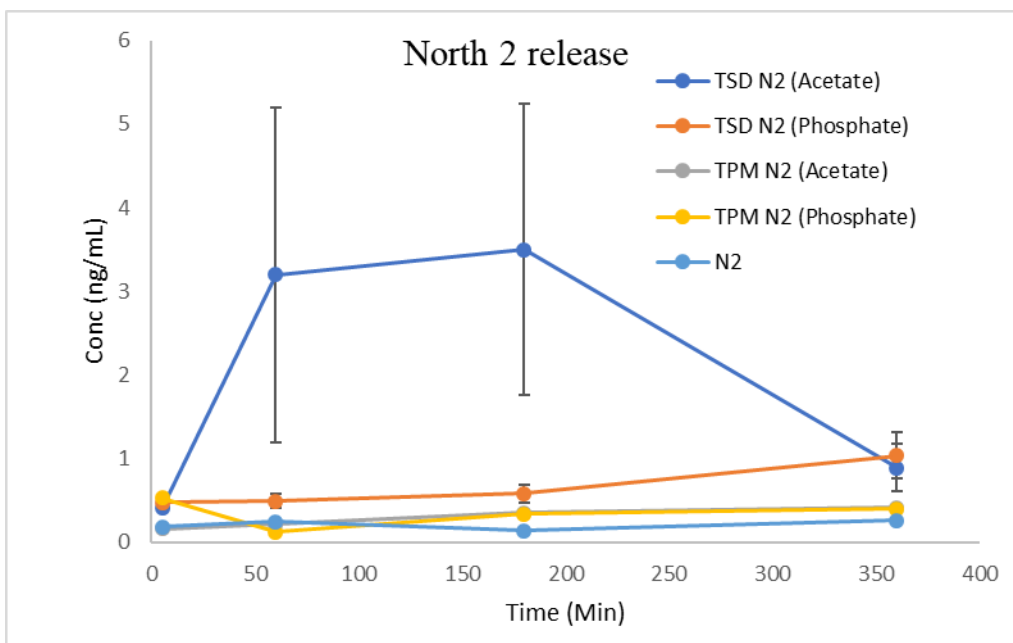


Figure 3.8(A): Release pattern of North 2 from North2: CFZ: EPO Ternary solid dispersion (TSD), Ternary Physical Mixture (TPM) and pure North 2 in presence of acetate and phosphate buffer

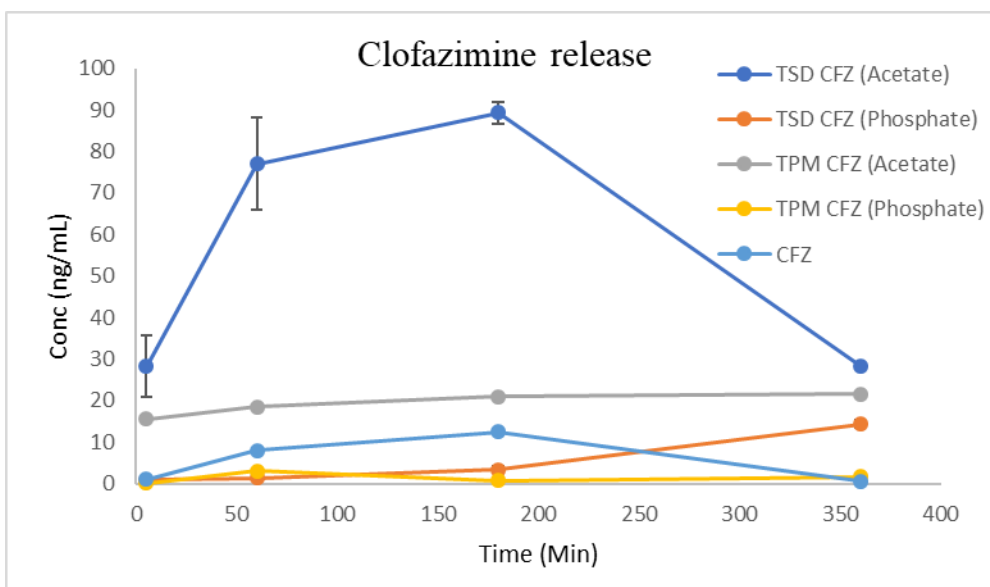


Figure 3.8(B): Release pattern of clofazimine from North2: CFZ: EPO Ternary solid dispersion (TSD), Ternary Physical Mixture (TPM) and pure North 2 in presence of acetate and phosphate buffer

3.2.1.8 *In vitro* dissolution studies:

Where people could get the increase in the aqueous solubility by formation of amorphous solid dispersions. There also have been studies where people have utilized one polymer to increase the solubility of multiple drugs simultaneously (Arca H. C.-G., 2017) (Alhalaweh, 2016). Riekes *et al.*, have studied ternary solid dispersion of hypolipidemic compounds, Lovastatin and Ezetimibe. They formulated ternary amorphous solid dispersions and were able to enhance the solubility. There was 25% of drug loading in 75% of polymer and they achieved around 50% release for both the compounds from 25% drug loading (Riekes M. K., 2016). Clofazimine was also formulated as solid dispersions to increase the solubility. Narang *et. al.*, formulated solid-solid dispersion of clofazimine with PEG and PVP. They could successfully improve the aqueous solubility of clofazimine as compared to the marketed capsule (Ajit S Narang, 2002). Clofazimine is not yet formulated as anti-tubercular solid dispersion but the combinations of clofazimine with other anti-tubercular drugs were studied (Alessandro De Logu, 2002). Current research study is focused on enhancement of dissolution of both North 2 and clofazimine for combination treatment of tuberculosis. Having confirmed that ternary SD North2:CFZ:EPO 1:1:2 was amorphous, the dissolution study was performed and the release behavior of clofazimine and North-2 was evaluated from EPO. As concluded from the aqueous solubility studies, acidic pH was maintained in the studies as Eudragit®EPO is soluble in aqueous medium at pH 5-5.5 (Rowe, 2006), hence in case North 2:CFZ:EPO, the medium used was acetate buffer of pH 5.5 for initial 30 minutes of dissolution and the phosphate buffer of pH 7.4 for rest of the time period of dissolution, this mimics the gastric and intestinal conditions of body (Arca H. Ç.-G., 2018). The dissolution studies showed that the concentration of the compounds released from the ternary SD North2:CFZ:EPO was significantly higher than their physical

mixtures. Initial amount of ternary solid dispersion was equivalent to 15 mg of each compound. Unlike North 2 and curcumin combination, in case of clofazimine and North 2, even if release was increased as compared to PM, the percent release was less. From North 2:CFZ:EPO, concentration of North 2 was $27.20 \pm 2.80 \mu\text{g/mL}$ at 4 hrs. (**Figure 3.9A**), which is around 14% of initial concentration. Similarly, concentration of clofazimine was $21.60 \pm 2.5 \mu\text{g/mL}$ at 4 hrs. (**Figure 3.9B**), which is around 10% of initial concentration. The release of North 2 and clofazimine from equivalent PM was 0.24% and 0.11% respectively. When this release was compared to PM, there was around 58 and 90 folds increase for North 2 and clofazimine respectively.

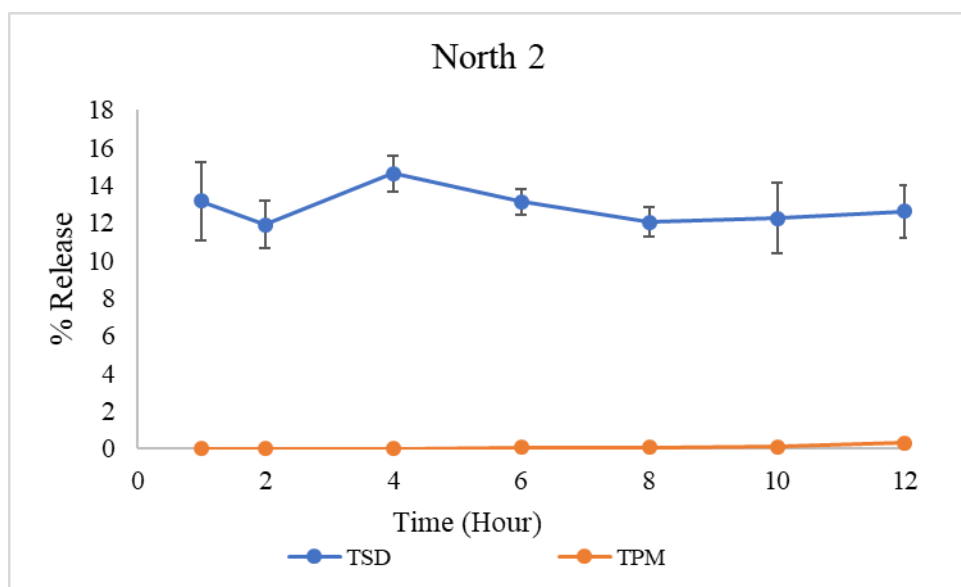


Figure 3.9(A): Dissolution pattern of North 2 from ATSD North2:CFZ:EPO 1:1:2 and PM.

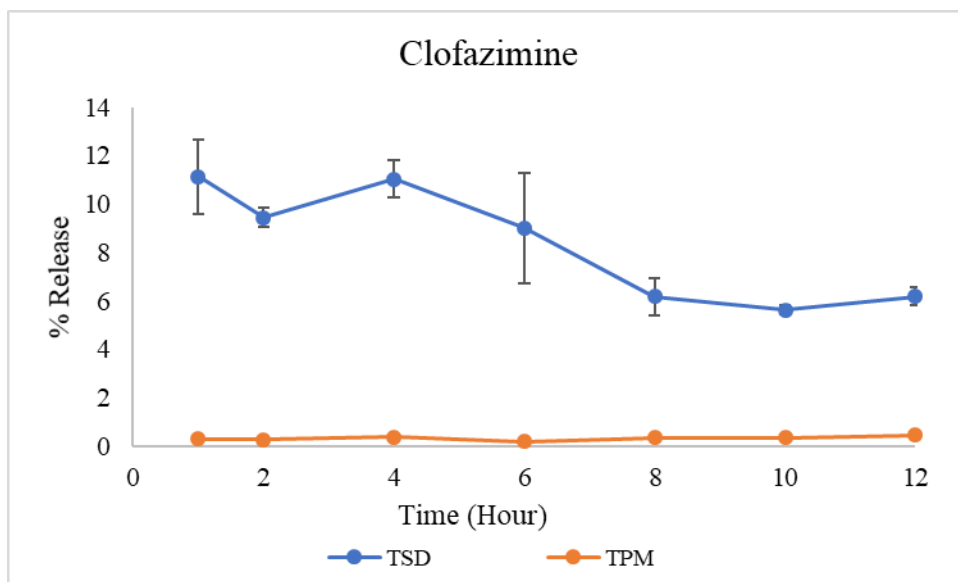


Figure 3.9(B): Dissolution pattern of Clofazimine from ATSD North2: CFZ: EPO 1:1:2 and PM.

The dissolution data was evaluated for different models for understanding of release mechanism of North 2 and clofazimine from EPO matrix. Release of both North 2 and clofazimine was observed to be increasing till 4 hours and then it started precipitating out from the dissolution medium after 4 hours, releasing highest amount at 4 hours. Hence the release data was distributed in two parts, one being dissolution phase and another being precipitation phase. The dissolution phase was plotted in models like first order, zero order, Higuchi model, Hixson Crowell model and Korsmeyer peppas model. The release of North 2 from North 2:CFZ:EPO was found to be following the Zero-order model for the release data (% release v/s time) of drug dissolution with the regression of 0.6336. This tells us that release of North 2 was independent of the initial concentration and the precipitation after 4 hours suggested that there might be a super-saturation of North 2 in the dissolution

medium. This super-saturation can be avoided if the drug loading is decreased. Release of clofazimine followed Hixson-Crowell model with regression of 0.7854. According to Hixson-Crowell model, larger the area faster is the dissolution and it describes the drug release with changes in surface area of particles (Ramteke K.H., 2014) (Radhakant Gouda, 2017). This tells us that clofazimine particles in polymer matrix were reached to very small size leading to the large surface area. This is because the linearity in Hixson-Crowell model suggests that the geometrical shape of the dosage form diminishes proportionally over the time (Hixson, 1931) (K. R. S. Sambasiva Rao, 2011).

3.2.1.9 Evaluation of Particles after Dissolution by using Scanning Electron Microscopy and X-Ray Diffraction:

SEM studies were performed after dissolution to evaluate the structural differences between particles of SD and PM after the dissolution. The dissolution media was filtered after the dissolution experiment and the dried particles trapped on the filter was evaluated for structural observation using SEM. In this study pure compounds particles were compared to the particles of SD and PM after dissolution. Pure North 2 and clofazimine particles were crystalline and with sharp edges. The particles of PM after dissolution was similar to the pure compounds, crystalline and sharp surfaces and within the range of 250-300 μm (**Figure 3.10**). There was no change in the surface morphology of the particles in PM even after the dissolution experiment of 12 hours. Whereas particles of SD after dissolution were smaller and the edges were blunt as compared to the particles of PM. The range of the particle size of SD in was about 25-30 μm (**Figure 3.10**), that is very less compared to PM and pure compounds. This indicated that the surface morphology of the

particles was changed significantly after the preparation of SD and this resulted into more surface area and better dissolution of the pure compounds from polymer matrix. To evaluate the crystallinity for the particles, these samples were also tested for XRD. Both SD and PM showed crystalline peaks representing the pure compounds. The intensity of peaks in SD sample was less than that of PM.

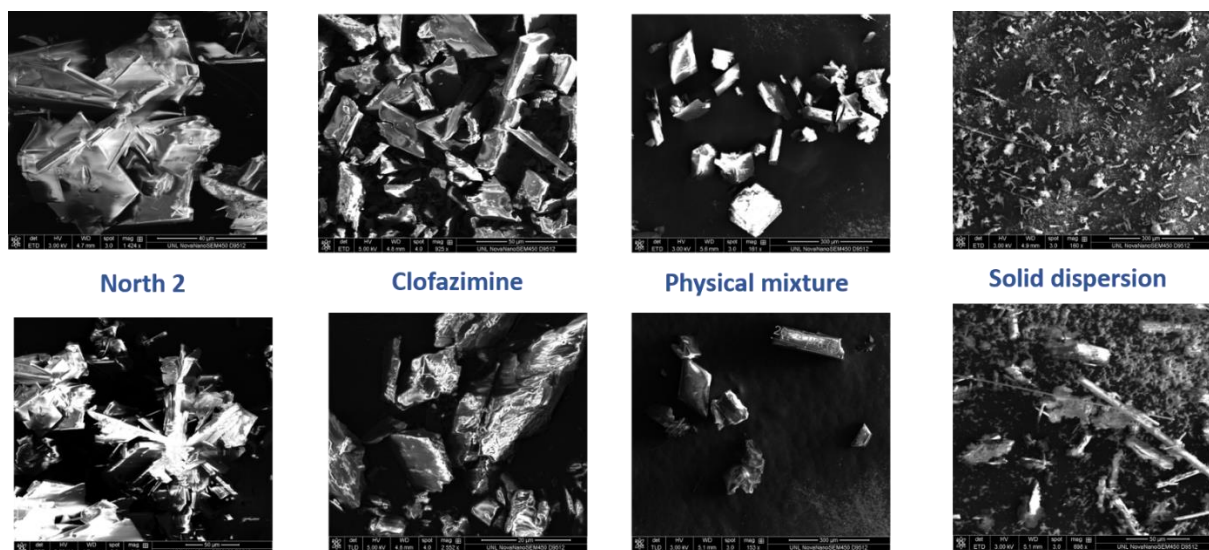


Figure 3.10: SEM showing surface morphology of precipitated particles of pure compounds and dried samples of ATSD and PM of North2: CFZ: EPO collected after dissolution study

3.3 Conclusion:

The novel amorphous ternary solid dispersion of novel, North 2 with enhanced aqueous solubility of both North 2 and clofazimine were successfully prepared and evaluated. The ternary dispersions having the high drug loading of 50%, i.e. 1:1:2 (North 2:Clofazimine:EPO) showed the increase in dissolution of both compounds as compared to physical mixtures. Possible reason for enhanced solubility was investigated by studying interactions of the compounds with the polymers by DSC, and FTIR. Like in case of North

2-Curcumin combination, these results concluded that interactions between two compounds and polymer in ternary dispersions is a useful technology for poorly soluble compounds.

3.4 Bibliography

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4 Conclusion and future directions

4.1 Conclusion:

North 2 can be successfully formulated with curcumin/clofazimine for the combination treatment of tuberculosis, with simultaneous enhancement in the aqueous solubility of both the compounds. Eudragit®EPO can successfully enhance the aqueous solubility of Both poorly water-soluble compounds, North 2, curcumin and clofazimine. EPO can be successfully used for the formation of ternary solid dispersions for simultaneous enhancement of solubility of two compounds. Techniques like differential scanning calorimetry, X-Ray diffraction can be used to evaluate the amorphicity, crystallization tendencies and miscibility of compounds and polymers. Fourier transform infrared spectroscopy, nuclear magnetic resonance and molecular modelling can be used to confirm the mechanism of interactions between the compounds and polymers. Aqueous solubility testing and dissolution experiments done for the ternary solid dispersions proved that solubility of North 2, Curcumin, and clofazimine was improved. Overall, it can be concluded that formation of ternary solid dispersion of North 2-curcumin and North 2-clofazimine with EPO could enhance the solubility of compounds and these combinations can be explored with different hydrophilic polymers to improve aqueous solubility of poorly water-soluble compounds.

4.2 Future studies

4.2.1 Addendum to studies performed:

In polymer selection/screening, different grades of Eudragit, HPMC, PVP and also other hydrophilic polymers like PEG, should be explored. And for optimization of drug loading,

various ratios can be studied for ternary combinations. The parameters such as stirring, sequence of adding the mixtures, use of different solvents, and different concentrations of compounds and polymers in the technique of screening of polymers can be explored to have a better idea of the compatibility of different ratios and the capacity of the polymers to keep the compounds in soluble state.

In evaluation of molecular interactions, solid dispersions can be compared with pure polymers FTIR data for the confirmation of involvement of functional group of polymers. Different ratios of the two compounds can be tried and evaluated for the interactions between two active compounds in the FTIR studies. Similarly, for NMR studies, different increasing ratios of active compounds can be evaluated for the confirmation of interactions between the compounds. Solid state NMR should be explored for the better understanding of the intermolecular interactions in solid dispersions. Additional molecular studied can be done such as Raman spectroscopy, NMR for the combination of North 2 and clofazimine.

For aqueous solubility studies and dissolution studies, more drug doses can be explored and compared with physical mixtures. ICH guidelines should be explored for detailed stability studies of amorphous ternary solid dispersions, at elevated temperatures, humidity.

4.2.2 Proceeding future studies:

For this research study about combination treatment against tuberculosis, we suggest following future studies for further development of amorphous ternary solid dispersions of North 2, curcumin, and clofazimine. For development of amorphous solid dispersions, North2:Cur:EPO and North2:CFZ:EPO, spray drying or melt extrusion techniques can be

explored after optimization of formulation. Techniques like spray drying would give better uniformity of the dispersion as compared to rota-evaporator. Developed formulations can be tested on the bacterial strains North 2-curcumin and North 2-clofazimine in combinations can be tested on the bacterial cell lines in different ratios for optimizing dose. The amorphous ternary solid dispersions can be tested for pharmacokinetic studies in mice. This will give the better idea of in-vivo absorption and efficacy of the formulations as compared to pure compounds. All the three compounds together can be formulated and tested for the pharmacokinetic studies, meaning, North 2, curcumin and clofazimine can be combined in the same formulation with hydrophilic polymer for solubility enhancement of all the three compounds together. This will reduce the pill burden from 3 to 1 at a time. Different potential, natural anti-oxidants instead of curcumin and other anti-TB agents instead of clofazimine can be formulated in the combination of North 2.

Looking at the potential of these ternary solid dispersions and the literature back up for the further development of the combination may lead to the preclinical and clinical studies of these amorphous ternary solid dispersions for the better and improved combination treatment of tuberculosis. This is the long-term goal of the research discussed in this thesis.

4.3 Global Impact of the research project:

Despite of the current treatment options TB continues to be the leading cause of death worldwide. According to the WHO report 2018, about 10.0 million people died from TB (Global Tuberculosis Report 2018, 2019). Increasing rates of mycobacterial resistant strains (both MDR and XDR) and an increase in the number of patients suffering from HIV-AIDS has made treatment of TB even more problematic. Furthermore, current treatment regimen for TB presents adherence problems due to high pill burden associated

with it (Global Tuberculosis Report 2018, 2019). Therefore, there is an urgent need for the development of a novel anti-tubercular drug/formulation active against the drug resistant strains.

Ternary solid dispersion of North-2 (novel anti-tubercular drug) with curcumin or clofazimine along with EPO as polymer, owing to its high solubility are our lead anti-tubercular formulations that can be used in combination with any currently approved anti-TB drugs. Moreover, successful combination of two anti-tubercular drugs in one formulation would decrease the pill burden, thus increasing the patient adherence.

In conclusion, development of ternary solid dispersions of North2:Curcumin:EPO and North2:Clofazimine:EPO as an anti-tubercular formulation may be an effective way for the treatment of TB, thus, contributing in the improvement of global health care.

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Appendix 1: LC-MS/MS Method Development and Validation

The novel amorphous ternary solid dispersions developed in this research study are intended for combination treatment with North 2-curcumin and North 2-clofazimine. Thus, it was essential to develop the analytical method for the simultaneous detection and quantification of North 2 and curcumin in first combination and North 2 and clofazimine in the second one. In this appendix, a detailed results and discussion of LC-MS/MS method development and validation for the combinations: 1) North 2-Curcumin and 2) North 2-Clofazimine is available.

After experimental observations it was determined that general conditions required for the methods was reverse phase HPLC column. For the better simultaneous detection with the release of the compounds in ng/mL ranges in dissolution experiments LC-MS/MS method was developed and validated as per USP guidelines.

Materials:

Indole-2-carboxamide derivative, North 2 was synthesized in the laboratory, synthesis protocol and characterization are mentioned in the appendix of this paper. Curcumin and internal standard, warfarin were purchased from Sigma-Aldrich Co. (St. Louis, MO). Clofazimine was purchased from Tokyo Chemical Industries Co., LTD. (Toshima, KITA-Ku, Tokyo, Japan). Solvents used were methanol, acetone, Formic acid (HPLC grade), Acetonitrile (HPLC grade) were purchased from Fischer Scientific (Fair Lawn, New Jersey).

Instrumentation:

An Agilent 1200 HPLC system (Agilent Technologies, CA, USA) coupled with AB Sciex

API 3200 Q Trap with an electrospray ionization (ESI) source (Applied Biosystems, Foster City, CA, USA) was used. The LC–MS/MS system was controlled by Analyst 1.4.2 software.

North 2-Curcumin

Method:

Chromatographic conditions:

Chromatographic separation was performed on Phenomenex Kinetex EVO C18 column (50 × 3.0 mm, 5 μm) with an isocratic mobile phase containing 30:70 v/v 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a flow rate of 0.250 mL/min for North 2-Curcumin (Table 1). The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. Positive mode was utilized for operation of Electrospray ionization (ESI) source. The source temperatures, ion spray voltage and gas pressures were optimized through flow injection analysis (FIA) by infusing mobile phase using LC. The source temperature, ion spray voltage and gas pressures (GS1 and GS2) were set at 400°C, 3000 V, 45 and 55 psi respectively. The optimization of mass spectrometer parameters such as de-clustering potential (DP), collision energy (CE), cell exit potential (CXP) and entrance potential (EP) was done by infusing each analyte and internal standard using 500 ng/mL solution in 80% methanol.

Table 1: Parameters for LC-MS/MS method

Parameters	Specifications

Column	Phenomenex Kinetex EVO C18 column (50 × 3.0 mm, 5 µm)
Mobile Phase	North2-Curcumin- 30:70 (A:B) North2- Clotazimine- 20:80 (A:B)
Flow Rate	250 µL/min
Source Temperature	4000C
Ion Spray Voltage	4000 V

Preparation of calibration curve and quality control samples:

Stock solutions of curcumin and North 2 were prepared in 80% methanol. Stock solutions were 1mg/mL. Internal standard (IS), warfarin stock solution was prepared and was 250ng/mL in 50% methanol. Calibration curve was prepared by serially diluting the stock solutions of each analyte from 4000 ng/mL to 1.9 ng/mL. 575 µL of IS solution was added to 400 µL of each serially diluted analyte solution. The volume was made up to 1000µL with phosphate buffer solution containing polymer. The final calibration curve obtained and used for the validation was from 400 ng/mL to 6.25 ng/mL. Similarly, quality control samples were prepared having concentrations of 304ng/mL, 14ng/mL, and 1ng/mL.

The unknown concentrations of the samples were determined by plotting the calibration curve with concentration on X-axis and peak area on Y-axis. The concentrations were determined by using the experimentally obtained peak area values in calibration curve equation obtained by linear regression.

Results and Discussion:

Specificity:

The specificity of compounds was tested by comparison of blank injection with standard solution injection under similar conditions. Considering the difference between the Log

P values of North 2 and Curcumin, i.e. 2.6 and 3.2 respectively, method development was started with isocratic system of 80% of Solvent B (acetonitrile, 0.1% formic acid) leading to the elution of all the compounds overlapping each other within 1 minute of the run. The method was changed to 70% of solvent B and 30% of solvent A leading to better resolution, and peak shape of North 2, curcumin, and warfarin. The retention time for North 2, curcumin and warfarin were 1.7, 1.15, and 1.2 minutes respectively. (Figure 1). The mass spectrometer was operated in electrospray ionization (ESI) positive mode with m/z 295.2(M+H) \rightarrow 135.2 for North-2 and 369.1(M+H) \rightarrow 177.0 for curcumin and 309.1(M+H) \rightarrow 163.0 for warfarin were selected for quantification. The optimized MRM parameters such as DP, EP, CE and CXP and retention times for all the analytes and internal standards are presented in Table 2.

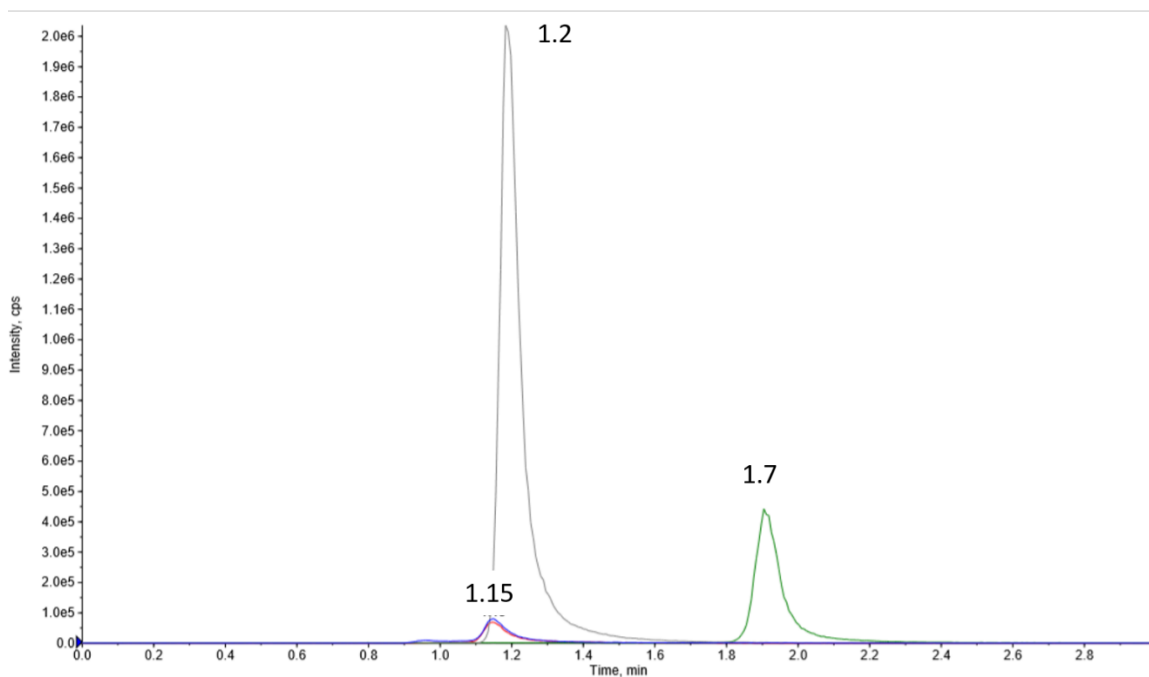


Figure 1: Chromatogram representing North 2, Retention time (Rt)= 1.7; curcumin, Rt= 1.15; and warfarin, Rt= 1.2

Table 2: Optimized MRM parameters of all the compounds in the LC-MS/MS method

Compound/Analyte	MRM Transition	DP (V)		EP (V)		CE (V)		CXP (V)	
		N2-Cur	N2-CFZ	N2-Cur	N2-CFZ	N2-Cur	N2-CFZ	N2-Cur	N2-CFZ
North 2	295.2(M+H)→135.2	250	100	10	10	30	30	15	20
Curcumin	369.1(M+H)→177.0	80		10		25		15	
Clofazimine	473.0 (M+H)→431.1		100		10		50		20
Warfarin (IS)	309.1(M+H) →163.0	80	100	10	10	20	20	20	20

Linearity:

ICH guidelines have defined the linearity as proportionality of test results produced by analytical method to the concentration of compound (Harmonization, 1996). The current LC-MS/MS method was tested for linear proportionality of peak area with increase in concentration of North 2 and curcumin. Linearity was evaluated by injecting eight samples from the concentration between the range from 6.25 ng/mL to 400 ng/mL for both North 2 and curcumin. Linearity was calculated by regression equation which uses the least square method and spearman rank coefficient. The regression equation and spearman coefficient are mentioned in the table 3.

Table 3: Linearity for North 2 and curcumin in LC-MS/MS method

Compound	Concentration range	Regression equation	Spearman Rank Coefficient
North 2	6.25-400 ng/mL	$y = 0.016x + 0.0588$	$R^2 = 0.9971$
Curcumin	6.25-400 ng/mL	$y = 0.0032x - 0.0066$	$R^2 = 0.9998$

The peak areas were plotted against corresponding concentration of compounds (figure

2). This was done separately for two compounds, North 2 (Figure 2a) and curcumin (Figure 2b).

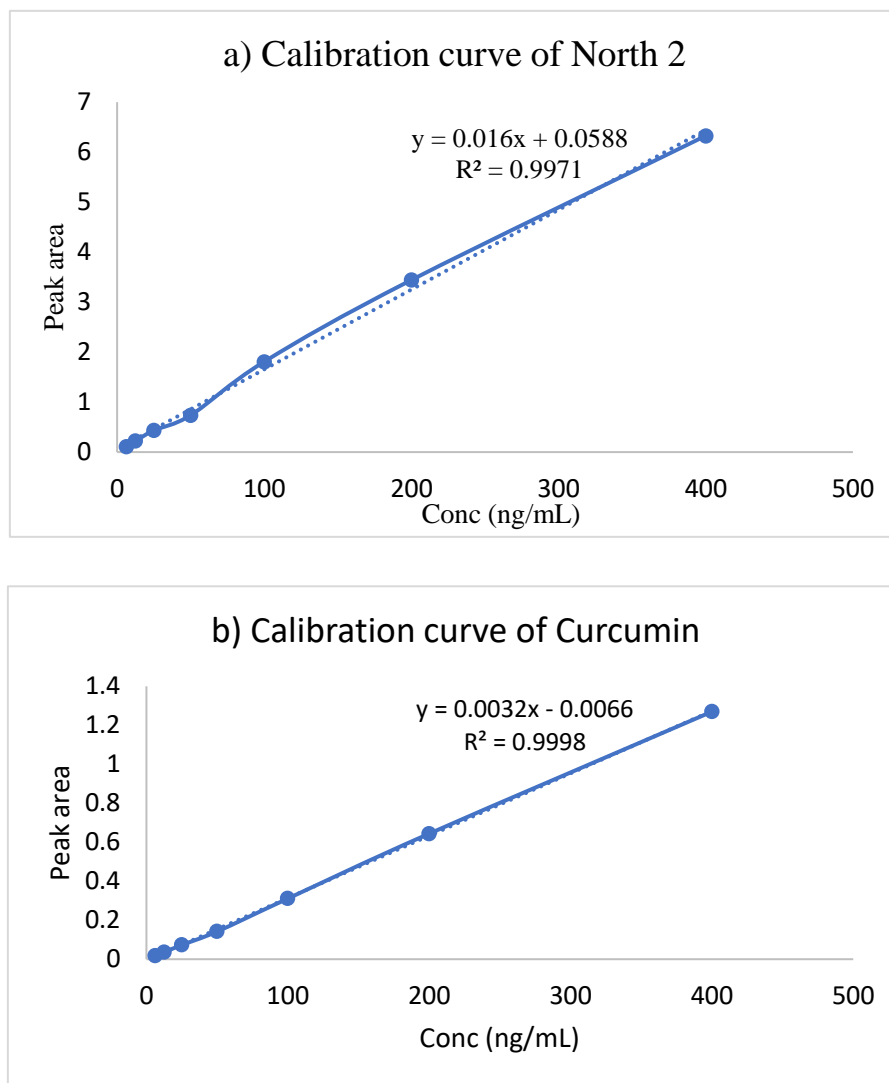


Figure 2: Calibration curve of a) North 2 and b) Curcumin in LC-MS/MS method of North 2-Curcumin

Both North 2 and curcumin had a good correlation between peak areas to the corresponding concentration in ng/mL.

Precision:

According to ICH guidelines, precision is closeness of the same test samples to give

homogeneous results at different times (Harmonization, 1996). Current North2-Curcumin LC-MS/MS method was tested for intra-day and inter-day precision. Intra-day precision was evaluated by injecting the same serially diluted samples multiple times within a day and under the same chromatographic conditions. The percent relative standard deviation was determined. The concentrations of North 2 and their mean value with %RSD are mentioned in Table 4 and that of curcumin in Table 5.

Table 4: Intra-day precision of North 2 for LC-MS/MS method

Concentration of North 2 (ng/mL)	Mean peak area	%RSD
6.25	0.165 ± 0.007	4.285
12.5	0.3475 ± 0.026	7.529
25	0.6515 ± 0.037	5.752
50	1.028 ± 0.049	4.815
100	2.264 ± 0.142	6.309
200	3.9205 ± 0.181	4.635
400	8.142 ± 0.256	3.152

Table 5: Intra-day precision of Curcumin for LC-MS/MS method

Concentration of Curcumin (ng/mL)	Mean Peak area	%RSD
6.25	0.018 ± 0	0.000
12.5	0.0355 ± 0	1.992
25	0.0705 ± 0.003	5.015
50	0.1355 ± 0.009	6.784
100	0.2985 ± 0.017	5.922
200	0.61 ± 0.048	7.883

400	1.217 ± 0.074	6.159
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Inter-day precision represents the coefficient of variation for the results of injected samples on different days. In this method, the stocks of pure compounds were stored at 4°C over 30 days. The standards were prepared from the same stock freshly on the day of injection. The results for inter-day precision for North2 and Curcumin are mentioned in the table 6 and table 7 respectively. Like intra-day, results for inter-day precision were tested for variability based on %RSD.

Table 6: Inter-day precision of North 2 for LC-MS/MS method

Conc of North 2 (ng/mL)	Mean peak area	%RSD
6.25	0.163 ± 0.001	0.868
12.5	0.3285 ± 0.017	5.381
25	0.668 ± 0.067	10.162
50	1.258 ± 0.011	0.899
100	2.203 ± 0.015	0.706
200	3.8725 ± 0.355	9.185
400	9.0015 ± 0.105	1.170

Table 7: Inter-day precision of Curcumin for LC-MS/MS method

Conc of Curcumin (ng/mL)	Mean peak area	%RSD
6.25	0.025 ± 0.0	1.916
12.5	0.039 ± 0.003	9.307

25	0.067 ± 0.006	10.051
50	0.147 ± 0.014	9.702
100	0.288 ± 0.013	4.828
200	0.632 ± 0.030	4.794
400	1.188 ± 0.093	7.873

This LCMS/MS method developed for North 2-Curcumin was validated for intra-day and inter-day precision over the period of 30 days. The coefficient of variation (%RSD) was below 10% for the samples and this was within the ICH guidelines.

Accuracy:

ICH guidelines have defined accuracy as the ability of method to accurately evaluate the quantity of analyte in a sample to its theoretical value (Harmonization, 1996). Accuracy of this method was evaluated by injecting the samples of known concentration, Quality Control samples, under the same chromatographic conditions. The concentration values were calculated for each quality control sample by using the equation obtained from linearity. The accuracy was calculated by using the formula below:

$$\% \text{ Accuracy} = \frac{\text{Measured Concentration}}{\text{Theoretical Concentration}} \times 100$$

According to the ICH guidelines, the measured concentration should be between 95% to 105% of the theoretical concentration (Harmonization, 1996). The accuracy of LC-MS/MS method for North 2 and curcumin is shown in Table 8 and Table 9 respectively.

Table 8: Accuracy of LC-MS/MS for quantification of North 2

Standard Conc (ng/mL)	Calculated Conc (ng/mL)	%RSD	% Accuracy
304	283.35 ± 3.53	1.24	93.21
14	13.61 ± 0.63	4.62	97.19
1	1.08 ± 0.04	3.34	108.000

Table 9: Accuracy of LC-MS/MS method for quantification of curcumin

Standard Conc (ng/mL)	Calculated Conc (ng/mL)	%RSD	% Accuracy
304	297.53 ± 12.95	4.35	97.87
14	13.69 ± 0.57	4.20	97.76
1	0.93 ± 0.03	3.23	93.03

This analytical method was tested for accuracy with three quality control samples, 304ng/mL, 14 ng/mL, and 1 ng/mL and their accuracy limit was found to be between 95% and 105%.

North 2-Clofazimine

Method:

Method Development:

The chromatographic method for North 2-Clofazimine was similar to North 2-Curcumin

method except mobile phase concentration, 20:80 v/v 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a flow rate of 0.250 mL/min for North 2-Curcumin (Table 1).

Preparation of calibration curve and quality control samples:

Method of preparation of all the stocks and serial dilutions was like North 2-Curcumin mentioned in first section of this appendix. The Final calibration curve samples tested were in range from 1.56 ng/mL- 200 ng/mL. The quality control samples were prepared of concentration, 150ng/mL, 75ng/mL, and 10ng/mL.

Results and Discussion:

Specificity:

The method development steps were similar to North 2-Curcumin method. This method was initially started with 80% of solvent B, since clofazimine has higher Log P value than curcumin. At 20:80 v/v 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a flow rate of 0.250 mL/min, better resolution and peak shape were obtained. The retention time for North 2, clofazimine and warfarin were 1.7, 0.91, and 1.16 minutes respectively (Figure 3). The optimized MRM parameters such as DP, EP, CE and CXP and retention times for all the analytes and internal standards are presented in Table 2.

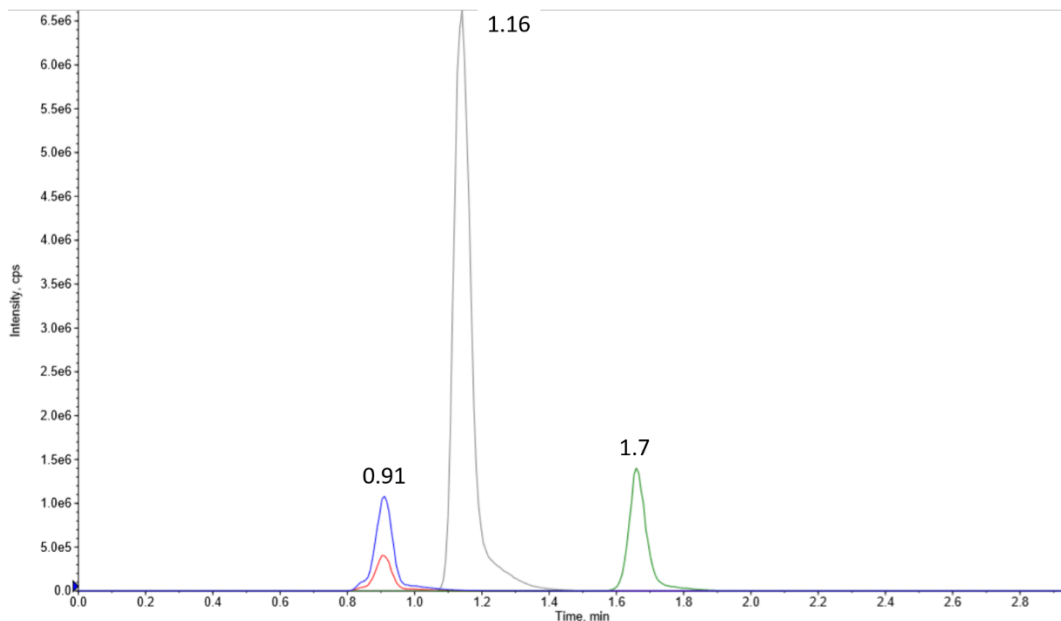


Figure 3: Chromatogram representing North 2, Retention time (Rt)= 1.7; clofazimine, Rt= 0.91; and warfarin, Rt= 1.16

Linearity:

Like North2-Curcumin, North 2-Clofazimine LC-MS/MS method was tested for linear proportionality of peak area with increase in concentration of North2 and clofazimine. Linearity was evaluated by injecting nine samples from the concentration between the range from 1.56 ng/mL to 200 ng/mL for both North 2 and clofazimine. Linearity was calculated by regression equation which uses the least square method and spearman rank coefficient. The regression equation and spearman coefficient are mentioned in the table 10.

Table 3: Linearity for North 2 and clofazimine in LC-MS/MS method

Compound	Concentration range	Regression equation	Spearman Rank Coefficient

North 2	1.56-200 ng/mL	$y = 0.0464x + 0.0721$	$R^2 = 0.9998$
Clofazimine	1.56-200 ng/mL	$y = 0.0115x + 0.107$	$R^2 = 0.9981$

Peak area was plotted for the two compounds, North 2 and clofazimine separately against corresponding concentration. Figure

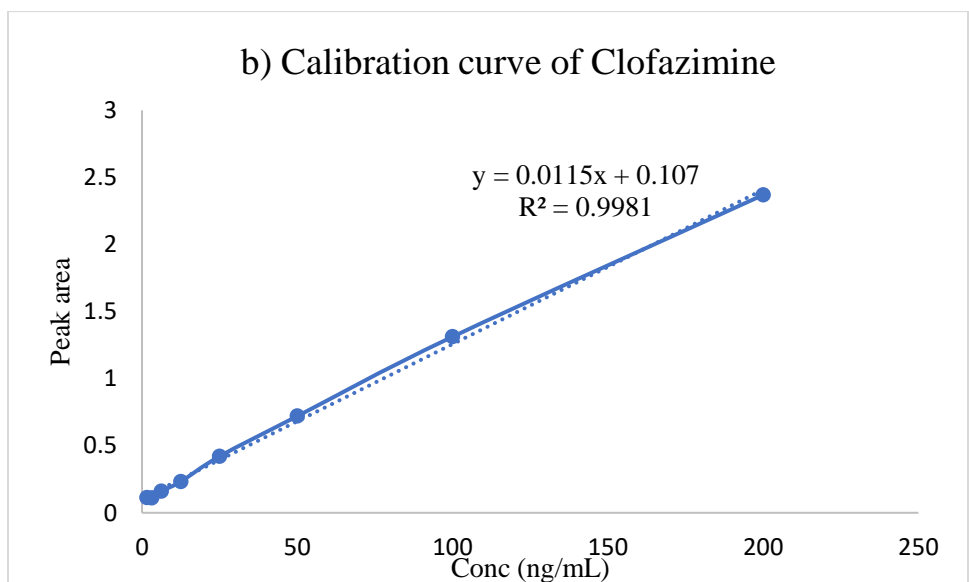
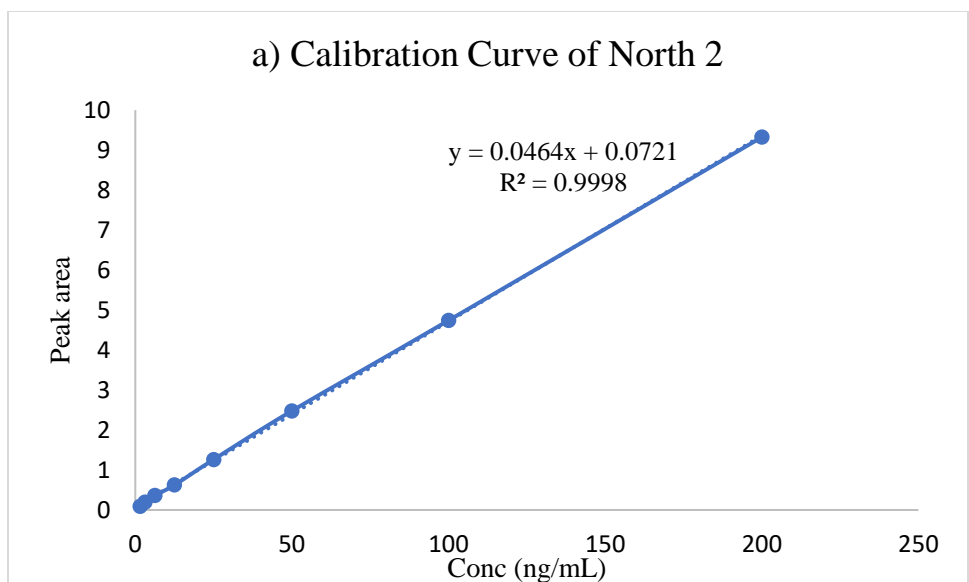


Figure 4: Calibration curve of a) North 2 and b) Clofazimine in LC-MS/MS method of North 2-Clofazimine

Both North 2 and clofazimine had a good correlation between peak areas to the corresponding concentration in ng/mL.

Precision:

Like North2-curcumin method, current North2-Clofazimine LC-MS/MS method was tested for intra-day and inter-day precision. Intra-day precision was evaluated by injecting the same serially diluted samples three times within a day and under the same chromatographic conditions. The percent relative standard deviation was determined. The concentrations of North 2 and their mean value with %RSD are mentioned in Table 11 and that of clofazimine in Table 12.

Table 11: Intra-day precision of North 2 for LC-MS/MS method

Concentration of North 2 (ng/mL)	Mean peak area	%RSD
1.56	0.097 ± 0.006	6.841
3.13	0.186 ± 0.0095	5.092
6.25	0.346 ± 0.011	3.220
12.5	0.635 ± 0.009	1.411
25	1.256 ± 0.014	1.115
50	2.428 ± 0.042	1.763
100	4.789 ± 0.149	3.116
200	9.12 ± 0.18	1.979

Table 12: Intra-day precision of Clofazimine for LC-MS/MS method

Concentration of North 2 (ng/mL)	Mean peak area	%RSD
1.56	0.110 ± 0.04	35.824
3.13	0.09 ± 0.001	1.111
6.25	0.108 ± 0.006	5.084
12.50	0.201 ± 0.011	5.688
25.00	0.375 ± 0.012	3.310
50.00	0.747 ± 0.045	6.030
100.00	1.330 ± 0.075	5.652
200.00	2.445 ± 0.092	3.777

For Inter-day precision, samples were injected on three different days. Like North 2-Curcumin method, in this method, the stocks of pure compounds were stored at 4°C over 30 days. The standards were prepared from the same stock freshly on the day of injection. The results for inter-day precision for North2 and Clofazimine are mentioned in the table 13 and table 14 respectively. Like intra-day, results for inter-day precision were tested for variability based on %RSD.

Table 13: Inter-day precision of North 2 for LC-MS/MS method

Concentration of North 2 (ng/mL)	Mean peak area	%RSD
1.56	0.088 ± 0.02	22.053
3.13	0.159 ± 0.019	12.036

6.25	0.309 ± 0.310	9.924
12.5	0.600 ± 0.038	6.265
25	1.199 ± 0.078	6.515
50	2.371 ± 0.073	3.067
100	4.734 ± 0.0175	3.693
200	8.92 ± 0.430	4.818

Table 14: Inter-day precision of Clofazimine for LC-MS/MS method

Concentration of Clofazimine (ng/mL)	Mean peak area	%RSD
1.56	0.139 ± 0.017	12.461
3.13	0.126 ± 0.033	26.310
6.25	0.121 ± 0.01	8.388
12.5	0.235 ± 0.02	8.544
25	0.392 ± 0.017	4.418
50	0.814 ± 0.026	3.192
100	1.492 ± 0.137	9.171
200	2.667 ± 0.207	7.749

This North 2-Clofazimine LCMS/MS method was developed and validated for intra-day and inter-day precision over the period of 30 days. The coefficient of variation (%RSD) was below 10% for the samples ranging from 6.25 ng/mL to 200 ng/mL. Variation for

first two samples of 1.56 ng/mL and 3.13 ng/mL was more than 10% and hence the range considered for the evaluation of results from the experiments (Dissolution and aqueous solubility experiments) was from 6.25 ng/mL to 200 ng/mL and this was within the ICH guidelines.

Accuracy:

Accuracy of North 2-Clofazimine LC-MS/MS method was evaluated by injecting the samples of known concentration, Quality Control samples, under the same chromatographic conditions. The concentration values were calculated for each quality control sample by using the equation obtained from linearity. The QCs used for this method were, The accuracy was calculated by using the formula below:

$$\% \text{ Accuracy} = \frac{\text{Measured Concentration}}{\text{Theoretical Concentration}} \times 100$$

According to the ICH guidelines, the measured concentration should be between 95% to 105% of the theoretical concentration. The accuracy of LC-MS/MS method for North 2 and curcumin is shown in Table 15 and Table 16 respectively.

Table 15: Accuracy of LC-MS/MS for quantification of North 2

Standard Concentration (QCs) ng/mL	Calculated Concentration ng/mL	% RSD	% Accuracy
10	9.803 ± 0.098	1.007	98.033
75	75.859 ± 2.417	3.187	101.146
150	149.307 ± 0.205	0.138	99.538

Table 16: Accuracy of LC-MS/MS for quantification of Clofazimine

Standard Concentration (QCs) ng/mL	Calculated Concentration ng/mL	% RSD	% Accuracy
10	10.281 ± 0.085	0.832	102.817
75	74.213 ± 0.372	0.502	98.951
150	156.262 ± 1.379	0.883	104.175

North 2-Clofazimine LC-MS/MS analytical method was tested for accuracy with three quality control samples, 150 ng/mL, 75 ng/mL, and 10 ng/mL and their accuracy limit was found to be between 95% and 105%. The accuracy of this method was within the limits according to ICH guidelines (Harmonization, 1996).

Conclusion:

It was the first time to develop and validate the analytical method for North 2 in combination with 1) Curcumin and 2) Clofazimine. Two LC-MS/MS methods were successfully developed and validated as per the guidelines of ICH for detection of compounds for the combination of 1) North 2-Curcumin and 2) North 2-Clofazimine. These two methods were validated for USP and ICH guidelines for their specificity, linearity, intra-day and inter-day precision and accuracy. From the results, it was concluded that both the methods were sensitive, precise and accurate according to ICH and USP guidelines. This method was used for the dissolution and aqueous solubility samples for the quantification of the concentration of compounds. In future, with necessary modifications, these methods can be utilized for the detection and quantification of combination of different compounds with North 2 after pharmacokinetic studies.

Bibliography:

1. International conference on harmonization. Guidance for Industry: Q2B Validation of Analytical Procedures: Methodology. ICH. Nov 1996.
2. USP 40 NF-35. VALIDATION OF COMPENDIAL PROCEDURES: First Supplement to USP 40–NF 35. General Information / Validation of Compendial Procedures.

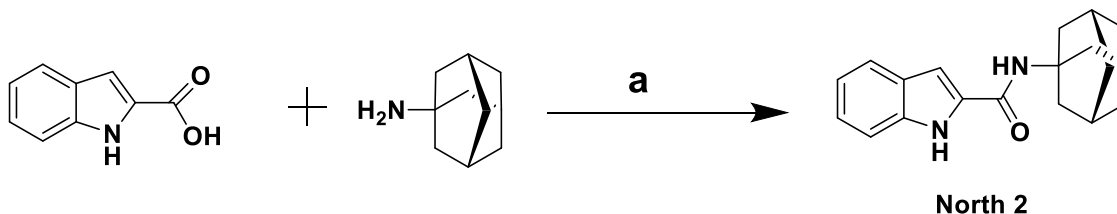
Appendix 2: North 2 Synthesis

General Experimental Details

Chemicals and Instrumentation:

All glassware were dried in a 150°C oven overnight. The commercially available reagents and solvents were used without further purification. Indole-2-carboxylic acid, 1-adamantaneamine, HOBt, EDC, TEA, and DMF were purchased from either Fisher Scientific (Pittsburg, Pennsylvania) or Sigma Aldrich (St. Louis, Missouri). TLC plates, silica gel column 100g was purchased from Biotage® (Charlotte, North Carolina). The chemical reaction was tracked using fluorescent silica gel-coated TLC plates and the separated components were visualized using UV light (254 nm). The compound was purified using flash-column chromatography on a Biotage Isolera™ with silica gel. The molecular weight of the compound is confirmed by Applied Biosystems 5500 Q trap LC/MS system. All ¹H and ¹³C NMR spectra were obtained on an Ascend™ 400 MHz Bruker system and chemical shifts were reported relative to TMS.

Synthetic Scheme:

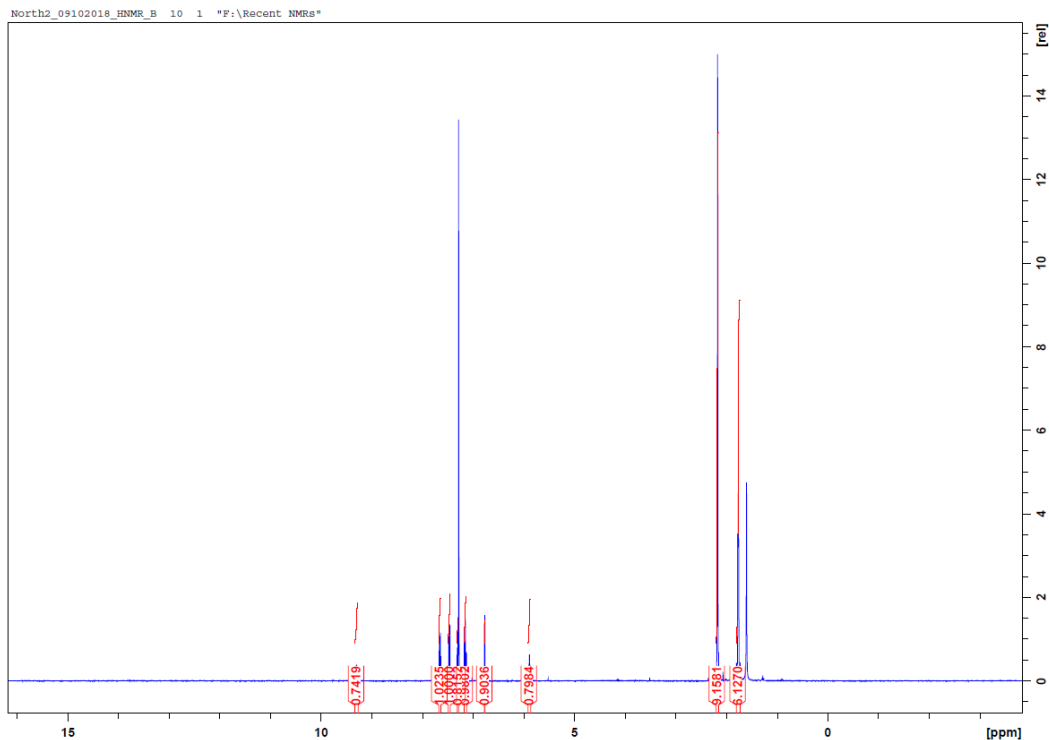


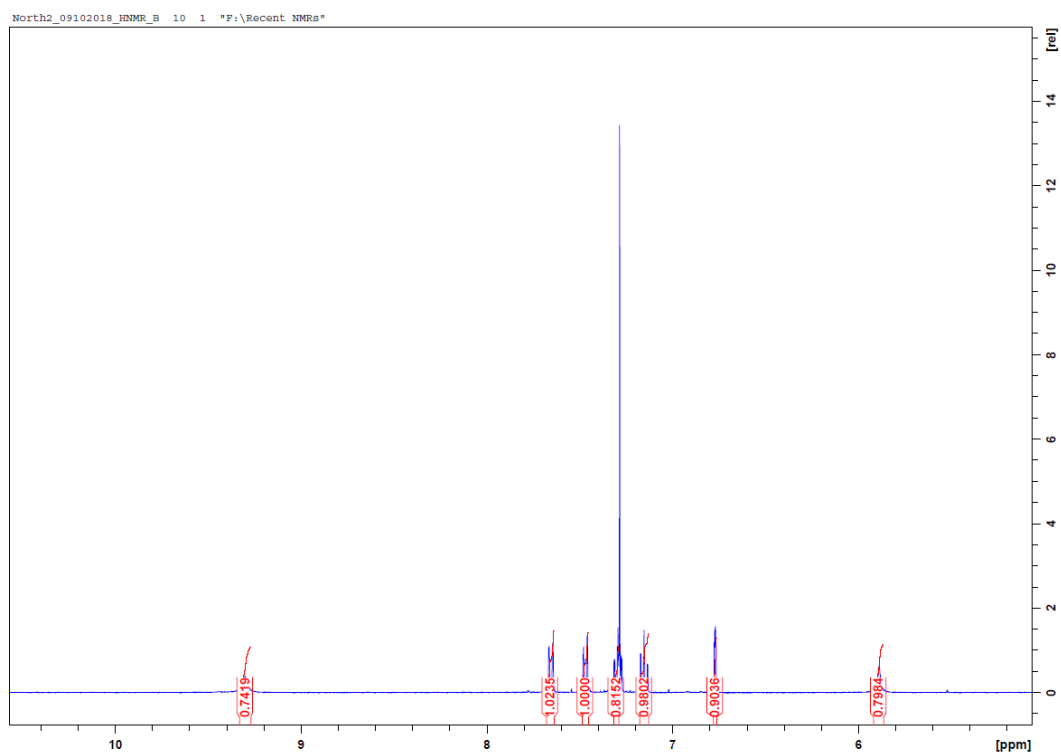
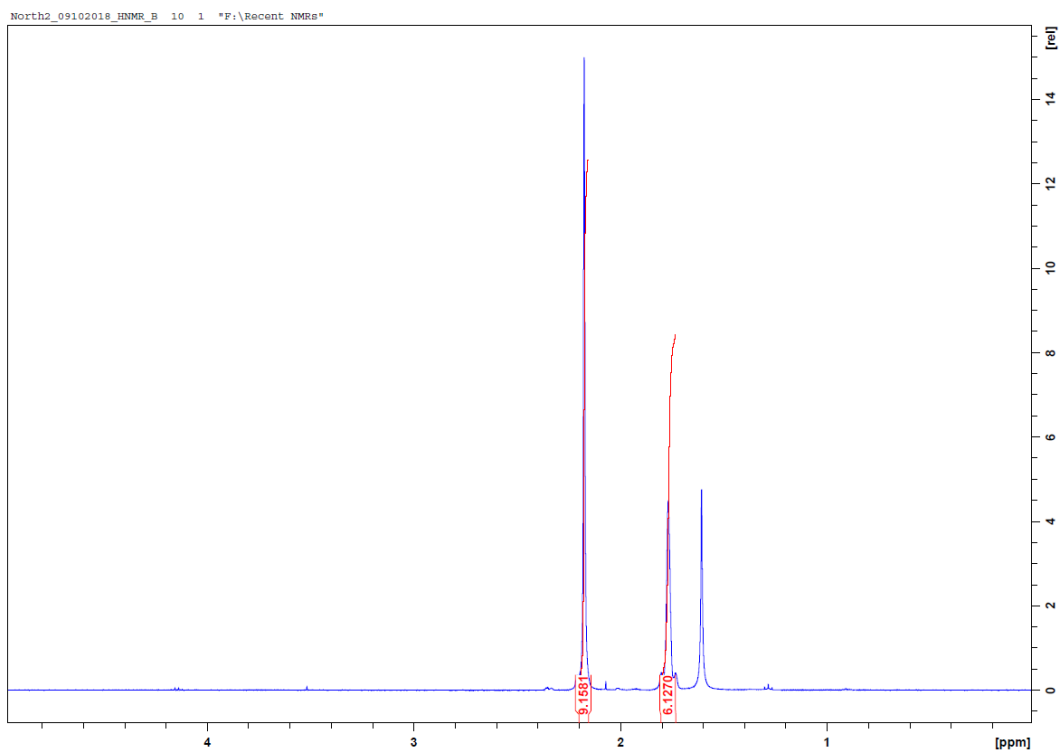
Scheme 1: Synthesis of North 2. Reagents and conditions: a) HOBt, EDC, TEA, DMF, r.t., overnight.

Procedure:

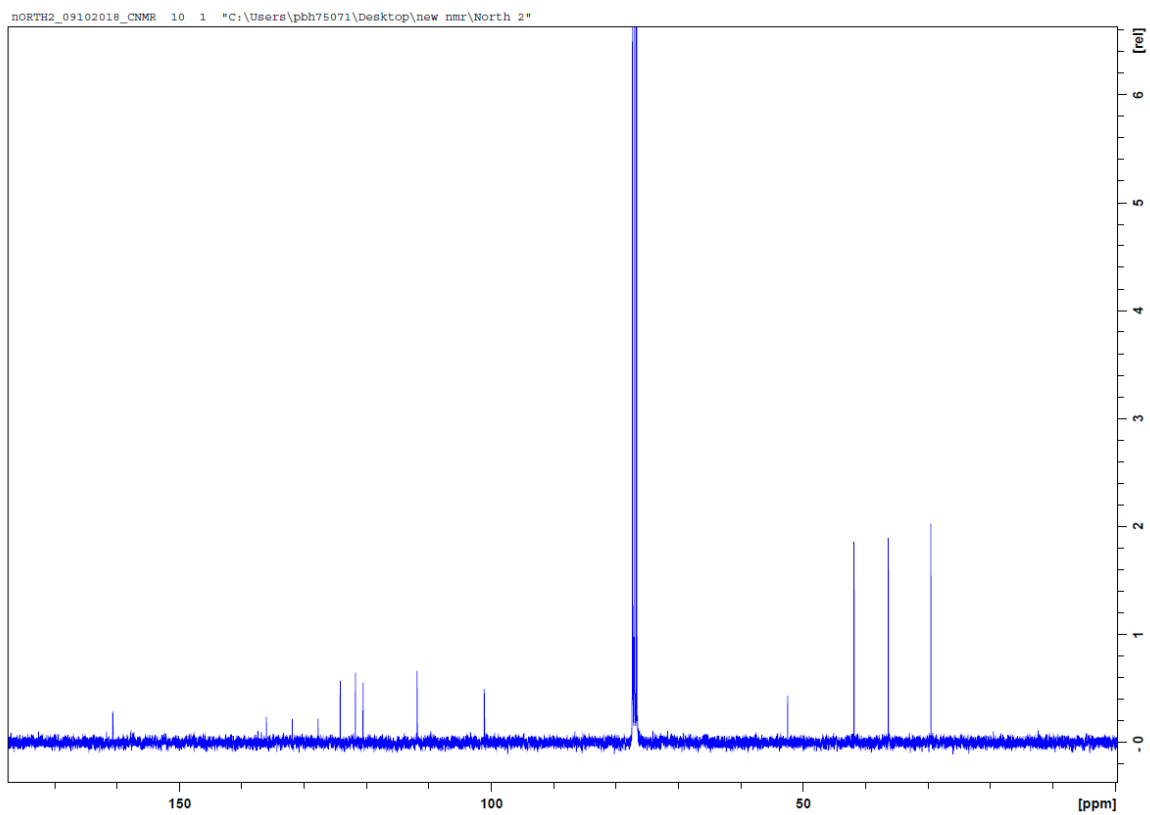
Indole-2-carboxylic acid (6.20 mmol) was dissolved in anhydrous DMF under nitrogen atmosphere and stirred at room temperature. HOBt (6.2 mmol) and EDC (7.4 mmol) were added to the RBF. The reaction was allowed to stir for 15 minutes. TEA (8.04 mmol) was added, and finally adamantane-1-amine was added. The reaction was allowed to continue at constant stirring overnight at room temperature. The completion of the reaction was monitored by TLC. The reaction mixture was extracted with ethyl acetate and dried over Na_2SO_4 . Then, the organic phase was concentrated in vacuum and purified by flash chromatography to get the desired product.

^1H NMR for North 2:

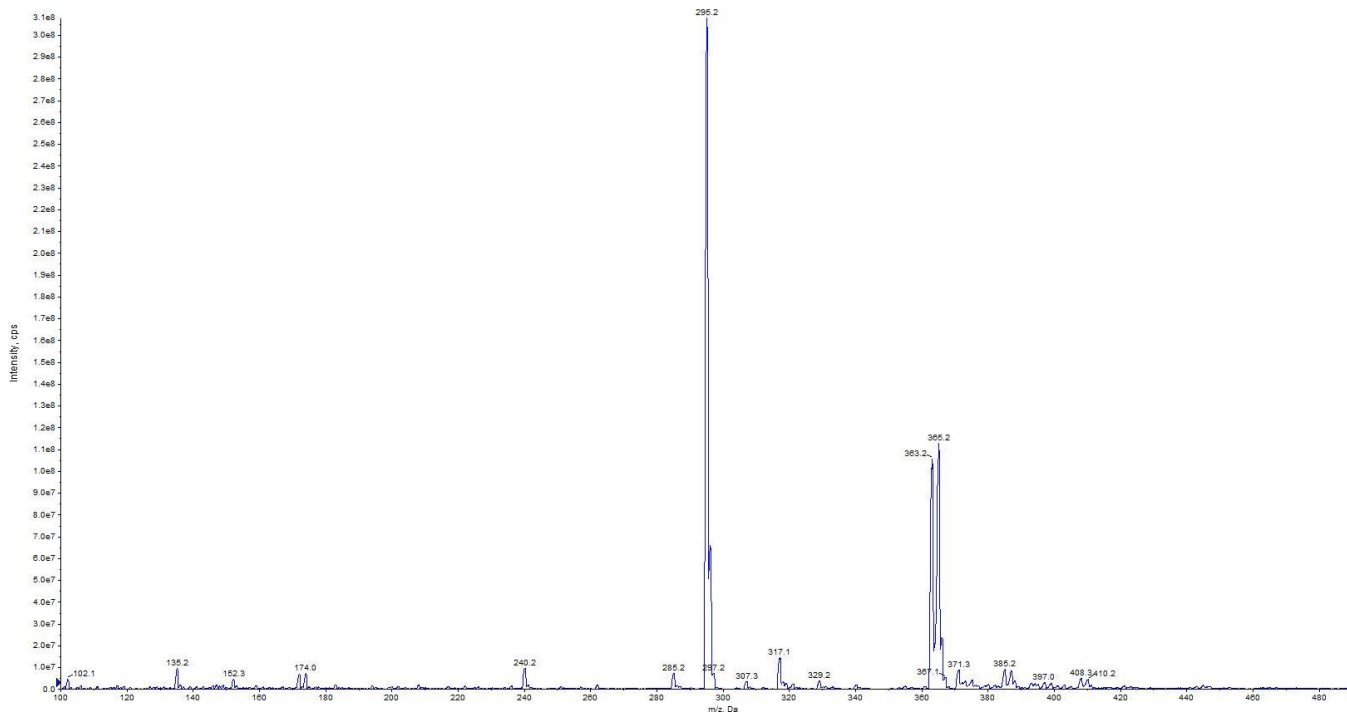




¹³C NMR for North 2:



Mass spectra for North 2:



Experimental data:

1123 mg (61% yield, >95% pure) of white powder; ^1H NMR (400 MHz, CDCl_3), $\delta = 1.76$ (s, 6H), 2.17 (br. s, 9H), 5.88 (s, 1H), 6.77 (s, 1H), 7.15 (t, $J = 8$ Hz, 1H), 7.29 – 7.31 (m, 1H), 7.45 (d, $J = 8$ Hz, 1H), 7.64 (d, $J = 8$ Hz, 1H), 9.30 (s, 1H); ^{13}C NMR (400 MHz, CDCl_3) $\delta = 29.51, 36.34, 41.84, 52.49, 52.49, 101.11, 111.89, 120.55, 121.78, 124.21, 127.76, 131.89, 136.06, 160.66$, calculated for $\text{C}_{19}\text{H}_{22}\text{N}_2\text{O}$: 294.4, found: 295.2 $[\text{M}+\text{H}]^+$