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PREPARATION, CHARACTERIZATION AND IN VITRO EVALUATION OF METFORMIN LOADED HYALURONIC ACID NANOPARTICLES FOR ORAL DELIVERY

By

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A THESIS

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Abstract

Metformin, an anti-diabetic drug, has a low oral bioavailability (50-60%) due to its poor intestinal absorption. Orally administered mucoadhesive nanoparticles have been shown to interact with the intestinal mucus layer and increase the intestinal permeability of the entrapped drug. The objective of the present study was to develop and characterize polymeric nanoparticulate system containing metformin with a mucoadhesive polymer hyaluronic acid. Blank and drug loaded nanoparticles were prepared using hyaluronic acid polymer by precipitation method with a high entrapment efficiency. The average particle size of the blank and drug loaded nanoparticles was found to be 155.52 ±18.70 nm and 114.53 ± 12.01 nm, respectively. Atomic force microscopy (AFM) revealed that the nanostructures were in form of nanofibres. Zeta potential was found to be in the range of -0.27 to 0.24 mV. Physical stability studies indicated that the particle size was maintained at < 200 nm, and no change in zeta potential values over a period of 65 days. Differential Scanning Calorimetry and X-ray diffraction analysis indicated that the drug was present in a crystalline state in the matrix. The in vitro drug release studies showed that >50% of metformin was released within one hour. Metformin nanoparticles were non-toxic at a concentration of ≤ 100 µM in Cacco-2 cells. Haemolysis studies indicated that these nanoparticles can also be safely administered via
intravenous route. Cellular uptake studies on Caco-2 cells indicated higher uptake of metformin from nanoparticle relative to that from metformin solution, up to first 45 minutes. Permeability studies indicated no passive permeation of the nanoparticles through a synthetic membrane lacking mucin. Thus requires further in vivo studies in the future with more bio-relevant environment like Caco-2 cells or porcine intestine. Diabetes is a disease affecting millions of people globally. The outcomes of this study could increase bioavailability, reduce dose and side effects of the metformin (widely used first line therapy for type 2 diabetes) which could impact humanity globally.
Dedicated to my parents, Mrs. Nalini Bhujbal and Mr. Vithal Bhujbal, and my sister Sheetal Bhujbal
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# Table of contents

Abstract iv

Acknowledgements vii

Table of contents List of figures ix

List of figures xvi

List of tables xix

List of equations xx

List of abbreviations xx

**CHAPTER 1: Introduction** 1

1.1. Diabetes 2

1.2. Oral therapy for type 2 diabetes 4

1.3. Drug Profile: Metformin 5

1.3.1. Physicochemical properties of metformin 5

1.3.2. Pharmacokinetics of metformin 6

1.3.2.1. Absorption and bioavailability 6

1.3.2.2. Distribution 7

1.3.2.3. Metabolism and elimination 8

1.3.3. Pharmacodynamics of metformin 9

1.3.3.1. Mechanism of action 9
1.3.3.2. Adverse effects

1.3.3.3. Drug interaction

1.4. Oral drug delivery

1.4.1. Overview of gastrointestinal tract (GIT)

1.4.2. Anatomy and physiology of small intestine

1.4.3. Oral drug absorption

1.4.3.1. Basic Structure of functional cell membrane

1.4.3.2. Mechanism of oral absorption

1.4.3.2.1. Passive diffusion

1.4.3.2.2. Active transport

1.4.3.2.3. Ionic – electrochemical diffusion

1.4.3.2.4. Endocytosis

1.4.4. Factors influencing drug absorption

1.4.5. Challenges of oral drug delivery

1.5. Nanoparticles to enhance intestinal absorption

1.5.1. Mucoadhesives

1.5.2. M Cells

1.5.3. Vitamin B₁₂ receptor

1.5.4. Neonatal Fc receptor

1.6. Objectives, hypothesis and specific aims
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Introduction</td>
<td>29</td>
</tr>
<tr>
<td>2.2</td>
<td>Materials</td>
<td>29</td>
</tr>
<tr>
<td>2.3</td>
<td>Methods</td>
<td>30</td>
</tr>
<tr>
<td>2.3.1</td>
<td>Chromatography</td>
<td>30</td>
</tr>
<tr>
<td>2.3.2</td>
<td>Preparation of solutions</td>
<td>30</td>
</tr>
<tr>
<td>2.3.2.1</td>
<td>Mobile phase</td>
<td>30</td>
</tr>
<tr>
<td>2.3.2.2</td>
<td>Standard solution</td>
<td>31</td>
</tr>
<tr>
<td>2.4</td>
<td>Calculations</td>
<td>31</td>
</tr>
<tr>
<td>2.5</td>
<td>Results and discussions</td>
<td>31</td>
</tr>
<tr>
<td>2.5.1</td>
<td>Specificity</td>
<td>31</td>
</tr>
<tr>
<td>2.5.2</td>
<td>Linearity</td>
<td>33</td>
</tr>
<tr>
<td>2.5.3</td>
<td>Precision</td>
<td>34</td>
</tr>
<tr>
<td>2.5.4</td>
<td>Accuracy</td>
<td>35</td>
</tr>
<tr>
<td>2.6</td>
<td>Applications of the HPLC method</td>
<td>36</td>
</tr>
<tr>
<td>2.6.1</td>
<td>Determination of drug load in the nanoparticles</td>
<td>36</td>
</tr>
<tr>
<td>2.6.2</td>
<td>Determination of drug entrapment efficiency</td>
<td>37</td>
</tr>
<tr>
<td>2.6.3</td>
<td><em>In vitro</em> release of metformin from the formulation</td>
<td>37</td>
</tr>
<tr>
<td>2.6.4</td>
<td>Cellular uptake of metformin in Caco-2 cells</td>
<td>38</td>
</tr>
<tr>
<td>2.6.5</td>
<td>Permeability studies</td>
<td>39</td>
</tr>
</tbody>
</table>
Chapter 3: Preparation, characterization and \textit{in vitro} evaluation of metformin loaded hyaluronic acid nanoparticles

3.1. Introduction

3.2. Materials

3.3. Methods

3.3.1. Formulation of the delivery system

3.3.2. Particle size and surface charge analysis

3.3.3. Determination of physical stability of the nanoparticles

3.3.4. Electron microscopy

3.3.4.1. Scanning electron microscopy (SEM)

3.3.4.2. Atomic force microscopy

3.3.5. Determination of the physical state of the drug in the nanoparticles

3.3.5.1. Differential scanning calorimetric studies

3.3.5.2. X-ray diffraction analysis

3.3.6. Thermogravimetric analysis

3.3.7. Karl Fischer titrimetry

3.3.8. HPLC analysis

3.3.9. Determination of drug load in the nanoparticles
3.3.10. Determination of drug entrapment efficiency 50
3.3.11. *In vitro* release studies 51
3.3.12. MTT toxicity assay 52
3.3.13. Haemolysis assay 53
3.3.14. Determination of cellular uptake 55
3.3.15. Permeability studies 55
3.3.16. Statistical data analysis 58

3.4. Results 58

3.4.1. Particle size and surface charge of the nanosuspensions 58
3.4.2. Particle size and surface charge of the nanoparticles 59
3.4.3. Physical stability of the nanoparticles 60
3.4.4. Electron microscopy 62
3.4.5. Physical state of the drug in the nanoparticles 65
  3.4.5.1. Differential scanning calorimetric studies 65
  3.4.5.2. X-ray diffraction analysis 67
3.4.6. Thermogravimetric analysis 69
3.4.7. Karl Fischer titrimetry 70
3.4.8. Drug load and drug entrapment efficiency of the nanoparticles 70
3.4.9. *In vitro* release of metformin 70
3.4.10. MTT toxicity assay 72
3.4.11. Haemolysis assay 73
3.4.12. Cellular uptake of metformin 75
3.4.13. Permeability studies 76

3.5. Discussion 77

3.5.1. Formulation of the delivery system 77
3.5.2. Particle size, zeta potential and surface morphology 82
3.5.3. Physical stability of the nanoparticles 83
3.5.4. Electron microscopy 84
3.5.5. Thermal analysis, moisture content and X-ray diffraction 84
3.5.6. HPLC analysis 86
3.5.7. Drug load in metformin nanoparticles 86
3.5.8. Drug entrapment efficiency 86
3.5.9. In vitro release of metformin 87
3.5.10. MTT toxicity assay 88
3.5.11. Haemolysis assay 89
3.5.12. Cellular uptake of metformin 89
3.5.13. Permeability studies 90

3.6. Conclusions 92

CHAPTER 4: Summary, global impact and future directions 94

4.1. Summary 95
4.2. Global impact
4.3. Future direction
Bibliography
List of figures

Figure 1: Metformin hydrochloride 5
Figure 2: GIT and different sites of drug absorption [22] 12
Figure 3: Three-dimensional view of layers of the small intestine [24] 14
Figure 4: Basic Structure of functional cell membrane 17
Figure 5: Classification of mechanisms of absorption 22
Figure 6: Chromatograms a) Blank Sample and b) Metformin Sample 32
Figure 7: Standard curve for metformin over a range of 15.6-500μg/mL 33
Figure 8: (A) Blank nanoparticles (B) Metformin nanoparticles 45
Figure 9: Set up for release studies 52
Figure 10: Set-up for passive metformin diffusion through an artificial membrane 56
Figure 11: Particle size results for blank nanoparticles at room temperature (n = 3), + Significant increase 60
Figure 12: Particle size results for metformin nanoparticles at room temperature (n = 3), + Significant increase 61
Figure 13: Zeta potential for blank and metformin loaded nanoparticles at room temperature (n = 9) 61
Figure 14: SEM of blank hyaluronic acid nanoparticles 62
Figure 15: SEM of metformin loaded hyaluronic acid nanoparticles 63
Figure 16: AFM images of blank hyaluronic acid nanoparticles (dilution 5 times) 63
**Figure 17:** AFM images of blank hyaluronic acid nanoparticles (dilution 50 times)  

**Figure 18:** AFM images of metformin loaded hyaluronic acid nanoparticles (dilution 5 times)  

**Figure 19:** AFM images of metformin loaded hyaluronic acid nanoparticles (dilution 10 times)  

**Figure 20:** An overlay of DSC thermograms of sodium hyaluronate, metformin hydrochloride, blank nanoparticles and metformin loaded nanoparticles analyzed with blank aluminium pan as reference  

**Figure 21:** An overlay of DSC thermograms of sodium hyaluronate, metformin hydrochloride, blank nanoparticles and metformin loaded nanoparticles analyzed with blank nanoparticles as reference  

**Figure 22:** An overlay plot of XRD patterns of blank nanoparticles and drug loaded nanoparticles  

**Figure 23:** An overlay of XRD patterns of blank nanoparticles and drug loaded nanoparticles along with reference pattern of the pure drug (metformin)  

**Figure 24:** TGA thermograms of the lyophilized blank and metformin loaded nanoparticles showing weight loss over a range of 25°C - 120°C
Figure 25: *In vitro* release profile of metformin

Figure 26: A plot of log cumulative of % drug remaining vs. time representing the first order release kinetics

Figure 27: Cytotoxicity profile of metformin solution, blank nanoparticles and metformin loaded nanoparticles after 24 hours incubation

Figure 28: Cytotoxicity profile of metformin solution, blank nanoparticles and metformin loaded nanoparticles after 72 hours incubation (+ Significant cell death)

Figure 29: Percent haemolysis at two wavelengths of detection (404 and 540nm) for drug solution, blank and drug loaded nanoparticles

Figure 30: The cellular uptake of metformin from the solution and nanoparticulate system in Caco-2 cells at different time points

Figure 31: Structure of hyaluronic acid, ADH and EDC

Figure 32: Reaction scheme for hyaluronic acid cross-linking

Figure 33: Hyaluronic acid - ADH cross-linked nanoparticles
List of tables

Table 1: Within day and day to day precision for HPLC analysis of metformin 35

Table 2: Accuracy results for the HPLC analysis of metformin 36

Table 3: Particle size and zeta potential of blank and drug loaded nanosuspensions (mean ± S.D; n = 3) 58

Table 4: Particle size and zeta potential of blank and metformin loaded nanoparticles (mean ± S.D; n = 3) 59

Table 5: Mean percent haemolysis of drug solution, blank and nanoparticles with Triton X as a positive control 74

Table 6: Permeability data for metformin solution (n = 9) 76

Table 7: Permeability data for metformin loaded nanoparticles (n = 9) 77
List of equations

**Equation 1:** Formula used for % accuracy calculation

*not defined.*

**Equation 2:** Formula for drug load calculation

37

**Equation 3:** Formula for drug entrapment efficiency calculation

37

**Equation 4:** Calculation of percent haemolysis

54

**Equation 5:** Calculation of effective permeability ($P_e$)

57

**Equation 6:** Calculation of fraction of compound in membrane ($R$)

58
**List of abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>Adenosine triphosphate binding cassette</td>
</tr>
<tr>
<td>ADH</td>
<td>Adipic acid dihydrazide</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BCS</td>
<td>Biopharmaceutics Classification System</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Media</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethyl formamide</td>
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<tr>
<td>DPP-4</td>
<td>Dipeptidyl peptidase-4</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>EDC</td>
<td>1-[3-(Dimethylamino) propyl]-3 ethylcarbodiimide hydrochloride</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon like peptide</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
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<tr>
<td>IF</td>
<td>Intrinsic factor</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug resistance-associated protein</td>
</tr>
<tr>
<td>OATP</td>
<td>Organic anion-transporting polypeptide</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>--------------------------------------------------</td>
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<tr>
<td>OCT</td>
<td>Organic cation transporter</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PMAT</td>
<td>Plasma membrane monoamine transporter</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>TGA</td>
<td>Thermogravimetric analysis</td>
</tr>
<tr>
<td>USP</td>
<td>United States Pharmacopeia</td>
</tr>
<tr>
<td>XRD</td>
<td>X-ray Diffraction</td>
</tr>
</tbody>
</table>
CHAPTER 1

Introduction
1.1. Diabetes

Diabetes is a chronic disease that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces. Globally, an increasing prevalence of people stricken with diabetes occurred with an estimated 422 million adults, living with diabetes in 2014, as compared to 108 million in 1980. The global prevalence of diabetes nearly doubled since 1980, rising from 4.7% to 8.5% in the adult population. In addition, diabetes caused 1.5 million deaths in 2012 [1]. In 2014, 29.1 million people in the United States (9.3% of the population) had diabetes and 8.1 million people were undiagnosed [2].

Diabetes occurs due to an imbalance between the demand and production of the hormone insulin. Insulin plays an integral role in glucose transport by stimulating the translocation of the glucose transporter GLUT4 (the major glucose transporter in skeletal muscle) from intracellular sites to the cell surface, thereby increasing the glucose uptake in cells and leading to a reduced blood glucose level [3]. Thus, diabetes is characterised by elevated levels of glucose in the blood. The most common types of diabetes include type 1, type 2, and gestational diabetes. Type 1 diabetes, previously known as insulin-dependent diabetes, is a chronic condition in which the pancreas produces little or no insulin. It is termed as an autoimmune disease where there are anti insulin or anti-islet cell antibodies present in blood which cause lymphocytic infiltration and destruction of the pancreas islets.
Type 1 diabetes requires daily administration of insulin, and does not respond to insulin-stimulating oral drugs. The cause of type 1 diabetes is not known and is currently not preventable [4].

Type 2 diabetes, formerly called non-insulin-dependent or adult-onset diabetes, is a chronic condition that results from the body’s ineffective use of insulin and affects the way the body processes blood sugar (glucose). This condition is caused by a relative deficiency of insulin and not an absolute deficiency because of beta cell deficiency coupled with peripheral insulin resistance [5]. This means that the body is unable to produce adequate insulin to meet the needs. Type 2 diabetes accounts for 90–95% of those with diabetes around the world, and is largely the result of excess body weight and physical inactivity [6]. Type 2 diabetes used to occur nearly entirely among adults, but now can be seen in children as well [1].

Gestational diabetes is caused when there are excessive counter-insulin hormones present during pregnancy. This leads to a state of insulin resistance and high blood sugar in the pregnant mother [5]. Its treatment can include both insulin therapy as well as oral anti-diabetic agents [7].

Diabetes of all types can lead to complications in various parts of the body leading to heart attack, stroke, kidney failure, leg amputation, vision loss, and nerve damage. In addition, it can increase the overall risk of dying
prematurely. In pregnancy, poorly controlled diabetes increases the risk of fetal death and other complications [1].

1.2. Oral therapy for type 2 diabetes

Patients affected with type 2 diabetes respond well to oral drug therapy. Oral treatment mostly consists of biguanides (metformin), sulfonylureas (tolbutamide, chlorpropamide, glibizide, glimepiride), meglitinide/phenyl alanine analogues (repaglinide nateglinide), thiazolidinediones (rosiglitazone, pioglitazone) and α-Glucosidase inhibitors (acarbose, miglitol). Biguanides enhance the action of insulin. Sulfonylureas and meglitinide/phenyl alanine analogues cause a brisk release of insulin from the pancreas. Thiazolidinediones tend to reverse insulin resistance by stimulating GLUT4 expression and translocation, improving the entry of glucose into muscle and fat. They also suppress hepatic gluconeogenesis. α-Glucosidase inhibitors reversibly inhibit α-glucosidases, the enzymes required for the digestion of carbohydrates in the brush border of small intestinal mucosa, thus slowing and decreasing the digestion and absorption of polysaccharides and sucrose [8]. In addition, there are newer classes like dipeptidyl peptidase-4 (DPP-4) inhibitors which prevent the inactivation of glucagon like peptide (GLP-1) by DPP-4. Thus, enhancing the insulin release stimulating activity of GLP-1 [9].
1.3. Drug Profile: Metformin

Metformin is generally recommended as a first line treatment for type 2 diabetes. One potential advantage of patients taking metformin includes weight loss or lack of weight gain [10]. Metformin is an official drug in United States Pharmacopoeia (USP), British Pharmacopoeia and European Pharmacopoeia, and is usually available in its salt form (metformin hydrochloride, CAS No of 1115-70-4) [11]. Figure 1 shows the chemical structure of metformin hydrochloride.

![Figure 1: Metformin hydrochloride](attachment:image.png)

1.3.1. Physicochemical properties of metformin

Metformin is also known as 1, 1-dimethylbiguanide, dimethylbiguanide or metiguanide. It’s IUPAC name is 3-(diaminomethyldiene)-1,1-dimethylguanidine. Some of the brand names are Glucophage, Glucophage XR, Glumetza, Fortamet [12]. Metformin has molecular formula of C\(_4\)H\(_{11}\)N\(_5\). It is available in its salt form as a white solid crystalline powder. Metformin and metformin hydrochloride have a
molecular weight of 129.16 g/mol and 165.62 g/mol, respectively [13]. Metformin hydrochloride has a melting point of 223 - 226° [11]. With a log p value of -2.64, it acts as a hydrophilic compound which is freely soluble in water (50 mg/mL), ethanol, and dimethyl sulfoxide (50 mM) [14, 15]. It is a basic drug. At a physiological pH, it exists as an organic cation, with a pKa of 12.4. Therefore, its passive diffusion through cells has been reported to be low [15].

1.3.2. Pharmacokinetics of metformin

In order to understand and control the therapeutic action of drugs in the human body, one must know how much drug will reach the site(s) of drug action and when this will occur. The absorption, distribution, metabolism (biotransformation), and elimination of drugs are the processes of pharmacokinetics. Understanding and employing pharmacokinetic principles can increase the probability of therapeutic success and reduce the occurrence of adverse drug effects in the body.

1.3.2.1. Absorption and bioavailability

The absolute bioavailability of a metformin hydrochloride 500 mg tablet given under fasting conditions is approximately 50-60%. Studies using single oral doses of metformin hydrochloride tablets (500-2550 mg) report a lack of proportional increase in drug absorption with increasing doses, indicating a saturable absorption mechanism. Thus, despite its high
solubility, metformin’s oral bioavailability is limited due to its saturable absorption. Hence, it must be administered in a high dosage. Food decreases the extent of and slightly delays the absorption of metformin [16]. These properties have classified metformin as a Biopharmaceutics Classification System (BCS) Class III drug [17]. Plasma membrane monoamine transporter (PMAT), expressed on the luminal side of enterocytes, primarily mediates the intestinal absorption of metformin. However, there are currently no *in-vivo* data to support the role of PMAT in the disposition and pharmacological effect of metformin. In addition, organic cation transporter (OCT) 1, expressed on the basolateral membrane of enterocytes, may transfer of metformin into the interstitial fluid. The role of OCT1 and OCT3 in the intestinal transport of metformin remains to be defined [18].

1.3.2.2. Distribution

The apparent volume of distribution of metformin following a single oral doses of metformin hydrochloride tablet (850 mg) on an average is 654 ± 358 L. In contrast to sulfonylureas, which are more than 90% plasma protein bound, metformin is negligibly bound to plasma proteins. Metformin partitions into erythrocytes, most likely as a function of time. At usual clinical doses and dosing schedules of metformin hydrochloride tablets, steady state plasma concentrations of metformin are reached within 24 to 48 hours and are generally <1 mcg/mL [16]. The hepatic uptake of metformin
is mediated primarily by OCT1 (SLC22A1) and possibly by OCT3 (SLC22A3). Metformin is widely distributed into body tissues including the intestine, liver, and kidney by organic cation transporters. OCT2 (SLC22A2), which is expressed predominantly at the basolateral membrane in the renal tubules, appears to primarily facilitate the uptake of metformin from circulation into renal epithelial cells [18].

1.3.2.3. Metabolism and elimination

Metformin is excreted unchanged in the urine and does not undergo hepatic metabolism (no metabolites have been identified in humans) or biliary excretion. Renal clearance is found to be approximately 3.5 times greater than creatinine clearance indicating that active tubular secretion is the major route for metformin elimination. Following oral administration, approximately 90% of the absorbed drug is eliminated via the renal route within the first 24 hours, with a plasma elimination half-life of approximately 6.2 hours. In blood, the elimination half-life is approximately 17.6 hours, suggesting that the erythrocyte mass may be a compartment for metformin distribution [16].

Renal excretion of metformin from the tubule cell to the lumen is mediated through MATE1 (SLC47A1) and MATE2-K (SLC47A2) transporters. MATE1 and MATE2-K are expressed in the apical membrane of the renal proximal tubule cells, and studies in healthy individuals suggest
that they contribute to the renal excretion of metformin. OCT1 also appears to be expressed on the apical and subapical domain side of both the proximal and the distal tubules in the kidney, and may play an important role in metformin reabsorption in kidney tubules [18].

1.3.3. Pharmacodynamics of metformin

1.3.3.1. Mechanism of action

The glucose lowering effect of metformin is the result of the drug action on liver, muscle, and adipose tissues. Effect of metformin on hepatic glucose production is considered to be dominant. Explanations offered for its hypoglycemic action are: suppressing of hepatic gluconeogenesis and glucose output from liver, enhancement of insulin-mediated glucose disposal in muscle and adipose tissues [8]. It seems to alter location of GLUT4 transport from intracellular site to plasma membrane [19]. In addition, metformin lowers the blood sugar level by increasing glucose uptake in peripheral tissues. Also, it inhibits intestinal glucose absorption by increasing anaerobic glucose metabolism, i.e. by stimulating glycolysis. It is important to note that it does not stimulate insulin secretion [20]. Hence, metformin requires insulin for its action, and acts as an anti-hyperglycaemic agent and does not cause hypoglycaemia.

The molecular mechanisms underlying metformin action appear to be complex and remain a topic of considerable debate. However, there is
general agreement that the administration of metformin results in the phosphorylation and activation of AMP-activated protein kinase in the liver, which in turn may lead to diverse pharmacologic effects, including inhibition of glucose and lipid synthesis. Peripheral effects include the stimulation of fatty acid oxidation and glucose uptake in skeletal muscle as well as a systemic increase in insulin sensitivity. However, the role of metformin in insulin-mediated glucose uptake has been debated [18].

1.3.3.2. Adverse effects

Abdominal pain, anorexia, nausea, metallic taste, mild diarrhoea and tiredness are the frequent side effects of metformin. It does not cause hypoglycaemia except in overdose. A by-product of glucose metabolism is lactate. Thus, lactic acidosis, which is a small increase in blood lactate, has been associated with metformin. However lactic acidosis is rare since metformin is poorly concentrated in hepatic cells. Alcohol ingestion may precipitate severe lactic acidosis. Vitamin $\text{B}_{12}$ deficiency can occur due to interference with its absorption on administration of high dose of metformin. In addition, biguanides are contraindicated in hypotensive states, cardiovascular, respiratory, hepatic, and renal diseases and alcoholics because of increased risk of lactic acidosis [8].

1.3.3.3. Drug interaction
Pharmacokinetic parameters were found to be altered upon co-administration of metformin with glyburide, furosemide or nifedipine. Cationic drugs (e.g., amiloride, digoxin, morphine, procainamide, quinidine, quinine, ranitidine, triamterene, trimethoprim, or vancomycin) that are eliminated by renal tubular secretion compete for common renal tubular transport systems. Certain drugs like corticosteroids, phenothiazines, thyroid products, estrogens, oral contraceptives, phenytoin, nicotinic acid, sympathomimetics, calcium channel blocking drugs, isoniazid, thiazides and other diuretics tend to produce hyperglycemia and may lead to loss of glycemic control [21].

1.4. Oral drug delivery

Generally, oral drug administration is the most common method of drug administration. It is the safest, most convenient, and most economical route of administration. Since, metformin is administered orally and its primary site of absorption is small intestine, anatomy and physiology of gastrointestinal tract has been discussed briefly.

1.4.1. Overview of gastrointestinal tract (GIT)

The major functional components comprising the GIT are stomach, small intestine (duodenum, ileum and jejunum) and large intestine (colon). Their primary functions are secretion, digestion, and absorption. These components differ from each other with respect to their anatomy, function,
secretions and pH. The entire mean length of GIT is approximately 450 cm and is lined by a thin layer of mucopolysaccharides (mucous/mucin), which primarily function as a barrier to the particulates such as bacteria, cells or food particles [22]. Figure 2 gives schematic representation of the GIT and different sites of drug absorption.

![Figure 2: GIT and different sites of drug absorption [22]](image)

1.4.2. Anatomy and physiology of small intestine

The small intestinal mucosa consists of four layers, first the muscularis, which is a thin layer of smooth muscle, second the submucosa,
which is the connective tissue layer underlying the mucosa, third the lamina propria, which is a connective tissue, and lastly a monolayer of epithelial cells. By contracting, the muscularis modulates the thickness of the unstirred water layer at the surface of the mucosa, affecting the absorption and helping to empty the luminal contents. Lamina propria is a structural support for the monolayer of epithelial cells and is folded to form villi. The epithelial cells are a heterogeneous population of cells which include: enterocytes or absorptive cells; goblet cells, which secrete mucin; endocrine cells, which produce hormones and peptides; Paneth cells, which secrete large amounts of protein rich materials; M cells, which transport antigens present in the intestinal lumen. In addition, there are crypt cells, which are undifferentiated cells, which start to differentiate into the above mentioned cells. The most common epithelial cell, enterocyte, is responsible for the majority of the digestion and absorption of both nutrients and drugs. The apical membrane has microvilli and contains the digestive enzymes such as disaccharides and peptidases. The apical membrane also expresses transport systems for the absorption. In contrast to the apical membrane, the basolateral membrane has smooth contours and no digestive enzymes. However, it expresses other transport systems including Na-K ATPase, glycosyltransferases, and adenyl cyclase. Molecules that have permeated through the monolayer of epithelial cells are transported by the blood and
lymphatic vessels located in the lamina propria [23, 24]. Figure 3 gives a three-dimensional view of layers of the small intestine.

![Figure 3: Three-dimensional view of layers of the small intestine](image)

The length of small intestine is about 285 cm. The folds in intestinal mucosa, called the folds of Kerckring, result in 3-fold increase in surface area. These folds have finger-like projections, called villi on their surface, which increases the surface area 30 times. These villi contain enterocytes, expressing microvilli, which results in 600 times increase in surface area. The blood flow to small intestine is 6 to 10 times more than that of stomach. Due to its large surface area, it is the major site of absorption for most of the drugs. This results in more than 200 square meters of surface. Also, the pH
range of 5 to 7.5 is most favourable for most of the drugs to remain unionized. In addition, the peristaltic movement in intestine is slow, transit time is long, and permeability is high. All these factors together, make small intestine the best site for absorption of most of the drugs [22].

A good oral bioavailability of a drug implies that the drug is able to reach the systemic circulation when taken orally. This makes the absorption of oral drug in the intestine an important factor to determine the drug bioavailability, especially for drugs like metformin which have low intestinal permeability. Hence, it has been a subject of intense and continuous investigation in the pharmaceutical industry [25] and has been discussed below.

1.4.3. Oral drug absorption

An ingested drug, if not degraded in the intestinal lumen by enzymes or bacteria, is absorbed by the intestinal epithelium (gut wall) and then transported by the blood stream (portal vein) to the liver. The drug can be metabolized here and the drug and its metabolites can be then cleared into the systemic blood circulation or into the bile. The compound (and metabolites) cleared in the bile re-enters the intestinal lumen and can be absorbed again. This is called the enterohepatic circulation. The fraction of the compound not absorbed in the intestine is excreted in the faeces. Compounds (and metabolites) that have entered the systemic circulation can
be excreted either by the kidneys in the urine or in the faeces in the large intestine [22].

A rapidly absorbed drug offers several advantages like lesser susceptibility of the drug for degradation or possible interaction with other drugs, higher blood levels of drugs rapid onset of action, and a more uniform and reproducible therapeutic response. In order to understand the absorption, a brief description of cell membrane structure and physiology is necessary.

1.4.3.1. Basic Structure of functional cell membrane

The plasma membrane consists of a bilayer of amphipathic lipids consisting of hydrophobic hydrocarbon chains and hydrophilic heads. The hydrophobic chains are oriented inward to the center to form a continuous hydrophobic phase and their hydrophilic heads are oriented outward. Membrane proteins are embedded in the bilayer, which function as structural anchors, receptors, ion channels, or transporters to transduce electrical or chemical signaling pathways and provide selective targets for drug actions. The hydrophobic core of the membrane is responsible for the relative impermeability of the polar molecules. The aqueous filled pores or perforations of 4-10 Å in diameter present in the membrane structure allow the passage of inorganic ions and small organic water-soluble molecules such as urea. Generally, this biomembrane acts like a semipermeable barrier
which permits rapid and limited passage of some compounds however restricting that of others compounds. This GI lining, which constitutes the absorption barrier, allows the rapid passage of most nutrients like glucose, amino acids, fatty acids, vitamins, etc. into the systemic circulation. However, it also prevents the entry of certain toxins and medicaments. Thus, for a drug to get absorbed after oral administration and reach the systemic circulation, it must first pass through this biological barrier [22]. Figure 4 represents this basic structure of functional cell membrane.

![Basic Structure of functional cell membrane](image)

**Figure 4:** Basic Structure of functional cell membrane

1.4.3.2. **Mechanism of oral absorption**

After oral dosing, drug molecules can cross the luminal membrane through various mechanisms. In the order of their importance (based on the frequency of occurrence), the principal mechanisms are passive diffusion, active transport, ionic or electrochemical diffusion, and endocytosis. Passive
diffusion involves the movement of the drug from its higher concentration to its lower concentration, i.e. along the concentration gradient or a downhill transport. Passive diffusion is comprised of two pathways viz. the paracellular pathway, in which drug diffuses through the aqueous pores at the tight junctions between the enterocytes (cells of the intestinal lining); and the transcellular (lipophilic) pathway, which requires drug diffusion across the lipid cell membrane of the enterocyte. Active diffusion involves the movement of drug from its lower concentration to its higher concentration, i.e. against the concentration gradient or an uphill transport and hence, energy is required for active transport. The active transport pathway is mediated by transporters and is divided into active drug influx and efflux. The relevance of each route is determined by the compound’s physicochemical properties and its potential affinity for various transport proteins [26].

1.4.3.2. 1. Passive diffusion

In paracellular diffusion, drug molecules are absorbed by diffusion and convective volume flow through the water-filled intercellular space [27]. Generally, drugs that are absorbed through this pathway have low molecular weight (MW < 250 g/mol) and are hydrophilic in nature (log p < -1). Because the junctional complex has a net negative charge, positively charged molecules pass through more readily, whereas negatively charged
molecules are repelled [28]. In addition, the paracellular pathway offers a very small window for absorption since it accounts only for < 0.01% of the total surface area of intestinal membrane. Pore transport, an example of paracellular drug transport, involves the convective/bulk flow transport of the drugs to narrow, aqueous-filled pores in the membrane following the paracellular pathway. Compounds like urea, water, and sugars are transported by this route.

In contrast the transcellular pathway is the major route of absorption for most of drug molecules. Generally, the rate of passive transcellular transport across the apical cell membrane is controlled by the physicochemical properties of the absorbed compound. Unlike the paracellular pathway, compounds that are absorbed through the transcellular pathway are unionised, with lipophilicity of log p > 0 and MW > 300 g/mole [26]. Facilitated diffusion and Ion-pair transport both follow the transcellular pathway.

Facilitated diffusion is a carrier-mediated system and hence, transports drug at a faster rate than that of a simple diffusion. Vitamin B₁₂ is transported by facilitated diffusion. It forms a complex with intrinsic factor (IF), a glycoprotein produced by gastric parietal cells and then transported across the intestinal membrane [22]. The movement of ionizable drugs across intestinal membrane can be facilitated by formation of ion pair. A
classic example of ion-pair transport is that of quaternary ammonium compounds and sulfonic acids. These agents form reversible neutral complexes with endogenous ions of GIT like mucin. Such complexes then have the required lipophilicity and aqueous solubility for passive diffusion. [22].

1.4.3.2.2. Active transport

Transporters expressed by the enterocytes belong to two main classes, viz. the adenosine triphosphate (ATP) binding cassette (ABC) and the solute carrier (SLC) classes. They are expressed on the apical and basolateral membranes for the influx or efflux of endogenous substances. As the name suggests, the ABC transporters utilize ATP as the energy source to drive the transport and are called primary active transporters. The SLC transporters, however, majorly use the ion gradients (H+, Na+ and Ca++ gradients) created across the membrane by primary active carriers (Na+/K+-ATPase, Na+/H+-ATPase). ABC transporters expressed in the intestine include multidrug resistance-associated protein 2 (MRP2; ABCC2), P-glycoprotein (P-gp, MDR1, ABCB1), breast cancer resistance protein (BCRP; ABCG2). MRP2, P-gp, and BCRP are localized on brush-border (apical) membrane while certain MRPs are expressed on the basolateral membrane. These efflux transporters functionally limit the enterocytic levels of their substrates by reducing uptake and facilitating efflux. Relevant SLC transporters at the
intestinal apical surface of epithelial cells include organic cation/zwitterion transporters (OCTs, SLC22A), novel organic cation transporters (OCTNs), organic anion-transporting polypeptide (OATP, SLCO), H+/peptide co-transporter. Substrates for OCT1 include endogenous compounds, such as choline, creatinine, and monoamine neurotransmitters; clinically used drugs, such as metformin, oxaliplatin and cimetidine [29]. Several other SLC transporters including organic anion or cation transporters (OATs or OCTs; SLC22) have also been identified in the intestine, but seem to be of less importance in oral drug absorption [30].

1.4.3.2.3. Ionic – electrochemical diffusion

The membrane charge is the governing factor of drug permeability. The permeation of ionized drugs, especially the cationic drugs depends on the electrical gradient, which acts as a driving force. At a given pH the rate of permeation of unionized molecules is the maximum, followed by anions and then cations [22].

1.4.3.2.4. Endocytosis

It is a minor transport mechanism. It involves engulfment of extracellular particle by a segment of the cell membrane to form a saccule or a vesicle, which is then pinched-off intacellularly. Two types of processes involved are phagocytosis (uptake of solid particulates, e.g.: fats and starch) and pinocytosis (uptake of fluid solute, e.g: Sabin polio vaccine) [22].
The classification of these absorption mechanisms has been summarized in Figure 5.

**Figure 5:** Classification of mechanisms of absorption

1.4.4. **Factors influencing drug absorption**

After oral administration, the extent of absorption of a drug in the gastrointestinal tract depends on physiological factors, physicochemical properties of the drug and the pharmaceutical characteristics of the dosage form. Physiological factors that will determine the extent of absorption of a compound include age, gastric emptying, gastrointestinal transit, gastrointestinal pH, diseased state, blood flow through GIT, and the composition of the intestinal lumen (e.g. pH, enzymes, food, etc), the intestinal epithelium and the liver. The physicochemical factors affecting absorption are drug solubility and dissolution rate, particles size and surface area, polymorphism, salt forms of drug, lipophilicity, hydrogen bonding
potential, pKa, and pH of the drug and its stability. The pharmaceutical factors include disintegration time (tablets/capsules), dissolution time, excipients, and the nature of dosage form [22].

1.4.5. Challenges of oral drug delivery

Disadvantages to the oral route include limited absorption of some drugs because of their physical characteristics (e.g. low water solubility or poor membrane permeability like in case of metformin), emesis as a result of irritation to the GI mucosa, destruction of some drugs by digestive enzymes or low gastric pH, irregularities in absorption or propulsion in the presence of food or other drugs. Hence, such oral drugs typically have to be administered at high doses to achieve therapeutic concentrations (e.g. metformin).

1.5. Nanoparticles to enhance intestinal absorption

Studies have indicated that polymeric nanoparticles are able to overcome several of the barriers to oral delivery and increase the oral bioavailability. For example, cyclosporine loaded PLGA nanoparticles showed sustained release of the drug in the plasma and a decrease in the cyclosporine associated nephrotoxicity. However, these two characteristics were absent in the commercial formulation (Sandimmune Neoral) [31, 32]. Chitosan nanoparticles have exhibited the ability to increase the permeability of drugs [33]. Mucoadhesive alginate/chitosan nanoparticles
were found to augment oral absorption and oral bioactivity of insulin [34]. Such nanoparticles, with a higher surface-to-volume ratio than that of microparticulate systems, can significantly increase their cellular contact, and/or provide greater area for dissolution and hence increase the absorption, thereby increasing the bioavailability. Also, PLGA polymeric nanocarriers have shown the ability to improve oral bioavailability of drugs with poor, enzymatic or metabolic stability like estradiol [35] and atorvastatin [36]. Thus, it is possible to prepare nanoparticles in such a manner that they can increase the intestinal absorption of a drug by offering mucoadhesive, permeation enhancing or sustained release characteristics leading to an increase in the intestinal absorption, and thereby increasing the oral bioavailability of the drug [37].

Various mechanisms to overcome the permeability barrier of the intestine and increase the absorption of drugs by oral administration nanoparticulate systems have been investigated and outlined below [38].

1.5.1. Mucoadhesives

Mucoadhesives are materials that interact with the intestinal mucus layer. It increases the residence time and contact of these materials with the intestinal epithelium. These mucoadhesive materials have been used to prepare mucoadhesive nanoparticles for enhanced oral bioavailability. Administration of these nanoparticles containing drug results in an increased
concentration of the drug at the absorption site that enhances the oral bioavailability and reduces the pharmacokinetic variability [39]. Some examples of mucoadhesive polymers include hyaluronic acid [40], chitosan, [41] and polyacrylic acid [42]. Many mucoadhesive materials can also act as permeation enhancers by reversibly opening tight junctions to enhance paracellular transport [43]. Although the nanoparticles are usually unable to pass through the paracellular pathway because of their particle size, drug molecules and biologics are able to cross through this pathway.

1.5.2. M Cells

M cells transport antigens across the intestinal barrier by transcytosis pathway for immune surveillance. They are associated with Peyer’s patches, an organized component of the gut-associated lymphoid tissue. M cells’ properties, such as reduced proteases, a lack of mucus secretion, and a sparse glycocalyx allow for adherence by nanoparticles. The active transcellular transport of nanoparticles begins with an endocytic process that occurs at the apical cell membrane and the particles then transport through the cells and release at their basolateral pole of the cells [44]. In order to target nanoparticles to M cells, many approaches have been tried for, e.g. more hydrophobic polymers improve M-cell transport [45]. In addition, targeting ligands conjugated to surface of the nanoparticle, such as bacterial adhesins, IgA antibodies, and toxins, increase M cell transport [38].
According to a study, the uptake of nanoparticles by enterocytes or M cells is dependent on the size of the nanoparticles. Small particles (<100 nm) can be taken up by enterocytes, while larger particles are more likely to be internalized by M cells [46].

1.5.3. Vitamin B$_{12}$ receptor

Ingested vitamin B$_{12}$ is bound by IF and the vitamin B$_{12}$–IF complex interacts with an IF receptor that traffics vitamin B$_{12}$ across the intestinal epithelium. Nanoparticles have been designed to target this pathway by conjugating vitamin B$_{12}$ to the surface of nanoparticles. Studies with insulin-loaded nanoparticles targeted to the vitamin B$_{12}$ receptor have shown enhanced absorption of insulin after oral administration.

1.5.4. Neonatal Fc receptor

Neonatal Fc receptor (FcRn) mediates IgG (Immunoglobulin G) transport across the intestinal epithelium. FcRn is present in the apical region of epithelial cells in the small intestine and colon. FcRn interacts with the Fc portion of IgG in a pH dependent manner, by binding with high affinity in acidic pH (< 6.5), but not physiological pH (7.4). Hence, targeted nanoparticles can interact with FcRn at the apical surface of the epithelial cells and be taken up by receptor-mediated endocytosis in acidic segments of the intestine such as the duodenum and jejunum. In the segments of the
intestine that are not acidic, the targeted nanoparticles still can be taken into the cells by fluid-phase pinocytosis and interaction with FcRn.

1.6. Objectives, hypothesis and specific aims

The objective of the present study was to develop and characterize polymeric nanoparticulate system containing metformin with a mucoadhesive hyaluronic acid polymer that can increase the intestinal absorption of the drug thereby increasing its oral bioavailability.

The hypothesis of this investigation was:
Metformin loaded hyaluronic acid nanoparticles can be prepared and characterized to improve its oral absorption leading to an increased bioavailability.

The specific aims for this investigation were:
1. Development and validation of a high pressure liquid chromatographic (HPLC) method for the analysis of metformin
2. Preparation and characterization of metformin loaded hyaluronic acid nanoparticles
3. In vitro evaluation of metformin loaded nanoparticles.
Chapter 2

Analytical method development and validation
2.1. Introduction

Metformin can be detected by spectrophotometric methods like UV-visible spectroscopic analysis [47], Fourier transform infrared spectroscopy [48] and colorimetry [49]. Several chromatographic techniques that include high performance thin-layer chromatography [50] and HPLC [51] have been developed for detection and quantification of metformin. HPLC is more popular and convenient method for metformin analysis. Some of these methods quantified metformin either in water [52] or in different mobile phases [50, 51]. When metformin was analyzed in the release media (phosphate buffer pH 7.4) using a previously reported method [53], co-elution of metformin with the buffer components was observed. Therefore, development of an HPLC method for such analysis was essential. The objective of this study was to develop and validate a sensitive HPLC method for the quantification of metformin in the release media (phosphate buffer pH 7.4).

2.2. Materials

Commercial grade of metformin was purchased from BioVision, Inc. (San Francisco, CA). Monobasic potassium phosphate was purchased from Spectrum Chemical Mfg. Corp (Brunswick, NJ). Dibasic potassium phosphate was purchased from Sigma Aldrich, (St. Louis, MO). Optima Grade Acetonitrile for HPLC analysis was purchased from Fischer Scientific
(Fair Lawn, NJ). Phenomenex Luna C18 5 μm (250x4.6 mm) column was purchased from Phenomenex (Torrance, CA).

2.3. Methods

2.3.1. Chromatography

The HPLC analysis was performed using a reversed phase LC-10ATvp HPLC, Shimadzu, Kyoto, Japan, equipped with a binary solvent pump, an autosampler and a photodiode array detector. The chromatographic separation was achieved on Phenomenex Luna C18 5 μm (250x4.6 mm) column using isocratic elution. The mobile phase consisted of 0.02M potassium phosphate buffer (pH 7.4): acetonitrile (60:40 v/v), at a flow rate of 1 mL/min. The injection volume was 20μL and the total run time was 6 minutes. The column effluents were monitored at the detector wavelength of 252 nm.

2.3.2. Preparation of solutions

2.3.2.1. Mobile phase

The aqueous solution of 0.02 M potassium phosphate buffer was prepared by dissolving Monobasic Potassium Phosphate (0.54 g) and Dibasic Potassium Phosphate (2.8 g) in 1000 mL water. It was filtered through 0.45 μm, 47 mm Nylon-66 filter (Chrom Tech, Inc., Apple Valley, MN) using vacuum. The organic phase consisting of 400 mL of acetonitrile
was added to 600 mL of aqueous phase to prepare 1 L of mobile phase. The apparent pH of the mobile phase was 8.3 and was degassed for 5 minutes before use.

2.3.2.2. Standard solution

Metformin stock solution with a concentration 500 µg/mL was prepared by dissolving 25 mg of metformin in 50 mL phosphate buffer (pH 7.4). The standard solutions were prepared from the stock solution by appropriate dilutions.

2.4. Calculations

The standard curve of metformin was obtained by plotting peak height of the standards against their appropriate concentrations. The unknown concentrations of metformin were determined from the regression equation obtained from the standard curve.

2.5. Results and discussions

2.5.1. Specificity

The USP defines specificity as the ability of a method to discriminate the analyte from all the potentially interfering substances such as impurities and degradation products. The specificity for this analytical method was assessed by comparing the chromatograms obtained by the injection of phosphate buffer (pH 7.4) with no drug (blank) and the chromatogram of
phosphate buffer containing the drug. Figure 6 (a) a representative chromatogram for phosphate buffer without any drug and Figure 6 (b) shows the representative chromatogram of phosphate buffer with the drug. A distinct and resolved peak was observed for metformin with a retention time of 2.4 minutes.

**Figure 6:** Chromatograms a) Blank Sample and b) Metformin Sample
2.5.2. Linearity

USP defines the linearity of an analytical procedure as its ability to elicit test results that are directly or with some suitable mathematical modification, proportional to the concentration of analyte in samples within a given range. Thus, in the given HPLC method, linearity refers to the linearity in the relationship of concentration and peak height. Linearity was tested by injecting six different standard solutions over a range of concentrations. The calibration curve was obtained by plotting concentration of the standards with their corresponding peak height as shown in Figure 7.

![Figure 7: Standard curve for metformin over a range of 15.6-500μg/mL](image)

By calculating slope, y-intercept and spearman rank coefficient ($r^2$) using least square regression, linearity was determined for the given...
concentration range. The standard curve was linear over a concentration range of 15.6 – 500 μg/mL. The linear regression equation for metformin was $y = 1161.6x + 3174.3$, $r^2 = 0.9996$. As per USP the spearman rank coefficient ($r^2$) of >0.99 is acceptable for an analytical procedure. A high $r^2$ value suggests that there is a strong relationship between the peak heights over the given range of concentration.

2.5.3. Precision

According to the USP, precision of an analytical method expresses the degree of agreement between a series of measurements obtained from multiple sampling of a homogenous sample. The higher the precision, the closer are the values to each other on repeated measurements under identical experimental conditions. The precision of the current analytical method was assessed using both within day and day to day precision. For performing within day precision, a set of metformin standard solutions were prepared and injected four times on the same day. Day to day precision on the other hand was performed by injecting the standards on six different days over a period of thirty days. The relative standard deviation (RSD) values were calculated for both within day as well as day to day precision and were found to be within USP acceptable limits (<10%) as shown in Table 1.
**Table 1:** Within day and day to day precision for HPLC analysis of metformin

<table>
<thead>
<tr>
<th>Concentration (μg/mL)</th>
<th>Intra-day</th>
<th></th>
<th>Inter-day</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean peak height (x 10^3)</td>
<td>RSD (%)</td>
<td>Mean peak height (x 10^3)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>15.625</td>
<td>17.3 ± 0.7</td>
<td>3.98</td>
<td>18.5 ± 1.4</td>
<td>7.4</td>
</tr>
<tr>
<td>31.25</td>
<td>35.7 ± 1.0</td>
<td>2.67</td>
<td>37.3 ± 2.5</td>
<td>6.65</td>
</tr>
<tr>
<td>62.5</td>
<td>71.0 ± 2.4</td>
<td>3.42</td>
<td>74.0 ± 4.9</td>
<td>6.51</td>
</tr>
<tr>
<td>125</td>
<td>137.9 ± 4.4</td>
<td>3.22</td>
<td>151.4 ± 11.9</td>
<td>7.83</td>
</tr>
<tr>
<td>250</td>
<td>267.5 ± 8.7</td>
<td>3.24</td>
<td>295.6 ± 20.4</td>
<td>6.89</td>
</tr>
<tr>
<td>500</td>
<td>537.1 ± 5.3</td>
<td>0.98</td>
<td>572.70 ± 44.4</td>
<td>7.76</td>
</tr>
</tbody>
</table>

### 2.5.4. Accuracy

Per the USP, the accuracy of an analytical procedure is the closeness of test results obtained by that procedure to the true value. The accuracy of an analytical procedure should be established across its range. The accuracy of the HPLC method was determined by injecting three quality control samples of concentrations 100, 200, 400 μg/mL, six times along with the standard solutions for metformin over a period of thirty days. Accuracy of
the analytical method was calculated the percentage of concentration measured by the assay to the known added amount of analyte in the sample as shown in Equation 1. The accuracy of this method was found to be within acceptable USP limits and is depicted in Table 2.

\[
\text{Accuracy (\%)} = \frac{\text{Measured concentration}}{\text{Theoretical concentration}} \times 100
\]

\text{Equation 1}

Table 2: Accuracy results for the HPLC analysis of metformin

<table>
<thead>
<tr>
<th>Actual concentration((\mu g/mL))</th>
<th>Measured concentration ((\mu g/mL))</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>94.61 ± 4.49</td>
<td>94.47</td>
</tr>
<tr>
<td>200</td>
<td>196.61 ± 2.74</td>
<td>98.79</td>
</tr>
<tr>
<td>400</td>
<td>363.62 ± 31.66</td>
<td>90.91</td>
</tr>
</tbody>
</table>

2.6. Applications of the HPLC method

2.6.1. Determination of drug load in the nanoparticles

The drug load in the nanoparticles was determined using this HPLC method. Drug loading was calculated as the percent ratio of the amount of drug extracted from the freeze dried formulation (nanoparticles) to the total amount of freeze dried formulation as shown in Equation 2. The extraction of metformin from the nanoparticles was carried out in phosphate buffer (pH
7.4) and the amount of drug in the extract was determined by HPLC analysis.

\[ Drug \ Loading = \frac{Amount \ of \ drug \ extracted \ from \ the \ freeze \ dried \ formulation}{Total \ amount \ of \ freeze \ dried \ formulation} \times 100 \]

Equation 2

2.6.2. Determination of drug entrapment efficiency

The drug entrapment efficiency of the nanoparticles was calculated as the percent ratio of the amount of drug entrapped in the nanoparticles to the amount of drug initially added to the nanoparticles as shown in equation 3. The extraction of metformin from the nanoparticles was carried out in phosphate buffer (pH 7.4) and the amount of drug in the extract was determined by HPLC analysis.

\[ Drug \ Entrapment \ efficiency = \frac{Amount \ of \ drug \ entrapped \ in \ the \ nanoparticles}{Amount \ of \ drug \ initially \ added \ to \ the \ nanoparticles} \times 100 \]

Equation 3

2.6.3. In vitro release of metformin from the formulation

The in vitro metformin release from the nanoparticles was determined using this HPLC method. The in vitro release studies were carried out over 8
hours at 37°C in phosphate buffer at pH 7.4. Dialysis tubing (Biotech CE Dialysis Tubing, 1.0 kD MWCO) containing nanoparticles were kept in the release medium. These studies were performed in 20 mL glass scintillation vials set up on a shaker (Thermo Scientific MaxQ 4000 orbital shaker, Waltham, MA). For sample collection, 100 µL of the solution was removed at specific time intervals and was replaced with equal volume of fresh buffer. The metformin content in those samples was analyzed using the HPLC method.

2.6.4. Cellular uptake of metformin in Caco-2 cells

The cellular uptake of metformin from the solution and nanoparticulate delivery system containing metformin was evaluated in Caco-2 cell lines obtained from American Type Culture Collection (Manassas, VA). Caco-2 cells were cultured in a standard Falcon™ 24-well tissue culture plates and incubated until confluency in a humidified chamber at 37°C. Confluent cell monolayers were treated with a single dose of metformin solution or nanoparticles loaded with equivalent amount of metformin in Roswell Park Memorial Institute medium (RPMI). The cells were washed thrice with ice cold RPMI and lysed with 1% (v/v) triton-X-100. A portion of the lysate was analyzed for protein content using BCA assay which was used for normalisation during the calculation of cellular uptake. The remaining portion of the cell lysates was centrifuged at 13,000
rpm for 10 minutes at 4°C (Accu-spin MicroR, Fisher Scientific, Fair Lawn, NJ). The amount of metformin in the supernatant was determined by the HPLC method.

2.6.5. Permeability studies

Parallel artificial membrane permeation assay (PAMPA), was used as an in vitro model to assess the passive permeability of the nanoparticles. This model used a 96-well filter-based donor plate which was placed upon a 96-well acceptor plate. The studies were carried out over 8 hours at 37°C in phosphate buffer pH 7.4. Metformin solution and metformin loaded nanoparticulate suspension containing Lucifer yellow (in order to confirm the intactness of the membrane) was added to the donor well. The acceptor well contained phosphate buffer of pH 7.4. The assembly was placed on a shaker (Thermo Scientific MaxQ 4000 orbital shaker, Waltham, MA). For sample collection, 150 µL of the solution was removed at specific time intervals from the donor as well as the acceptor wells and analyzed using HPLC. For the simultaneous detection and quantification of metformin and Lucifer yellow from the samples, the above mentioned HPLC method was modified in terms of the detector wavelength. The column effluents were monitored at the detector wavelength of 234 nm (instead of 252 nm).

The results for drug loading, entrapment efficiency, in vitro release and permeability studies would be discussed in detail, in chapter 3.
2.7. Conclusions

A sensitive, specific and reproducible HPLC analytical method was developed and validated successfully for quantitative analysis of metformin using isocratic reverse phase HPLC. The method was capable of giving faster elution of metformin with a good resolution and accurately detected concentrations as low as 6.4 μg/mL. The results are within the acceptable USP limits when validated for accuracy, precision, linearity and specificity. Overall, a suitable analytical method was developed for rapid and accurate quantification of metformin in aqueous solutions in the presence of phosphate buffer.
Chapter 3

Preparation, characterization and *in vitro* evaluation of

metformin loaded hyaluronic acid nanoparticles
3.1. Introduction

This project was designed with the aim of increasing the oral bioavailability of metformin by preparing nanoparticles with mucoadhesive polymer like hyaluronic acid. Metformin loaded hyaluronic acid nanoparticles were prepared using nanoprecipitation technique. Particle size and surface charge of nanoparticle can play an important role in the permeation across the intestinal membrane. Therefore, particle size and zeta potential was measured for the system for the nanoparticles using Brookhaven particle size analyser. Electron microscopy (scanning and atomic force) was used to study the surface morphology as well as the particle shape of the nanoparticles. Physical state of the drug in polymer matrix can greatly affect drug release as well as the stability of the drug in the formulation. Physical state was determined using differential scanning calorimetry (DSC) and X-ray diffraction (XRD) methods. Presence of moisture can affect stability of the drug in the nanoparticulate system. The moisture content was determined by Karl Fischer titrimetry and confirmed using Thermogravimetric analysis (TGA). The amount of drug being entrapped in the system was determined using HPLC method as described earlier. In vitro release studies were performed to understand the release kinetics of metformin from this nanoparticulate system. The toxicity of this formulation was tested on Caco-2 cell lines using MTT (3-(4, 5- Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) cytotoxicity assay.
Nanoparticles smaller than 500 nm can enter the circulatory system [54]. Since the particles size of the nanoparticles was < 200 nm, blank and drug loaded nanoparticles were tested for their haemolytic potential by in vitro haemolysis assay. This assay evaluates hemoglobin release in the plasma (as an indicator of red blood cell lysis) following test agent exposure to red blood cells. Cellular uptake and permeability of the nanoparticles were analysed and the results were compared to that of drug solution. These studies were designed to understand the metformin permeation and absorption from nanoparticles as compared to the drug solution through the intestine.

3.2. Materials

Commercial grade of metformin was purchased from BioVision, Inc. (San Francisco, CA). Sodium Hyaluronate (MW=1.0×106 Da) was purchased from Bloomage Freda Biopharm Co., Ltd. (Jinan, China). Adipic Acid Dihydrazide (ADH), 1-[3-(Dimethylamino) propyl]-3 ethylcarbodiimide hydrochloride (EDC), Optima Grade Acetone and Acetonitrile for HPLC analysis were purchased from Fischer Scientific (Fair Lawn, NJ). Caco-2 cells were purchased from American Type Culture Collection (Manassas, VA). Dulbecco's Modified Eagle Media (DMEM), RPMI, Trypsin, penicillin/streptomycin, L-glutamine, sodium pyruvate, and non-essential amino acids were purchased from Cellgro Mediatech Inc.
(Corning, NY). MTT reagent, sodium dodecyl sulfate (SDS), sodium bicarbonate, magnesium chloride and dimethylformamide (DMF) were purchased commercially from Sigma Aldrich, (St. Louis, MO). Fetal Bovine Serum (FBS) albumin was acquired from Atlanta Biologicals, (Lawrenceville, GA). Whole human blood was obtained from Innovative Research Inc. (Novi, MI). PAMPA assay plates (donor and acceptor) were from Millipore Corporation (Billerica, MA).

3.3. Methods

3.3.1. Formulation of the delivery system

Blank and drug loaded nanoparticles were prepared by precipitation method. Briefly, 1% (w/v) sodium hyaluronate solution was prepared by overnight hydration of 750 mg sodium hyaluronate in 75 mL filtered (0.45 µM) and de-ionized water. EDC (34 mg) was added to the hyaluronic acid solution and stirred on a magnetic stirrer at 100 rpm for 30 minutes. Metformin hydrochloride (200 mg) was added to the above mixture and stirred on a magnetic stirrer at 100 rpm. Finally, ADH (30 mg) was added and the mixture was probe sonicated at 50 watts in a pulse mode (on time 1 sec, off time 0.5 sec) for 2 hours to ensure complete cross-linking. The assembly was placed in an ice bath during the sonication step to prevent overheating of the system. The cross-linked aqueous phase was added to 225 mL acetone (aqueous phase: organic phase; 1:3 v/v) with continuous
magnetic stirring. The colloidal dispersion was centrifuged (Accu-spin MicroR, Fisher Scientific, Fair Lawn, NJ) at 13,000 rpm and at 4°C for 10 minutes to isolate the nanoparticles. The organic solvent was evaporated under reduced pressure using a rotatory evaporator. The resultant suspension was lyophilized using a lyophilizer (Millrock Technology, Kingston, NY). Batches were prepared in triplicates. Figure 8 shows the blank (a) and the metformin loaded nanoparticles (b) obtained after lyophilisation.

![Figure 8: (A) Blank nanoparticles (B) Metformin nanoparticles](image)

3.3.2. Particle size and surface charge analysis

The particle size and the zeta potential were determined for the nanouspensions as well as nanoparticles obtained after freeze drying using the Brookhaven Zetameter (ZetaPlus, Brookhaven Instruments Corporation, Holtsville, NY). The dilutions were optimized before the actual measurement of particle size and zeta potential. For the particle size measurement 2 mL of nansuspension was diluted with 8 mL of acetone. In
case of the nanoparticles, 2 mg of nanoparticles were suspended in 10 mL of solvent system containing 2 mL of deionized water and 8 mL of acteone. For zeta potential measurement, 5 mg of nanoparticles were suspended in 10 mL of deionized water. For each sample, five readings were recorded and each reading was taken in triplicate (n = 3).

3.3.3. Determination of physical stability of the nanoparticles

For the physical stability studies, the blank as well as metformin loaded nanoparticles were stored at room temperature (25°C) and in a container containing desiccant (Drierite) at 0% relative humidity. The particle size and zeta potential were measured over a period of 65 days. The particle size and zeta potential of the nanoparticles were measured in triplicates at specific time points (0, 7, 14, 21, 28, 35, 65) as described earlier.

3.3.4. Electron microscopy

3.3.4.1. Scanning electron microscopy (SEM)

SEM was performed on the blank and metformin loaded nanoparticles at Xavier University, New Orleans, LA.

3.3.4.2. Atomic force microscopy

AFM was performed on the blank and metformin loaded nanoparticles at University of Nebraska Medical Centre, Omaha, Nebraska. For all the samples, freshly cleaved mica was used. The samples were
diluted with water 5 or 50 times from the original stock (1 mg/mL) solution. A droplet of a sample (5-10 µL) was immediately deposited on 1-(3-aminopropyl) silatrane (APS) modified mica for total of 2 min of incubation time. Excess samples were washed with de-ionized water and dried under a flow of Argon gas. AFM images in air were acquired using MultiMode AFM NanoScope IV system (Bruker Instruments, Santa Barbara, CA) operating in tapping mode with 1.5 Hz scanning rate. TESPA probes from Bruker were used for tapping mode imaging. The probes had a spring constant of about 40 N/m and a resonance frequency between 300 and 320 kHz.

3.3.5. Determination of the physical state of the drug in the nanoparticles

To determine the physical state (amorphous or crystalline) of metformin in the polymeric nanoparticulate matrix, DSC and XRD analysis were performed.

3.3.5.1. Differential scanning calorimetric studies

Sodium hyaluronate, pure drug, blank and drug loaded nanoparticles were analyzed using the DSC (Shimadzu, DSC 60, Kyoto, Japan). About 3 to 5mg of each sample was crimped in an aluminum pan, which was treated as the sample pan and was analyzed from room temperature to 300°C at a rate of 10°C using nitrogen purge (flow rate of 20 mL/minute). A separate
pan consisting of air crimped in an aluminum pan was used as the reference pan (n = 3). In another set of DSC study, blank nanoparticles were used in the reference pan.

3.3.5.2. X-ray diffraction analysis

XRD analysis was performed on the blank, the blank and metformin loaded nanoparticles at University of Minnesota, Minneapolis, MN. A powder X-ray diffractometer (D8 ADVANCE; Bruker AXS, Madison, WI) equipped with a Si strip one dimensional detector (LynxEye) was used. Samples were exposed to Cu Kα radiation (40 kV×40 mA) over an angular range of 5 - 40° 2θ with a step size of 0.02° and a dwell time of 0.5 seconds.

3.3.6. Thermogravimetric analysis

The weight loss as a function of temperature for blank as well as metformin loaded nanoparticles was analyzed by TGA (Shimadzu Thermogravimetric Analyzer, TGA 50, Kyoto, Japan). About 3-5 mg of sample was filled into aluminium pans and heated from room temperature to 300°C at a rate of 10°C using nitrogen purge (flow rate of 20 mL/minute). The percent weight loss was analyzed from 25°C to 120°C (n = 3).

3.3.7. Karl Fischer titrimetry

The moisture content in the lyophilized, blank and drug loaded nanoparticles was determined by Karl Fisher titration (Mettler DL18 Karl
Fischer titrator, NJ, USA). A biamperometric titration was carried out with about 25-30 mg of sample. Percent moisture content was determined for the known weight of the sample (n = 3).

3.3.8. HPLC analysis

The HPLC method was developed for determining metformin content in the nanoparticles. The chromatographic separation of metformin was achieved by isocratic elution on Phenomenex Luna C18, 5 μm (250x4.6 mm) column. The mobile phase composition was Acetonitrile: potassium phosphate buffer in water (40:60 v/v). The flow rate of 1 mL/min was maintained for a total run time of 6 minutes. The column effluents were monitored at the detector wavelength of 252 nm.

3.3.9. Determination of drug load in the nanoparticles

Drug load was calculated by the determination of the amount of the drug in the freeze dried nanoparticles to the weight of the nanoparticles. A 7.5mg sample of freeze dried nanoparticles was weighed and dispersed in 9 mL of extracting solvent, potassium phosphate buffer (pH 7.4). The dispersion was stirred for 24 hours by magnetic stirrer at 100 rpm. The dispersion was centrifuged at 13,000 rpm for 10 minutes at 4°C (Accu-spin MicroR, Fisher Scientific, Fair Lawn, NJ) to settle the polymer matrix. From the supernatant, a sample of 1 mL was withdrawn and filtered using 0.45 micron syringe filters and transferred into HPLC vials. The amount of
metformin in the sample was determined by HPLC analysis. Drug loading was calculated as:

\[
Drug \ Loading = \frac{\text{Amount of drug extracted from the dried formulation}}{\text{Total amount of dried formulation}} \times 100
\]

### 3.3.10. Determination of drug entrapment efficiency

The drug entrapment efficiency was calculated by determination of the amount of drug entrapped in the nanoparticles to the amount of drug initially added to the nanoparticles. Potassium phosphate buffer (pH 7.4) was used as an extracting solvent. A sample of 7.5 mg of drug loaded nanoparticles was dispersed in 9 mL of extracting solvent and stirred for 24 hours by magnetic stirrer at 100 rpm. The dispersion was centrifuged at 13,000 rpm for 10 minutes at 4°C (Accu-spin MicroR, Fisher Scientific, Fair Lawn, NJ) to settle the polymer matrix. From the supernatant, a sample of 1 mL was withdrawn and filtered using 0.45 micron syringe filters and transferred into HPLC vials. The amount of metformin in the sample was determined by HPLC analysis. Drug entrapment efficiency was calculated as:

\[
Drug \ Entrapment \ efficiency = \frac{\text{Amount of drug entrapped in the nanoparticles}}{\text{Amount of drug initially added to the nanoparticles}} \times 100
\]
3.3.11. **In vitro release studies**

The *in vitro* release of metformin from the freeze dried nanoparticles was performed in phosphate buffer solution (pH 7.4) at 37°C. Dialysis tubing (Biotech CE Dialysis Tubing, 1.0 kD MWCO, 16 mm Flat-width) was used as a sample holder. The dialysis tubing was cut into 4-5 cm pieces and wetted in phosphate buffer solution for 3 hours. A sample amount of 20 mg of nanoparticles dispersed in 2 mL of phosphate buffer solution was introduced into the dialysis bag using a blunted needle and a syringe. The dialysis bag was sealed from both ends using custom made dialysis bag clippers. The sealed dialysis bag was introduced in 17 mL phosphate buffer solution in a 20mL glass scintillation vial. The release assemblies were placed into an incubated shaker (Thermo Scientific MaxQ 4000 orbital shaker, Waltham, MA) for a total of 8 hours at a speed of 150 rpm and kept at a temperature of 37°C. Samples (100 µL) of the total volume of release medium (19 mL) were removed at various time points and analyzed using the HPLC analysis method as described previously. At definite time intervals (0, 5, 15, 30, 60, 90, 120, 180, 240, 300, 360, and 420 in minutes) samples were collected and replaced with equal volume of phosphate buffer solution (100 µL). Figure 9 shows the *in vitro* release
setup. The collected samples were analyzed on the HPLC using the method described previously.

![Image](image)

**Figure 9:** Set up for release studies

### 3.3.12. MTT toxicity assay

The cytotoxicity of the metformin solution, blank and metformin loaded nanoparticles was determined in Caco-2 cell lines using the MTT assay. DMEM media supplemented with 20% FBS, 10% L-glutamine, 10% sodium pyruvate, 10% nonessential amino acids and 10% penicillin streptomycin was used for growing Caco-2 cells. Twenty-four hours prior to treatment cells were seeded 100μL media/well into 96 well culture plates. The cells were incubated overnight in a humidified chamber with 5% CO₂ atmosphere at 37°C. Twenty-four hours after the original plating of cells, cells were treated different concentrations of three different treatments prepared in RPMI which were: treatment 1- metformin solution; treatment
2- metformin loaded nanoparticles containing equivalent amount of metformin of treatment 1; treatment 3- blank nanoparticles equivalent to the amount metformin loaded nanoparticles in treatment 2. The treatments were applied on top of existing media in equal volume to the media in the wells (100μL:100 μL) and allowed to incubate for 8 hours. Following the 8 hours of exposure time, the treatments and the media were removed, 100 μL fresh media was added to each well, and plates were returned to incubation chamber for 24 and 72 hours. After the incubation period, the cells were treated with 30 μL solution of MTT reagent (5 mg/mL) in phosphate buffer solution (pH 7.4) and incubated for additional 4 hours. The treatment was removed and the cells were lysed using 100 μL of lysing reagent, a solution of 20% (w/v) SDS: DMF in 1:1 ratio was used to solubilize the formazan crystals formed during the study. The plates were mixed on an incubated shaker (Thermo Scientific MaxQ 4000 orbital shaker, Waltham, MA) for 1 hour at 37°C and the absorbance was analyzed on a Thermo Multiskan MCC Unit microplate reader at 540 nm.

3.3.13. **Haemolysis assay**

Red blood cells (RBCs) were isolated from whole human blood by centrifuging it at 1000 rpm for 5 minutes (Thermo Scientific Sorvall® Legend RT, Waltham, MA). The supernatant serum fraction was discarded. The volume was raised up to the initial volume of the whole blood using 0.9
% (w/v) normal saline. The blood sample was centrifuged for 4-6 times until a clear supernatant was obtained. After the last cycle, the RBCs were diluted 1:10 with normal saline. This RBC solution was tested for haemolysis with drug solution (500 µM), drug loaded hyaluronic acid nanoparticles (500 µM) and blank nanoparticles (amount equivalent to drug loaded nanoparticles). The treatment volume was 200 µL of the RBC solution and 800 µL of the test solution. A 1% (v/v) solution of Triton-X in normal saline was used as a positive control whereas 0.9% (w/v) saline solution was used as a negative control. The treated RBCs were placed on the incubator shaker for 1 hour (Thermo Scientific MaxQ 4000 orbital shaker, Waltham, MA). After the incubation period, the samples were centrifuged at 13,000 rpm for 5 minutes on the microcentrifuge (Accu-spin MicroR, Fisher Scientific, Fair Lawn, NJ). The supernatant was collected and was analyzed on the microplate reader (Thermo Scientific Multiskan MCC, Waltham, MA) at 404 nm and 540 nm using normal saline as blank (n = 3). The percent haemolysis was calculated using the following formula Equation 4. The percent haemolysis was calculated as a mean of two wavelengths.

\[
\% \text{ Hemolysis} = \frac{\text{Sample absorbance} - \text{Blank absorbance}}{\text{Absorbance of positive control} - \text{Blank absorbance}}
\]

**Equation 4**
3.3.14. Determination of cellular uptake

The cellular uptake of metformin from the solution and nanoparticulate delivery system containing was evaluated in Caco-2 cells (epithelial cells from colon tissue). Caco-2 cells were cultured in a standard Falcon™ 24-well tissue culture plates at a seeding density of approximately 3x10^5 cells per well. Cells were incubated until confluency in a humidified chamber at 37°C. Confluent cell monolayers were treated with a single dose of 500 µL of metformin solution (1000 μM) or nanoparticles loaded with equivalent metformin in RPMI for 15 ,30, 45, 60, 90 and 120 minutes. The cells were washed thrice with ice cold RPMI and lysed with 200 µL of 1% (v/v) triton-X-100. The cell lysates were collected in microcentrifuge tubes, and 25 µL of the lysate was assayed for the total cellular protein content using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Remaining portion of the lysate was centrifuged at 13,000 rpm for 10 minutes at 4°C (Accu-spin MicroR, Fisher Scientific, Fair Lawn, NJ). The amount of metformin in the supernatant was determined by the HPLC method. The cellular uptake was reported as the mean ± SD of metformin content (in μg) per mg of total cellular protein (n = 3).

3.3.15. Permeability studies

PAMPA was used as an in vitro model to assess the passive permeability of the nanoparticles. This model consists of a 96-well
polyvinylidene fluoride (PVDF) filter-based donor plate which is placed upon a 96-well acceptor plate. The setup is represented in Figure 10.

Figure 10: Set-up for passive metformin diffusion through an artificial membrane [55]

One percent (w/v) solution of lecithin in dodecane was prepared and sonicated to ensure complete dissolution. Phosphate buffer pH 7.4 (300 µL) was added in to the acceptor wells (bottom). The PAMPA membrane was generated by adding 5 µL of the 1% (w/v) lecithin in dodecane solution to each filter in donor wells (top plate). Test samples consisted of metformin solution (1000 µM) or metformin loaded nanoparticles containing equivalent amount of metformin prepared in phosphate buffer pH 7.4 containing Lucifer yellow (1 µM). One fifty microlitres of the test samples was added to the donor wells (n = 9). The drug-filled donor plate was placed into the
acceptor plate ensuring the underside of the membrane was in contact with
the solution in the acceptor plate. Sealing tape was placed on the top of the
donor plate and the assembly was placed on an incubated shaker at 150 rpm
(Thermo Scientific MaxQ 4000 orbital shaker, Waltham, MA) to achieve
rapid equilibrium [56]. These studies were carried out over 8 hours at 37°C.
For sample collection, 150 µL of the solution was removed at specific time
intervals from the donor and the acceptor wells and analyzed using HPLC.
Equation 5 was used to for the calculation of the apparent permeability (Pe)
[55], where, (V_D) is the volume of the donor compartment, (V_A) is the
volume of the acceptor compartment, (Area) is the area of the membrane
times the porosity, (Time) is the incubation time, (C_A(t)) is the concentration
of the acceptor compartment after incubation, and (Ceq) is the equilibrium
concentration.

\[ P_e = \frac{V_D X V_A}{(V_D + V_A) X Area X Time} X \left\{ -\ln \left[ 1 - \frac{C_A(t)}{C_{eq}} \right] \right\} \]

Equation 5

The fraction of the compound that is retained in the membrane (R) is
calculated using Equation 6 [57], where (C_D(0)) and (C_D(t))are the
concentrations in the donor compartment before and after incubation,
respectively. Low R values suggest a lower tendency for accumulation in the
biological membranes and consequently a lower accumulation in fatty tissues.

\[ R = 1 - \left( \frac{C_D(t)}{C_D(0)} \right) - \left\{ \left[ \frac{V_A}{V_D} \right] X \left[ \frac{C_A(t)}{C_D(0)} \right] \right\} \]

Equation 6

3.3.16. Statistical data analysis

The experimental data was statistically analyzed for the purpose of comparison using a student’s t-test. The differences were termed statistically significant at P<0.05.

3.4. Results

3.4.1. Particle size and surface charge of the nanosuspensions

The particle size and zeta potential measurements were performed on blank and metformin loaded nanosuspensions and are shown in Table 3.

Table 3: Particle size and zeta potential of blank and drug loaded nanosuspensions (mean ± S.D; n = 3)

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Blank Nanoparticles</th>
<th>Metformin Loaded Nanoparticles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle Size (nm)</td>
<td>76.99 ±10.24</td>
<td>178.89 ± 20.45</td>
</tr>
<tr>
<td>Polydispersity Index</td>
<td>0.24 ± 0.02</td>
<td>0.11 ± 0.03</td>
</tr>
</tbody>
</table>
The data represented is the average of three samples. Significant difference (P<0.05) was observed in the particle size of blank and drug loaded nanosuspension.

### 3.4.2. Particle size and surface charge of the nanoparticles

The particle size and zeta potential measurements were also performed on blank and metformin loaded nanoparticles after freeze drying. The data in Table 4 represents the average particle size and zeta potential of three batches of nanoparticles.

**Table 4:** Particle size and zeta potential of blank and metformin loaded nanoparticles (mean ± S.D; n = 3)

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Particle Size (nm)</th>
<th>Polydispersity Index</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank Nanoparticles</td>
<td>155.52 ±18.70</td>
<td>0.30 ± 0.05</td>
<td>0.06 ± 0.34</td>
</tr>
<tr>
<td>Metformin Loaded Nanoparticles</td>
<td>114.53 ± 12.01</td>
<td>0.34 ± 0.01</td>
<td>-0.14 ± 0.39</td>
</tr>
</tbody>
</table>

There was a significant difference (p<0.05) between particle size of blank and drug loaded nanoparticles, however, both the types of nanoparticles were observed to be in the desired particle size range. Zeta potential of both blank as well as drug loaded nanoparticles was found to be in desirable neutral range.
3.4.3. Physical stability of the nanoparticles

The physical stability of blank and drug loaded nanoparticles at room temperature was monitored in terms of particles size and zeta potential over a period of 2 months. Particle size results for blank and metformin loaded nanoparticles are represented in Figure 11 and Figure 12. Although significant increase (p<0.05) in particle size was observed at certain time intervals, for blank as well as drug loaded nanoparticles, the particle size remained below 200 nm throughout the tested period. Figure 13 indicates the zeta potential for blank and metformin loaded nanoparticles. No significant increase was observed in zeta potential of blank as well as drug loaded nanoparticles.

Figure 11: Particle size results for blank nanoparticles at room temperature (n = 3), + Significant increase
**Figure 12:** Particle size results for metformin nanoparticles at room temperature (n = 3), + Significant increase

**Figure 13:** Zeta potential for blank and metformin loaded nanoparticles at room temperature (n = 9)
3.4.4. Electron microscopy

Representative SEM images of blank and metformin loaded hyaluronic acid nanoparticles have been shown in Figure 14 and Figure 15, respectively. Images of blank nanoparticles from AFM studies have been depicted in Figure 16 and Figure 17. The images of metformin loaded nanoparticles from AFM are shown in Figure 18 and Figure 19.

![SEM of blank hyaluronic acid nanoparticles](image)

**Figure 14:** SEM of blank hyaluronic acid nanoparticles
Figure 15: SEM of metformin loaded hyaluronic acid nanoparticles

Figure 16: AFM images of blank hyaluronic acid nanoparticles (dilution 5 times)
Figure 17: AFM images of blank hyaluronic acid nanoparticles (dilution 50 times)

Figure 18: AFM images of metformin loaded hyaluronic acid nanoparticles (dilution 5 times)
3.4.5. Physical state of the drug in the nanoparticles

3.4.5.1. Differential scanning calorimetric studies

An overlay of DSC thermograms of sodium hyaluronate, metformin hydrochloride, blank nanoparticles and metformin loaded nanoparticles analysed with blank aluminium pan as reference is shown in Figure 20. Metformin hydrochloride showed a sharp endothermic peak at 231.5°C. Sodium hyaluronate showed an exothermic peak at 234.81°C. An exothermic peak at 235.8°C was observed for blank nanoparticles. Sodium hyaluronate, blank as well as drug loaded nanoparticulate formulations
displayed broad endothermic peaks around 100°C. The DSC curve of drug loaded nanoparticles showed no other peak except the broad peak at 100°C.

Figure 20: An overlay of DSC thermograms of sodium hyaluronate, metformin hydrochloride, blank nanoparticles and metformin loaded nanoparticles analyzed with blank aluminium pan as reference

Another overlay of DSC thermograms, is shown in Figure 21. In this study, blank nanoparticles were used in reference pan for the analysis of metformin loaded nanoparticles. A sharp endothermic peak was observed at 234.2°C for metformin loaded nanoparticles.
Figure 21: An overlay of DSC thermograms of sodium hyaluronate, metformin hydrochloride, blank nanoparticles and metformin loaded nanoparticles analyzed with blank nanoparticles as reference.

3.4.5.2. X-ray diffraction analysis

An overlay of powder XRD patterns of blank nanoparticles and drug loaded nanoparticles is shown in Figure 22. An overlay of XRD patterns of blank nanoparticles and drug loaded nanoparticles along with reference pattern of the pure drug (metformin) is shown in Figure 23. The XRD analysis of metformin indicates a unique fingerprint pattern for the drug. The metformin loaded nanoparticles indicated several
identical characteristic peaks of metformin. These characteristic fingerprints of metformin were absent in the blank nanoparticles.

**Figure 22:** An overlay plot of XRD patterns of blank nanoparticles and drug loaded nanoparticles

**Figure 23:** An overlay of XRD patterns of blank nanoparticles and drug loaded nanoparticles along with reference pattern of the pure drug (metformin)
3.4.6. Thermogravimetric analysis

The TGA thermograms of lyophilized blank and drug loaded nanoparticles are shown in Figure 24. In case of lyophilized blank and drug loaded nanoparticles, a weight loss of 8.45 ± 0.72% and 7.20 ± 2.08% was observed, respectively, on heating up to 120°C. There was no significant difference (p<0.05) between weight loss of lyophilized blank and drug loaded nanoparticles.

![TGA thermograms](image)

**Figure 24:** TGA thermograms of the lyophilized blank and metformin loaded nanoparticles showing weight loss over a range of 25°C - 120°C
3.4.7. Karl Fischer titrimetry

The moisture content of the blank and drug loaded nanoparticles were found to be 13.85 ± 1.21% w/w and 6.81 ± 0.54% w/w, respectively, as determined by Karl Fisher titrimetry. The moisture content of blank nanoparticles was found to be significantly (p<0.05) higher than the drug loaded nanoparticles.

3.4.8. Drug load and drug entrapment efficiency of the nanoparticles

The drug load and drug entrapment efficiency of metformin nanoparticles as determined by HPLC was 22.84 ± 0.74% (w/w) and 91.4 ± 1.29% (w/w), respectively.

3.4.9. In vitro release of metformin

The in vitro release of metformin nanoparticles was studied over a period of 8 hours. Figure 25 represents the in vitro release profiles of metformin from nanoparticulate system and from metformin solution plotted as % cumulative release over time. The release pattern was similar in both the cases. A rapid release of metformin was seen from nanoparticulate system. In order to understand the mechanism of drug release from the formulation, the release data was plotted into several kinetic models. The best linearity was found by fitting the data in first order kinetic model as shown in Figure 26. The plot of log cumulative of % drug remaining vs.
time was found to be linear with $r^2$ value of 0.9851 for metformin nanoparticles.

![Graph](image)

**Figure 25:** *In vitro* release profile of metformin

![Graph](image)

**Figure 26:** A plot of log cumulative of % drug remaining vs. time representing the first order release kinetics
3.4.10. MTT toxicity assay

The percentage cell survival of the intestinal Caco-2 cells after treatment with metformin solution, blank nanoparticles and metformin loaded nanoparticles is depicted in Figure 27 and Figure 28. For the tested metformin concentration range (0.001-1000 µM), the dose response curve demonstrated no significant (p<0.05) cell death for the cells treated with metformin solution and blank nanoparticles at any point. Also, the cells treated with metformin nanoparticles at a concentration of ≤ 100 µM, demonstrated no significant cell death at any point. Highest concentration of metformin nanoparticles (1000 µM) caused significant cell death after 72 hours of incubation, but none at 24 hours.

![MTT assay on Caco-2 cells (24 hours)](image)

**Figure 27:** Cytotoxicity profile of metformin solution, blank nanoparticles and metformin loaded nanoparticles after 24 hours incubation


**Figure 28**: Cytotoxicity profile of metformin solution, blank nanoparticles and metformin loaded nanoparticles after 72 hours incubation (+ Significant cell death)

3.4.11. **Haemolysis assay**

The results of haemolysis assay at two wavelengths of detection (404 and 540nm) are shown in Figure 29. The mean percent haemolysis at the two wavelengths is presented in Table 5. Haemolysis by Triton X was used as a positive control and normal saline was read as negative control. The haemolysis caused by the drug solution and nanoparticles was lower than the vehicle control. There was no significant difference (P<0.05) in the haemolysis caused by blank and drug loaded nanoparticles.
Figure 29: Percent haemolysis at two wavelengths of detection (404 and 540nm) for drug solution, blank and drug loaded nanoparticles.

Table 5: Mean percent haemolysis of drug solution, blank and nanoparticles with Triton X as a positive control.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean % haemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Control</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>Negative Control</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Metformin Solution</td>
<td>-0.12 ± 0.13</td>
</tr>
<tr>
<td>Blank nanoparticles</td>
<td>-0.38 ± 0.03</td>
</tr>
<tr>
<td>Metformin nanoparticles</td>
<td>-0.29 ± 0.19</td>
</tr>
</tbody>
</table>
3.4.12. Cellular uptake of metformin

The *in vitro* cellular uptake of metformin in Caco-2 cell line following the treatment with metformin solution, blank nanoparticles and metformin loaded nanoparticles is presented in Figure 30. The total amount of metformin per mg of protein content taken up by the Caco-2 cells was significantly (p<0.05) higher in case of metformin loaded nanoparticles as compared to metformin solution up to first 45 minutes. The total amount of metformin per mg of protein content taken up by the Caco-2 cells from metformin solution was significantly (p<0.05) higher as compared to the uptake from metformin nanoparticles from 90 minutes and onwards.

![Cellular uptake of metformin](image)

**Figure 30:** The cellular uptake of metformin from the solution and nanoparticulate system in Caco-2 cells at different time points
3.4.13. **Permeability studies**

Effective permeability (Pe) and the fraction retained in the membrane (R) along with the acceptor and donor concentrations of metformin and metformin loaded nanoparticles at specific time points, that have been used for the calculation are represented in Table 6 and Table 7, respectively. The equilibrium concentrations (Ceq) of metformin and metformin loaded nanoparticles were found to be $41.6 \pm 3.82 \mu g/mL$ and $36.09 \pm 0.97 \mu g/mL$, respectively.

**Table 6:** Permeability data for metformin solution (n =9)

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Acceptor concentration (µg/mL)</th>
<th>Donor Concentration (µg/mL)</th>
<th>Pe (cm/hour)</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0</td>
<td>168.39 ± 1.31</td>
<td>0</td>
<td>-0.02 ± 0.01</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>169.95 ± 3.34</td>
<td>0</td>
<td>-0.03 ± 0.02</td>
</tr>
<tr>
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<td>170.64 ± 1.62</td>
<td>0</td>
<td>-0.03 ± 0.01</td>
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<tr>
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<td>169.86 ± 1.12</td>
<td>0</td>
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<tr>
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<td>171.44 ± 3.22</td>
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<td>-0.06 ± 0.02</td>
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</table>
Table 7: Permeability data for metformin loaded nanoparticles (n = 9)

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<tr>
<th>Time (hours)</th>
<th>Acceptor concentration (µg/mL)</th>
<th>Donor Concentration (µg/mL)</th>
<th>Pe (cm/hour)</th>
<th>R</th>
</tr>
</thead>
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<tr>
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<td>0</td>
<td>0.1 ± 0.03</td>
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<tr>
<td>30</td>
<td>0</td>
<td>147.08 ± 4.41</td>
<td>0</td>
<td>0.11 ± 0.03</td>
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<td>0</td>
<td>145.85 ± 5.05</td>
<td>0</td>
<td>0.12 ± 0.03</td>
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<tr>
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<td>144.49 ± 2.61</td>
<td>0</td>
<td>0.12 ± 0.02</td>
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<td>0</td>
<td>0.11 ± 0.01</td>
</tr>
</tbody>
</table>

3.5. Discussion

3.5.1. Formulation of the delivery system

The aim of this study was to increase metformin permeability and subsequently increase its oral bioavailability. Hence, we synthesized metformin loaded hyaluronic acid nanoparticles with a novel nanoprecipitation approach. Hyaluronic acid, as represented in Figure 31, is a naturally occurring linear glycosaminoglycan, biocompatible, nonimmunogenic and biodegradable [58]. Hyaluronic acid was the polymer of choice because: firstly, hyaluronic acid exhibits mucoadhesive properties in an intestinal environment, unlike chitosan, which does not show any
mucoadhesivity at a pH close to neutrality [60, 61]. These mucoadhesive properties of hyaluronic acid were found to increase residence time of the formulation on mucosa by producing an intimate contact with it resulting in an increased bioavailability [61]. Hyaluronic acid has also been associated with an increase in penetration across the Caco-2 cell monolayer [60], pointing to an occurrence of a deeper interaction between hyaluronic acid and the mucosal surfaces. Secondly, a polyanionic polysaccharide like hyaluronic acid cannot open the tight junctions between contiguous epithelial cells which has a an overall negative charge, unlike other mucoadhesive polycationic polymers like chitosan, which suggest that hyaluronic acid exhibits low toxicity to the intestinal epithelial cells and promises long-term safety for chronic diabetes treatment [40]. Thirdly, its solubility characteristics (soluble in water, insoluble in acetone) made it an ideal candidate for the nanoprecipitation method of nanoparticles synthesis. Lastly, hyaluronic acid nanoparticles could increase cellular uptake of metformin in intestinal epithelial cells via hyaluronic acid-CD44 interaction [58]. CD44 receptor is a structurally variable and multifunctional cell surface glycoprotein expressed on most cell types [62]. Thus, hyaluronic acid could be used as both the matrix and ligand. Therefore, the hyaluronic acid nanoparticles would be of low cost in comparison with either lectin or vitamin B_{12} conjugated nanoparticles. In order to couple and cross-link the hyaluronic acid molecules, EDC and ADH were used as coupling agent and
cross-linking agent, respectively. Figure 31 indicates the structure of EDC and ADH.

**Figure 31:** Structure of hyaluronic acid, ADH and EDC

Once metformin, hyaluronic acid, EDC and ADH were dispersed in the water, following reactions as illustrated in Figure 32 occurred: first hyaluronic acid and EDC react to form an O-Acylisourea intermediate, thereby converting the –OH of the carboxyl group of hyaluronic acid to a better leaving group and activating it towards the nucleophilic attack of ADH. It is then followed by a nucleophilic attack by ADH and formation of cross-linked hyaluronic acid nanoparticles.
This carbodiimide mediated cross-linking was continued by probe sonicating for 2 hours at room temperature until the formation of amide bonds between the carboxylic acid groups of glucuronic acid units of hyaluronic acid and the hydrazide groups of ADH. The power of the probe sonicator was optimized such that it ensured adequate continuous mixing of the mixture without overheating the system. The assembly was kept in an ice-bath to absorb the excess heat generated by the system. Figure 33 represents the final cross-linked hyaluronic acid molecules.
The cross-linking reaction was followed by precipitation step. For this, aqueous phase was added into acetone such that the volume of the system was now four times with respect to the initial aqueous phase. Water diffused in acetone caused the precipitation of insoluble hyaluronic acid nanoparticles. Metformin, which does not participate in the cross-linking reaction, is also insoluble in acetone, and hence is physically entrapped in the cross-linked hyaluronic acid molecules during this step. The colloidal dispersion was centrifuged to isolate the nanoparticles by settling down unreacted polymer and heavy particles. The organic solvent was evaporated.

**Figure 33:** Hyaluronic acid - ADH cross-linked nanoparticles
by using a rotatory evaporator. The dispersion was freeze-dried to obtain the nanoparticle powder.

### 3.5.2. Particle size, zeta potential and surface morphology

One of the important problems with lyophilization is the aggregation of nanoparticles which involves the formation and growth of tight clusters [63]. The process of aggregation is usually not reversible. This aggregation is primarily due to various stresses that the product encounters during freeze drying. This stress also leads to an increase in the particle size [64]. However, there was no significant (p<0.05) increase the particle size of metformin loaded nanoparticles after freeze drying, which could be attributed to non-occurrence of aggregation during the process of freeze drying.

Agglomeration is a process where the particles are loosely held together in a mass in solid state, and is easily reversible. The nanoparticles indicated formation of large agglomerates on freeze drying. However, these agglomerates were easily broken down into individual nanoparticles when dispersed in water, as indicated by the particle size analysis of such nanosuspensions.

For oral delivery, particle size of particulate system is an important factor governing the drug uptake and permeability across the intestine [65, 66]. Research has indicated that nanoparticles with a small particle size
(about 100 nm) have significantly greater uptake in Caco-2 cells as compared to larger particles (1 μm, and 10 μm) [67]. The obtained particle size of metformin loaded nanoparticles (114.53 ± 12.01 nm) could be optimal for cellular uptake phenomenon.

3.5.3. Physical stability of the nanoparticles

Physical instability (aggregation or fusion of nanoparticles) is a major obstacle that limits the use of nanoparticles [68]. The stability issues of drug nanoparticles could arise during the long periods of storage. Hence, metformin loaded nanoparticles were studied for their stability over a period of 65 days. Physical stability studies comprised of measuring the particles size and zeta potential. The particle size for both blank as well as drug loaded nanoparticles remained fairly constant in the size range of 100-200 nm at room temperature. The zeta potential was found to be neutral (± 1mV) throughout the course of the experiment suggesting that nanoparticles are physically stable over a period of two months. Retention of particle size is generally expected in case of solid nanoparticles. The reason behind this is that freeze drying converts the liquid nanosuspension into solid nanoparticles, thereby ensuring that there is no Ostwald ripening, which generally is the cause for physical instabilities in nanosystems [69].
3.5.4. Electron microscopy

As discussed previously, the nanoparticles formed loose agglomerates on freeze drying which dispersed easily into individual nanoparticles on the addition of water. When freeze dried solid samples were imaged using SEM, similar agglomeration was seen for the nanoparticles. In order to study the morphology of individual nanoparticles, a liquid sample containing nanoparticles in their dispersed state was analyzed using AFM. The AFM analysis indicated that the nanostructures were present in the form of nanofibres for both blank as well as metformin loaded nanoparticles. The particles size of the nanofibres, as indicated by AFM, was found to be between 100-200 nm. These particle size results are in good agreement with the results obtained from particle size analysis by Brookhaven zetasizer.

3.5.5. Thermal analysis, moisture content and X-ray diffraction

The sharp endothermic peak in DSC thermogram of metformin at 231.5°C corresponds to the melting of metformin. Both blank as well as drug loaded nanoparticulate formulations displayed diffused (broad) endothermic peaks around 100°C. To characterize these broad endothermic peaks obtained in the DSC thermograms, the weight loss of these formulations was analyzed up to a temperature of 120°C by TGA analysis. However, the weight loss could have been due to dehydration or desolvation. Therefore, Karl Fischer titration was performed to determine
the moisture content of these formulations. As there was a good degree of agreement in the % weight loss detected by TGA analysis and % moisture content determined by Karl Fischer titration, the broad endothermic peak in DSC thermograms of the formulations was confirmed to be from the moisture in the formulation. An exothermic peak was observed in blank nanoparticles around 235.08°C due to possible degradation of the polymer. The melting (endothermic) peak at around 231°C of metformin as well as the polymer degradation (exothermic) peak at around 234.81°C were absent in the thermogram of metformin loaded nanoparticles. As these were two opposite thermal events around the same temperature range, there is a possibility that the net effect is a result of cancellation of the opposite heat flow. In order to test this hypothesis and determine the physical state of the drug in the formulation, the DSC analysis of metformin loaded formulations was performed using blank nanoparticles as the reference. In such a case, any difference in heat flow would be due to presence of drug. When blank nanoparticle was used as reference material for DSC analysis, the metformin loaded nanoparticles showed an endothermic peak around 234.2°C indicating the melting of metformin. The powder XRD studies showed metformin fingerprints in the drug loaded nanoparticle which were absent in blank nanoparticulate system. This confirms that the drug (metformin) was present in a crystalline state in drug loaded nanoparticulate systems. These observations clearly indicate that data obtained from DSC of polymeric
nanoparticle needs careful evaluation and interpretation otherwise the results could be misleading.

3.5.6. HPLC analysis

The HPLC method was developed and validated for the detection of metformin from phosphate buffer (pH 7.4). The retention time of metformin was 2.4 minutes. The samples were quantified by analyzing the metformin peak of the chromatogram. The method was validated and was used for analyzing metformin content to determine drug load and entrapment efficiency, in vitro drug release and cellular drug uptake.

3.5.7. Drug load in metformin nanoparticles

Theoretical drug load of metformin with respect to its excipients was 19.72% (w/w) which was significantly less (p<0.05) compared to the experimental drug load of 22.84 ± 0.74% (w/w). Since drug load is indicative of the ratio of drug to its excipients, this difference in drug load could be due to the loss of excipients and not drug, during the multi-step preparation process, most likely during the centrifugation of the nanoparticulate dispersion.

3.5.8. Drug entrapment efficiency

During the nanoparticle preparation, hyaluronic acid nanoparticles were dispersed in an organic phase in which both hyaluronic acid and metformin were insoluble. The excess organic solvent was removed from
the system by rotatory evaporator. Thus, there was minimal loss of the drug during the process resulting in a formulation with more than 90% of metformin content. This high metformin content could be due to drug entrapment as well as due to the presence of drug in free or adsorbed form in the system.

3.5.9. *In vitro release of metformin*

*In vitro* release studies were performed on metformin loaded hyaluronic acid nanoparticles using phosphate buffer (pH 7.4). The release pattern of drugs from a system is largely governed by factors like drug properties, nature of the polymers used, and drug polymer interactions. As discussed previously, metformin is a highly hydrophilic drug (log p -2.64) which is freely soluble in water (50 mg/mL). The release of metformin from hyaluronic acid was observed over a period of 8 hours after which the release seemed to be complete and had reached a plateau. The release is seen to be rapid as exhibited by high metformin release (over 90% (w/w)) in first 2 hours. The release of the drug from the particles is seen to occur within 5 minutes of the beginning of the study. This quick release could be due to the presence of free as well as adsorbed drug on the surface of nanoparticles. The high hydrophilic nature of metformin as well as the aqueous medium used for these studies could also contribute to the rapid drug release. Even though hyaluronic acid does not completely solubilize in water, it behaves as
a porous matrix for release of entrapped metformin. Thus, another possible explanation for the rapid release of the drug could be the tendency of the hyaluronic acid to swell in aqueous media [70, 71] which might lead to an increased water penetration into the matrix. The first order kinetics of drug release from this formulation indicates that the release of the drug is governed by one factor, which is probably the concentration of metformin present in the matrix.

3.5.10. MTT toxicity assay

The MTT toxicity assay on Caco-2 cells indicates the higher toxicity of the metformin loaded nanoparticulate system as compared to the metformin solution or blank nanoparticles at higher concentration. The increased toxicity of metformin nanoparticles may be attributed to the higher accumulation of metformin within the Caco-2 cells as compared to the solution. The blank nanoparticles indicated no toxicity even at highest concentrations up to 72 hours. This suggests that the hyaluronic acid nanoparticulate formulation by itself is safe and non-toxic. The percent survival of Caco-2 cells was found to decrease with time for treatment with highest concentration of metformin loaded formulation. The percent cell death was significant (p<0.05) only after 72 hours of incubation, and not at 24 hours, suggesting apoptosis caused by metformin in Caco-2 cells.
3.5.11. **Haemolysis assay**

Haemolysis assay was performed on drug solution, blank and drug-loaded nanoparticles. No haemolysis was observed in blood samples treated with nanoparticle suspensions of both kinds. The percent haemolysis by the nanoparticles was lesser than the vehicle indicating no haemolysis from nanoparticles. This study ensured that the formulation was safe in the event of its entry into the circulatory system. Additionally, it confirms that this nanodelivery system could be administered intravenously, if necessary.

3.5.12. **Cellular uptake of metformin**

The cellular uptake of metformin from the solution and nanoparticulate delivery system was investigated at different time points. The results indicate that the uptake of metformin from the nanoparticulate system was significantly higher compared to that from drug solutions at p<0.05, up to 45 minutes. The maximum metformin uptake from the nanoparticles was found to be at 30 minutes, after which it somewhat decreased with time. The trend observed during uptake studies showed a gradual increase, followed by a decrease in the amount of drug uptake by the cells, with respect to time for the drug loaded nanoparticles, which could possibly be due to the rapid drug release from the nanoparticles (>50 % in 1 hour).
3.5.13. Permeability studies

PAMPA is a widely used *in vitro* model for evaluating GIT permeability as it has a high correlation with the *in vivo* permeability studies [72]. The compound of interest can passively permeate through the membrane from the donor to the acceptor plate. The PVDF filter was converted to an artificial membrane by the application of 1% (w/v) lecithin in dodecane solution. Lecithin is a mixture of amphiphilic molecules that have hydrophilic heads and lipophilic tails. The amphiphilic molecules in lecithin are arranged uniformly over the filter support forming a membrane-like phospholipid bilayer, thereby mimicking membrane phospholipids in mammalians [73]. One of the shortcomings of using this membrane is that it can mimic passive diffusion only. Since it does not have transporters/receptors embedded in the filter membrane, it cannot transport molecules by active transport. As stated previously, metformin requires active transporters for its passage through a membrane [74, 75]. However, since metformin nanoparticles have drug entrapped inside the polymeric system, and have an overall neutral charge, it is not necessary that the nanoparticles would require active transport (like metformin) to permeate across the membrane. In fact, studies have asserted the ability of nanoparticles to increase the absorption of a drug, by increasing its uptake via passive diffusion [76, 77]. Hence, in order to test our hypothesis that formulating metformin in the form of neutral nanoparticles could possibly
lead to an increased permeation by passive diffusion, thereby bypassing the saturable active transport mechanism of the drug molecule, PAMPA was chosen as an in vitro model.

The R values (<0.1), for metformin solution as well as nanoparticles, indicate that there was no drug retention in the membrane. The Pe values for metformin solution suggested an absence of passive permeation for metformin. These results are consistent with the conclusions of previous studies which have asserted that metformin uptake in the intestine is primarily governed by active transport involving organic cation transporters and not by passive transport mechanism. The Pe values for metformin loaded nanoparticles indicate an absence of passive diffusion for metformin nanoparticles as well. A possible explanation for this could be lack of mucin in the in vitro model used. Intestinal epithelium is lined with a mucous layer. Mucoadhesive polymers like hyaluronic acid have been known to interact with mucus layer and increase the residence and contact time of nanoparticles with the epithelium [78, 79]. Mucoadhesive materials can act as permeation enhancers by reversibly opening tight junctions to enhance paracellular transport [60, 43]. Moreover, the increased concentration of drug at the absorption site due to the mucin binding effect of the polymer results in an increased oral bioavailability and reduces the pharmacokinetic variability [39].
The results of this study emphasize a need for the presence of mucin to simulate more accurate in-vivo conditions in the permeation testing model for characterizing mucoadhesive hyaluronic acid nanoparticles. Alternative models such as Caco-2 cells [80], rat intestine [81], and porcine intestine [82], can be used to provide a better simulation of the in vivo conditions.

3.6. Conclusions

Hyaluronic acid nanoparticles containing metformin were developed and characterized. The particle size of the lyophilized nanoparticles, tested by dynamic light scattering, showed a uniform size distribution and was found to be less than 200 nm which was desirable for nanoparticulate delivery for enhancing GI permeability. The results from electron microscopy indicated that the particles were present in the form of nanofibres and were in good agreement with the results obtained from particle size analysis using Brookhaven zetasizer. The blank as well as drug loaded particles were found to be physically stable over a period of 2 months. The drug was present in crystalline state in the nanoparticulate system with low moisture content. The quantitative estimation of metformin in the nanoparticles was successfully done using a HPLC method. Metformin content in the nanoparticulate system was found to be high (>90%). The release of metformin from the nanoparticles was found to be rapid. The metformin loaded nanoparticles were found to be non-toxic at
therapeutic concentrations (≤ 100µM). The haemolysis assay indicated that the formulation does not produce haemolysis of red blood cells. The artificial membrane permeation assays indicated lack of passive diffusion for metformin solution as well as metformin nanoparticles, entailing the need for further evaluation with more bio-relevant membranes containing mucin.
CHAPTER 4

Summary, global impact and future directions
4.1. Summary

Hyaluronic acid nanoparticles were successfully prepared by nanoprecipitation method. Metformin, an anti-diabetic agent was incorporated into the mucoadhesive hyaluronic acid polymeric system. This approach was designed to improve the oral bioavailability of metformin by increasing its permeability across intestinal epithelium. The nanoparticles were formulated with the aim of delivering them to the intestine via oral delivery.

First, a sensitive HPLC method was developed and validated for the accurate and precise detection of metformin content as low as 6.4 μg/mL. This method allowed detection of the effluents at a rapid rate thereby minimizing the use of organic solvents. This HPLC method was further used for metformin quantification in drug content analysis, in vitro drug release, cellular drug uptake, and permeability studies.

The nanoparticles were synthesized by nanoprecipitation and yielded water dispersible metformin loaded hyaluronic nanoparticles in the size range of 150-200 nm, which is estimated to be optimum for permeation across the intestinal epithelium [67]. The particle size and the neutral zeta potential was found to be stable over a period of 2 months. The AFM images confirmed the particle size of the nanoparticles. Further, it revealed that the particles were present in the form of nanofibres. DSC and XRD
analysis demonstrated that the metformin existed in a crystalline state in the nanoparticles. By using blank nanoparticles as a reference in the DSC studies, the present research work illustrated an example of the fact that absence of any thermal events in DSC thermograms sometimes can be misleading if two opposite thermal events occur at the same temperature range in a formulation. The results of water content analysis by Karl Fischer titrimetry were in good degree of agreement with the % weight loss as determined by TGA indicating a moisture content of <10% (w/w). The metformin content in the system was as high as >90% (w/w) indicating minimal drug loss during the nanoparticle preparation.

The in vitro release studies showed a rapid drug release of metformin from the polymer matrix with more than 90% of metformin release over 2 hours. Metformin nanoparticles indicated cellular toxicity on Caco-2 cells at a concentration of 1000 µM, however, they were found to be non-toxic at therapeutic concentrations (≤ 100 µM). In Caco-2 cells, metformin uptake was higher from metformin nanoparticles as compared to that from metformin solution up to first 45 minutes. The haemolysis assay showed that the system does not cause RBC lysis, and thus, is safe for oral as well as intravenous administration. The permeability results indicate the absence of passive diffusion of mucoadhesive metformin nanoparticles across the synthetic membrane model in the absence of mucin necessitating the need of
further *in vitro* studies with a more bio-relevant environment to provide higher *in vivo* correlation.

4.2. Global impact

Globally, 422 million adults were affected by diabetes in 2014 and type 2 diabetes accounted for 90-95% of these cases. The ever increasing numbers of new cases is a cause for concern. Metformin is a highly effective first line drug for type 2 diabetes therapy. However, it has a poor permeability and administered in high dosage. Also, it has been associated with side effects like gastric irritation, nausea, weight loss, and diarrhea, etc. A formulation which could improve the permeability of metformin could lead in drug dose reduction, potentially reducing the side effects, and thereby increasing patient compliance. Also, improvement in permeability by this mechanism is not limited to metformin. After suitable *in vitro*, pre-clinical and clinical testing of metformin loaded nanoparticles, this system may also provide a new direction for improving permeability of other BCS III drugs which have a high solubility, but are limited in their action by their poor permeability.

4.3. Future direction

In order to improve the adherence to and permeation through the intestinal epithelium, targeting ligands like vitamin B_{12}, bacterial adhesins, IgA antibodies etc. can be conjugated to the nanoparticle surface [38]. These
ligands could potentially increase the permeability of the particles by their specific mechanism. For example, vitamin B$_{12}$ conjugated nanoparticles can be taken up by the formation of vitamin B$_{12}$–IF complex, which interacts with an IF receptor that traffics the complex across the intestinal epithelium [83]. Bacterial adhesins, IgA antibody conjugated nanoparticles can be transported by the transcytosis pathway used by M cells to transport antigens across the intestinal barrier [84].

The nanoparticulate system designed in this study entrapped metformin, an anti-diabetic agent as a drug. However, since it was entrapped physically into the system and not conjugated chemically, other water soluble drugs can be easily incorporated into this hyaluronic acid nanoparticulate polymeric system. The present study dealt with the loading of nanoparticles with one anti-diabetic agent. Future studies may include incorporation of more than one anti-diabetic agent in the nanocarriers.

Hyaluronic acid is considered to be mucoadhesive polymer, and it is important for the system to be mucoadhesive which helps in retaining the drug at the absorption site for a longer period of time and act as a permeation enhancer. Hence, these nanoparticles could be further analysed to test their mucoadhesivity on Caco-2 cells or rat or porcine tissue.

Although the prepared nanoparticles dispersed easily when suspended in water, they were found to have a tendency to agglomerate in their powder form after freeze drying. Typically, dry powder formulations include
excipients to improve their powder flow properties performance. Excipients could be added to the formulation to reduce the particle agglomeration.

The nanoparticles prepared in this study were tested for their permeability on synthetic membrane. In order to get a better understanding of the effect of particles on drug permeability, nanoparticles should be tested for their permeability across the Caco-2 cells monolayer or on rat or porcine intestinal tissue.
Bibliography


