Development of Biorelevant In Vitro Release Testing Methods for Periodontal Microparticles

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Dissolution testing is one of the primary methods employed not only as a quality control tool but also to evaluate bioequivalence and assess the effect of process and formulation parameters on drug release. Currently, there is no compendial-level method to assess dissolution of particulate systems administered in the periodontal pocket. Therefore, the goal of this work is to develop and demonstrate the utility of dissolution methods for microparticles applied in the periodontal pocket. Arestin®, a clinically used extended release periodontal system was used as the reference product. It is composed of poly(lactic-co-glycolic acid) (PLGA) microparticles loaded with an antibacterial drug, minocycline hydrochloride (MIN). Two methods were evaluated in this study. The first method utilized a standard USP IV apparatus modified to include a dialysis tube with dispersed microparticles, which was developed as a rapid screening method. In the second method, a novel apparatus was designed by our group to simulate the in vivo environment of the periodontal pocket. The applicability of these methods was evaluated by rigorously testing for reproducibility and discriminatory ability. To test the discriminatory ability of the developed methods, a panel of MIN-loaded PLGA microparticles that differed in composition and process conditions were utilized. The method utilizing the USP IV apparatus was equipped with online UV-analysis and was carried out at a flow rate of 10mL/min for 3 days. Microparticles that showed different dissolution profiles in this method were tested in the novel, more biorelevant small volume apparatus, which was designed to hold 250µL of gingival crevicular fluid simulant (sGCF), where the particles are
dispersed. sGCF was continuously delivered to the device at a biorelevant flow rate and collected daily for dug content analysis using a stability-indicating UPLC method. Both the developed methods could discriminate between Arestin® and the comparators. Release of MIN was largely dependent on molecular weight of PLGA used, with higher molecular weight showing higher release. The methods evaluated in this work can be used in routine quality control analysis to detect batch-to-batch variability. Future studies can evaluate the applicability of the developed methods to assess bioequivalence of MIN-loaded periodontal systems.
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Preface

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1.0 Introduction

Dissolution is the process by which a solute in a solid, liquid or gaseous state dissolves in a solvent, yielding a solution [1]. In the pharmaceutical context, dissolution is the process by which the drug in a drug product dissolves in body fluids and becomes available for absorption. There are a variety of factors that affect the rate and extent of dissolution. Dissolution testing is a pivotal quality control test that assesses the impact of formulation and process variables on the drug product. In addition, dissolution testing has implications for better design of drug products and prediction of \textit{in vivo} performance. This chapter discusses the concept of dissolution testing, followed by the applications of dissolution testing, development of dissolution methods and ultimately the currently used methods for dissolution testing of particulate systems.

1.1 The Concept of Dissolution Testing

The concept of dissolution testing was first explored at the end of the 19\textsuperscript{th} century. However, the use of \textit{in vitro} dissolution testing methods in the pharmaceutical industry gained importance only in the mid-20\textsuperscript{th} century [2]. Since then, dissolution testing has experienced a phenomenal increase in importance and is currently one of the most important characterization tests employed in the pharmaceutical industry. With around 84 \% of the top 50 drug products in the US and European markets administered orally [3], dissolution testing methods for tablets and capsules are understandably the most advanced. However, with the advent of a vast number of novel drug delivery systems, development of dissolution testing methods for modified-release
dosage forms has gained increased recognition. Interest has concurrently developed in correlating in vitro dissolution data to in vivo product performance, creating the need for development of predictive in vitro dissolution tests.

According to the International Union of Pure and Applied Chemistry (IUPAC), the rate of dissolution can be defined as the change in concentration of a dissolved substance over a certain period of time [4]. The concept of rate of dissolution was first introduced in 1897 by Arthur A. Noyes and Willis R. Whitney [5]. They studied the dissolution of two sparingly soluble salts in water and observed that the rate of dissolution of a compound is proportional to the difference between the instantaneous concentration of the solution and the saturation solubility. This observation, stated mathematically, forms the Noyes and Whitney equation (Equation 1).

\[
\frac{dC}{dt} = k(C_s - C_t)
\]  

(1)

Where,

\(dC\) = change in concentration over time \(dt\)

\(C_t\) = concentration at time \(t\)

\(C_s\) = saturation solubility of substance

\(k\) = constant

Noyes and Whitney proposed the mechanism of dissolution to the formation of a thin layer of unstirred liquid forming around the particle. They called this the diffusion layer and particles were proposed to diffuse from the diffusion layer to the bulk fluid [5] . To quantify the diffusion that occurs through the diffusion layer, Fick’s first law of diffusion was used [6] (Equation 2).

\[
\frac{dM}{dt} = -SD \frac{dC}{dx}
\]  

(2)

Where,

\(dM\) = mass diffused in time \(dt\)
As reviewed by Dokoumetzidis, A. and Macheras, P., the next development in the field took place at the dawn of the 20th century. In 1900, Erich Brunner and Stanis-laus von Tolloczko proposed further conditions under which Equation 1 holds [2]. According to their paper, properties of the exposed surface, including surface area and structure, rate of agitation, temperature and arrangement of the apparatus influenced the rate of dissolution. They modified Equation 1 to include a term for exposed area (S) available for dissolution (Equation 3).

\[
\frac{dC}{dt} = k_1 S (C_S - C_t)
\]  

(3)

In 1904, Erich Brunner and Nernst Walther combined the diffusion layer theory with Fick’s first law of diffusion (Equation 2) to give the Nernst-Brunner equation (Equation 4) [6].

\[
\frac{dC}{dt} = \frac{D S}{V h} (C_S - C_t)
\]

(4)

Where,

\[V = \text{volume of dissolution media}\]

\[h = \text{thickness of diffusion layer}\]

In the above equations, surface area is considered to be constant during the experiment. However, in most practical cases, the surface area of a particle reduces as it continues to dissolve in a liquid [6]. In 1931, A. W. Hixson and J. H. Crowell studied the dissolution of solids under perfect sink conditions [7]. Under sink conditions, the change in concentration of the bulk fluid is negligible and thus can be considered to be a constant. Incorporating these changes in Equation 2,
the diffusional mass transport can be considered to be a function of the surface area, \( S \) (Equation 5).

\[
\frac{dM}{dt} = -k_2S_t
\]  

(5)

Where,

\( S_t \) = surface area of the particle at time \( t \)

For a perfectly spherical particle, the surface area \( S \) is proportional to its mass, \( M^{2/3} \). Substituting surface area for mass in Equation 5 and integrating the resulting equation gives Hixson-Crowell’s “cube root law” (Equation 6) [7].

\[
\sqrt[3]{M_t} = \sqrt[3]{M_0} - k_3t
\]

(6)

Where,

\( M_t \) = mass of substance at time \( t \)

\( M_0 \) = initial mass of substance

This equation holds true for non-disintegrating, perfectly spherical particles undergoing dissolution under sink conditions. The Hixson-Crowell equation is one of the most commonly used equations to model drug release from pharmaceutical dosage forms. However, application of the equation becomes challenging as it is difficult to provide perfectly sink conditions and have non-disintegrating dosage forms in a practical setting. Thus, various other models have been developed over the years in order to model different types of dosage forms.

Although the concept of \textit{in vitro} dissolution had advanced considerably in the first half of the 20\textsuperscript{th} century, its applications were limited to the field of chemical engineering [2]. During this time, the \textit{in vivo} availability of the drug was thought to be determined by the disintegration time of tablets [8]. However, a number of bioavailability concerns were raised in the 1950s. These incidences brought to light the insufficiency of disintegration to test the equivalence of two
products [9-11]. The importance of in vitro dissolution testing as a quality control tool was recognized in 1970, when dissolution testing was adopted as a routine quality control test in 6 monographs in the United States Pharmacopoeia (USP) and National Formulary (NF) [2].

According to the USP, a dissolution test evaluates the rate and extent to which a compound (drug) forms a solution (dissolves) under a predetermined set of carefully controlled conditions [12]. There are multiple variables which need to be considered while developing a dissolution method. These are mentioned in the USP general chapter on development and validation of dissolution methods [13] and would be highlighted later in the document. As with any other analytical method, a dissolution method should be robust, reproducible and discriminatory in nature. However, with the increasing applications of in vitro dissolution testing, new paradigms are being set to define an ideal dissolution test.

1.1.1 Characteristics of an Ideal Dissolution Test

The ideal characteristics of a dissolution test vary according to their intended application. In general, dissolution tests are expected to be discriminatory in nature, robust and reproducible [14]. Dissolution tests intended to be used for predicting in vivo release should display certain level of correlation.

1.1.1.1 Discriminatory Potential

One of the core characteristics of a dissolution test is its discriminatory potential. A dissolution method should be able to differentiate between products that are not equivalent to one another. However, the method should not be so sensitive that it discriminates between products that are clinically equivalent [15]. The discriminatory potential of a dissolution method is
influenced by the type of apparatus, media composition, temperature and stir/flow rate. Of note, the criteria chosen to decide equivalence also determines the discriminatory potential of a method. For example, in Figure 1 the test is discriminatory when the one-point acceptance criteria states that more than 90% of the drug should dissolve within 90 minutes. However, the same test is non-discriminatory when the acceptance criteria states that more than 90% of the drug should be dissolved at 120 minutes.

![Figure 1. Representative Figure Showing Dependence of Discriminatory Potential on Acceptance Criteria](image)

1.1.1.2 Robustness

Robustness is the ability of the dissolution method to remain unaffected by small, deliberate changes made to the method [16]. Like any other analytical procedure, a dissolution test should be robust and not be sensitive to small changes in method parameters. There should not be
differences in results obtained by different labs or analysts. The dissolution method should not be sensitive to small changes in rate of agitation, media composition and temperature. [15].

1.1.1.3 Transferability/ Reproducibility

The method should be simple enough to enable adoption by different laboratories [14]. The results should be independent of the source of materials, quality of solvent and brand of equipment. Anyone with the standard operation procedure (SOP) for the method should be able to run the test provided they have the required equipment at hand. Easy transferability of the test becomes important as it is likely that the same dissolution method would be used for routine quality control analysis in the future.

1.1.1.4 Ability to Predict In-vivo Response

*In vitro* dissolution tests are one of the major characterization methods used to simulate *in vivo* drug release. Ideally, there should be a strong correlation between a dissolution test and *in vivo* response (absorption/plasma drug concentration) of the drug product. Thus, an *in vitro* test could serve as a predictor of *in vivo* bioavailability [17]. Use of biorelevant media, reservoir volumes and agitation rates that mimic *in vivo* environments help in enhancing the predictive power of dissolution tests. As we will see further, a dissolution test which predicts *in vivo* response plays an important role in determining bioequivalence. The use of predictive dissolution testing in obtaining biowaivers increases its importance exponentially.
1.1.2 Applications of Dissolution Testing

Dissolution testing has evolved from being a routine quality control method to an important evaluation technique used in the pharmaceutical industry. Reliable dissolution tests find applications in various areas of the product life-cycle, from drug development and optimization to quality assurance post marketing, as described below.

1.1.2.1 Product Development

An important role of in vitro dissolution testing in the early stages of drug development is in product development. A good dissolution test can discriminate between products that are different due to differences in formulation and/or manufacturing conditions. Thus, dissolution testing is used to screen products prepared using different excipients and using different processing parameters. Drug products that show superior and reproducible dissolution profiles are further optimized to produce a marketable formulation [18]. The use of in vitro dissolution testing in product development has become more important as manufacturers are shifting to developing products using the quality by design (QbD) approach. For example, dissolution testing can provide information on the effect of process parameters on product performance. Such knowledge can be utilized to determine a design space, as explained by Dickinson, P. A. and colleagues [19]. Use of a design space reduces the regulatory burden on manufacturers and provides them some flexibility in modifying manufacturing conditions post approval.

1.1.2.2 Routine Quality Control Analysis

The most common use of a validated dissolution method is for ensuring batch-to-batch reproducibility. The Food and Drug Administration (FDA) requires dissolution testing to be
performed on all batches before they are released to the market. In case the produced batches fail to meet the acceptance criteria for dissolution, the batches will be rejected. Dissolution testing is also a required characterization test for assessing shelf-life stability of drug products [20]. If a stability sample fails in the dissolution test, the batch must be recalled from the market.

1.1.2.3 Biowaiver Application for Drugs containing Lower Strength of Actives

Another important application of dissolution testing is for the request of biowaivers. If a manufacturer has got an approval for a higher strength of dosage form, in vivo bioequivalence studies for lower strength products can be waived [21]. Such biowaivers are accepted if the relative ratios of API-to-excipients of the lower strength product and approved product are similar. Instead of single-point comparisons routinely used in the industry, biowaiver applications require dissolution profile comparisons. The dissolution profiles are compared at each timepoint for similarity and differences. If the difference factor \( f_1 \) between the two dissolution profiles is below 15 (0 – 15) and the similarity factor \( f_2 \) is above 50 (50 – 100), the dissolution profiles are said to be similar and in vivo bioequivalence studies can be waived. The \( f_1 \) and \( f_2 \) values can be calculated as per equations 7 and 8 respectively.

\[
f_1 = \left[ \frac{\sum_{t=1}^{n} |R_t - T_t|}{\sum_{t=1}^{n} R_t} \right] \times 100 \tag{7}
\]

\[
f_2 = 50 \times \log\left[ \frac{1}{\sqrt{\left[ \frac{1}{n} \sum_{t=1}^{n} (R_t - T_t)^2 \right]}} \right] \times 100 \tag{8}
\]

Where,

\( n \) = number of time points

\( R_t \) = dissolution value of the reference at time \( t \)

\( T_t \) = dissolution value of the test at time \( t \)
1.1.2.4 Scale-Up and Post Approval Changes

The FDA allows for *in vitro* dissolution testing data to substitute for *in vivo* bioequivalence tests for most scale-up and post approval changes. Minor changes in excipient concentrations, changes in batch size or manufacturing unit can be approved based on similarity in dissolution profiles of the new and old products [22].

1.1.2.5 Generic Product Bioequivalence

*In vivo* bio-equivalence studies can be waived for most immediate-release (IR) formulations containing highly soluble and highly permeable APIs. Extended-release (ER) formulations usually require *in vivo* bioequivalence studies to be performed only on the highest strength. *In vitro* data is acceptable for the other strengths, provided that *in vivo* bioequivalence studies are performed on the highest strength of drug product [14]. However, biowaivers for generic drug products of low therapeutic index drugs are understandably not accepted. FDA also requires modified release (MR) dosage forms to be tested for alcohol effects. Different levels of ethanol are added to the dissolution media to see if burst release occurs from MR formulations. Burst release or “dose dumping” from MR formulations is unacceptable. If an *in vitro-in vivo* correlation has been established, *in vitro* dissolution testing can be substituted for *in vivo* bioequivalence studies for IR/ER formulations administered orally or non-orally, but these cases are dealt on a case basis. Thus, dissolution testing is the most important *in vitro* test to determine bioequivalence and in some cases bioavailability of drug products.
1.1.3 Role of Dissolution Testing in Assessing Bioavailability and Bioequivalence

Bioavailability (BA) can be defined as the fraction of the drug from the dosage form that reaches the systemic circulation unchanged [23]. Comparative BA studies compare the bioavailabilities of two or more formulations of the same drug [24]. Consequently, drug products are said to be bioequivalent (BE) when there is no significant difference in their BAs. In order to be BE, the drug products need to be either pharmaceutical equivalents or pharmaceutical alternatives. Pharmaceutically equivalent drug products contain identical amounts of the same API. Pharmaceutical alternatives are drug products that contain the same API but it may be in a different dosage form, amount or chemical form. The various criteria to determine BE and the use of in vitro dissolution tests as surrogate to clinical studies to prove BE has been discussed in the following sections.

1.1.3.1 Criteria for Bioequivalence

The currently employed method for determining bioequivalence for two drug products is the 80/125 criteria after log transformation [25]. Accordingly, FDA considers two drug products to be bioequivalent if the 90 % confidence interval of the ratio of geometric means of key pharmacokinetic properties after log-transformation is within 80 % to 125 %. The key pharmacokinetic properties that are required to meet this criterion include the $C_{\text{max}}$ (maximum plasma concentration), area under the curve (AUC) from time 0-t and AUC from time 0-$\infty$ [26]. Figure 2 illustrates the criteria required in order to prove BE.
1.1.3.2 Tests for Determining Bioequivalence

When BA and BE regulations were first introduced, there was confusion as to whether \textit{in vivo} studies were required to be performed on drugs that were approved prior to introduction of these regulations. In 1974, the Drug Bioequivalence Study Panel which was a part of the Office of Technology Assessment stated that it was not practical to perform BE studies on all drugs or drug products that were approved before the inclusion of BA guidelines [28]. It was proposed that a certain group of drugs be identified where evidence of BA is crucial. \textit{In vivo} studies should be required to determine BA and BE of only these drug products. These suggestions were incorporated in the guidelines in 1977 and \textit{in vivo} efficacy studies were waived for some drugs that were approved before 1962 [29].
The current tests that can be used to determine BA and BE are listed in Title 21 of the Code of Federal Regulations (CFR). BA and BE are covered under part 320 of the regulations. According to the section, the following test procedures, in descending order of preference are acceptable as evidence to determine the BA and BE of a drug product [30]:

A. *In vivo* (clinical) studies determining the concentration of drug (and/or its active metabolites) in the blood/plasma/serum or another appropriate biological fluid as a function of time. If there is an *in vitro* test that has been correlated with and is predictive of human *in vivo* bioavailability data, it can be used in lieu of *in vivo* studies.

B. *In vivo* (clinical) studies where the concentration of API/active metabolites are measured in the urine as a function of time can also be used as a measure of BA and BE. FDA recommends the time intervals to be as small as possible in order to effectively estimate the rate of elimination of the drug. This approach is not appropriate for drugs that are not excreted primarily in the urine.

C. A third way to determine BA and BE is by using pharmacological end points. This *in vivo* approach is used when there is no method to determine the concentration of API in biological fluids or excretory products. There should, however, be a well-established method to determine the acute pharmacological effect. BA determination by pharmacological studies is suitable for use of drugs that are administered for local use.

D. The least accurate method as described by the FDA is that of well-controlled clinical trials comparing the safety and efficacy of two drug products. This method is accepted when previous approaches cannot be used. Accepted
application of this technique is for locally administered products which are not intended to be absorbed, e.g.: topical preparations and inhaled bronchodilators. Periodontal microparticles such as Arestin® and their generic products can also be considered in this category.

E. An in vitro test (usually the dissolution test) which is currently available and accepted by the FDA. This test should ensure that the drug product has a suitable in vivo human BA.

F. Any other approach that the FDA deems fit to establish BA and BE of drug product.

1.1.3.3 Bioequivalence Testing According to the Biopharmaceutics Classification System

In the past two decades, the role of dissolution testing in determining BA and BE has increased considerably. This is a result of the introduction of the Biopharmaceutics Classification System (BCS). The BCS classification of drug substances was introduced by G. L. Amidon. According to the BCS, drug substances can be divided into four categories as follows: Class I – high solubility, high permeability; Class II – low solubility, high permeability; Class III – high solubility, low permeability; and Class IV – low solubility, low permeability [31]. In context of biowaiver applications, a drug is said to be ‘highly soluble’ when the highest strength of the API in a drug product is soluble in 250 mL or less of aqueous media at 37 ± 1 °C within the pH range of 1 – 6.8 [32]. A ‘highly permeable’ drug has a systemic BA (or extent of absorption) of 85 % or more of an administered dose, provided the drug is stable in the gastrointestinal (GI) tract. Besides solubility and permeability, the FDA considers dissolution as a third parameter when assessing biowaiver requirements. The FDA suggests that it will consider applications to grant biowaivers to manufacturers for BCS Class I or Class III drugs when formulated in an IR product.
1.2 Development and Validation of Dissolution Methods

Development of reliable dissolution method is therefore an important step in the product development and approval process. Dissolution method development involves optimization of a number of variables, such as the type of dissolution apparatus, dissolution media and volume, agitation/flow rate, sampling times, temperature and analytical procedure [13]. The most important steps in development of a dissolution method are the selection of an apparatus and the determination of which dissolution media will be used, as described in the following paragraphs.

1.2.1 Types of Dissolution Testing Apparatus

Dissolution tests can be carried out in a variety of apparatuses, depending on the type of drug product that needs to be tested. However, to standardize the dissolution tests worldwide, regulatory agencies compiled a list of official dissolution apparatuses. There are seven types of dissolution apparatuses that are listed in the USP. The first four types of official dissolution apparatuses (Apparatus I – IV) are listed in USP general chapter 711 [33]. This chapter is harmonized with the Japanese Pharmacopoeia and European Pharmacopoeia. USP Apparatus V – VII are listed in general chapter 724 [34]. These three apparatuses are intended for testing of transdermal systems and are not accepted by the European and Japanese pharmacopoeias. The seven official USP apparatuses are tabulated in Table 1 and an illustration of their agitation assembly is presented in Figure 3.
### Table 1. List of Official Dissolution Apparatus, their Properties and Applications [35]

<table>
<thead>
<tr>
<th>Apparatus Type</th>
<th>Agitation Rate</th>
<th>Media Volume/ Flow Rate</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apparatus I – Basket</td>
<td>25 – 50 rpm</td>
<td>500 – 4000 mL</td>
<td>Capsules, tablets</td>
</tr>
<tr>
<td>Apparatus II – Paddle</td>
<td>25 – 150 rpm</td>
<td>500 – 4000 mL</td>
<td>Capsules, tablets, suspensions</td>
</tr>
<tr>
<td>Apparatus III – Reciprocating Cylinder</td>
<td>9.9 – 10.1 cm (reciprocating amplitude)</td>
<td>250 mL</td>
<td>Capsules, tablets, suspensions, granules</td>
</tr>
<tr>
<td>Apparatus IV – Flow Through Cell</td>
<td>NA</td>
<td>Up to 50 mL/min</td>
<td>Any dosage form, with appropriate modification</td>
</tr>
<tr>
<td>Apparatus V – Paddle Over Disk</td>
<td>25 – 150 rpm</td>
<td>500 – 4000 mL</td>
<td>Transdermal patch</td>
</tr>
<tr>
<td>Apparatus VI – Cylinder</td>
<td>50 rpm</td>
<td>500 – 4000 mL</td>
<td>Transdermal patch</td>
</tr>
<tr>
<td>Apparatus VII – Reciprocating Holder</td>
<td>2 cm (reciprocating amplitude)</td>
<td>Variable</td>
<td>Transdermal patch, non-disintegrating tablets</td>
</tr>
</tbody>
</table>
1.2.2 Types of Media Used for Dissolution Testing

The FDA recommends all dissolution tests to be carried out using aqueous media [36]. However, the choice of media depends on the purpose of dissolution testing. In general, types of dissolution media used for dissolution testing can be classified as non-biorelevant and biorelevant.

1.2.2.1 Non-Biorelevant Dissolution Media

When a dissolution test is carried for routine quality control purposes, non-biorelevant media are generally used [37]. Such dissolution tests are conducted mainly to obtain dissolution profiles of drug products and are not intended for determining *in vitro-in vivo* correlations.
(IVIVC). Usually, the limited buffering capacity of pure water discourages its use as a dissolution media. Many a times, researches use pH-adjusted aqueous solutions that mimic the pH of stomach (1.2) for dissolution testing. Thus the use of 0.1 N HCl solution recommended by the FDA to simulate the gastric pH is a non-biorelevant dissolution medium [21]. The volume of media employed is usually either 500, 900 or 1000 mL in compendial methods. Surfactants such as sodium lauryl sulfate and tween are often used to maintain sink conditions for drugs exhibiting poor aqueous solubility.

1.2.2.2 Biorelevant Dissolution Media

Biorelevant media are those media which mimic the fluid present at a particular site, at least to some extent. In 2015, Markopoulos, C. and colleagues classified biorelevant media into four categories [38]. Level 0 biorelevant media are those which mimic just the pH of the system. Such media were proposed to have some buffer capacity (not necessarily biorelevant) that could maintain the pH of the system. Under this classification, a pH 6.8 buffer media that is recommended by the FDA to mimic the pH of the intestine can be considered to be biorelevant [21]. Level I biorelevant media simulate the pH and buffering capacity present in vivo. Consideration of fasted and fed states comes into account as the fed conditions influence buffering capacity. Level II biorelevant media contain bile salts, lipids and other digestion products. The osmolality of the solution, along with composition is mimicked. Level III biorelevant media most closely mimic the fluid present in the body. They are similar in pH, buffer capacity, composition, osmolality, protein and enzyme content as well as viscosity [38].

The most commonly used biorelevant media simulate the gastro-intestinal environment. There are biorelevant media which mimic both the fasted and fed states of the stomach and small intestine [37, 39]. Over time, media mimicking the fluids present in various regions, such as
synovial fluid [40], vaginal fluid [41], colonic fluid [42] and lung fluid [43] among others have been reported. Marques, M. R. C. and colleagues reviewed the various biorelevant dissolution media that can have potential applications in dissolution testing of drug products [43].

1.2.3 Validation of Dissolution Methods

The guidelines for validation of dissolution methods are more general than those for chromatographic methods. The USP Chapter 1225 on Validation of Compendial Procedures [16] categorizes analytical procedures into four different categories. Each category has different requirements for validation. According to this chapter, dissolution methods need to be validated for precision. Other parameters such as accuracy, specificity, limit of detection and quantitation, linearity and range may be done, depending on the specific dissolution test. USP Chapter 1092 on The Dissolution Procedure: Development and Validation [13] gives a more detailed guideline on validation of dissolution tests. Some parameters that should be checked while validating dissolution methods include the discriminatory potential, specificity, linearity, accuracy, precision, robustness, filter compatibility and stability of sample.

1.3 Dissolution Testing of Micro/Nano-particulate Systems

A closer look at the various apparatuses and media would suggest that most of the methods are designed to test drug release from oral solid dosage forms. These methods are usually not suitable to test drug release from micro- or nanoparticulate systems. Thus, there have recently been efforts to develop methods to assess drug release from particulate dosage forms.
methods used for particulate systems were first classified by D’souza S. S. and DeLuca P. P. to be of the following three types: sample and separate, dialysis-based methods and continuous flow methods [44].

1.3.1 Sample-and-Separate Type Methods

Sample and separate based methods are the most commonly used dissolution methods for release testing of particulate systems [44, 45]. In sample and separate procedures, micro- or nanoparticles are added to tubes containing a suitable dissolution medium. These tubes are exposed to the desired temperatures and may be agitated during the test. Sampling of media is conducted at predetermined times by separating the particles from the media. Separation of microparticles is carried out by either filtration or centrifugation of the solution. After sampling, new media is added to the tubes to maintain sink conditions and dissolution volume [46].

Sample-and-separate methods provide flexibility in the amount of media that can be used for drug release testing. Methods reported in literature use either tubes or bottles and vary greatly in volume of media that is used for testing [45]. Media volumes of 1 mL [47, 48] to more than 100 mL [49, 50] can be used for drug release testing. Multiple modes of agitation (if desired) can be used in the methods. This is a simple method and can be carried out in almost every laboratory setting without the need of specialized equipment. However, the most common problem with this method is the loss and/or destruction of particles during sampling. Both filtration and centrifugation are unsuitable techniques for separation of particles from the dissolution media. Another issue that is often encountered with this technique is aggregation of microparticles [51]. Hydrolyzed polymers can clog filters and centrifugation can disrupt particle structure, giving unreliable results.
1.3.2 Dialysis-Based Methods

Dialysis methods were initially used for drug release testing of oily and liquid formulations [44]. Using dialysis-based methods to test drug release from microparticles helps in overcoming the problems involved with separation in the sample-and-separate methods. Briefly, in dialysis methods, the formulation to be tested is physically separated from the sampling media with the help of a dialysis membrane. Originally, the formulation to be tested was added to dialysis sacs, which was exposed to the dissolution media and maintained at an appropriate temperature [51]. The bulk media is generally agitated to assist drug diffusion from inside the dialysis sac [44]. Sink conditions are better maintained when the ratio of bulk fluid to fluid inside the dialysis sac is 5-10 [45]. In order to avoid interference of dialysis membrane in drug release, it is recommended that the molecular weight cut off (MWCO) of the membrane should be at least 100 times the size of the compound [52].

Although dialysis methods are convenient to measure drug release of particulate systems, they are often criticized for inadequate agitation and violation the sink conditions within the dialysis sacs [51]. In order to avoid sink violations, reverse dialysis methods were introduced, where microparticles are suspended in the outer bulk media and samples are taken from inside the dialysis sac. Reverse dialysis methods have been shown to have lesser variability than dialysis methods [53] but may lead to loss of sample [45]. In general, both dialysis and reverse dialysis membranes require a large amount of media to maintain sink conditions.
1.3.3 Continuous Flow Methods

Continuous flow methods involve the use of a flow through cell. Typically, particles are added to a cell and the desired media is made to flow through the cell with the help of pumps. Media can be allowed to flow either without re-circulation (open loop) or with re-circulation (closed loop). Like the previous methods, continuous flow methods provide flexibility to use the desired media volume for drug release studies [46]. As reported in literature, continuous flow methods can be used in various configurations. One of the earliest tests involved dispersing microparticles in filtration cells and passing media continuously through the cells at a constant flow rate [54]. Aubert-Pouëssel, A. and coworkers developed a method where microparticles were dispersed in unpacked high-performance liquid chromatography (HPLC) tubes and incubated at 37°C. Media was continuously circulated through the cells at 5 µL/min and was collected in a refrigerated chamber where samples were stored prior to analysis [55]. The microparticles tested in this method were not separated from the dissolution media. Since microparticles are not separated from the continuously flowing media, the released polymer and/or the particles themselves may block the filter, causing irregular of flow rates during the test.

The most recently applied continuous flow dissolution method used to measure drug release from particulate systems utilizes the USP IV apparatus. It is currently the preferred method for drug release testing [46] and provides several advantages such as greater lab-to-lab uniformity, decreased aggregation of particles, and flexibility in the flow rate, volume and type media used. The USP IV apparatus can also be run in various configurations. In some configurations, microparticles are added to flow through cells and glass beads are used to prevent aggregation of particles, achieve a laminar flow and decrease the dead volume inside the cell [56]. Use of a filter on the exit side of the cell allows for separation of particles from the released drug. However,
polymers can settle in the tubes, blocking tubes and causing irregularities in the flow [46]. More recently, a dialysis enclosure has been used to physically separate microparticles from the dissolution media [57]. This technique combines the advantages of the dialysis-based techniques with those of continuous flow methods.

Another advantage of continuous flow-based methods is that a broad range of flow rates can be used for media circulation. Depending on the flow rate required, three types of pumps are used for continuous flow dissolution methods. HPLC pumps are used to achieve low flow rates of 0.4 mL/min [58] and peristaltic pumps are used for re-circulation and higher flow rates up to 30 mL/min [59]. Syringe pumps are generally used when extremely low flow rates are required. One of the lowest flow rates that has been used for drug release of microparticles is 5 µL/min [55].

Most of the methods for drug release testing of particulate systems have been developed for testing injectable micro- or nanoparticles. They are usually carried out at high flow rates and relatively low media volumes to simulate the site of injection [60]. Since parenteral microparticles are designed to release the drug over extended periods of time, most of the research efforts focus on developing accelerated methods to study drug release from microparticles in a shorter time span. Researchers have developed accelerated continuous-flow methods for parenteral microparticles by using elevated temperatures [61], acidic [62] or alkaline [63] pH and changing the ionic strength [64].

There have been minimal efforts to design drug release testing methods for microparticles under biorelevant conditions. The dispersion releaser technology was recently introduced to test drug release from micro- and nanosized carriers. It is a dialysis-based device comprising of a slotted sample holder which is wrapped with a dialysis membrane [65]. This device is magnetically attached to the mini-paddle set-up of the mini-vessel assembly [66]. Jung, F. and coworkers
compared the dispersion releaser technology to filtration-based drug release testing using biorelevant release media [67]. A physiologically-based pharmacokinetic model (PBPK) was developed and tested for both the methods. Although the filtration-based method was faster, the dispersion releaser technology could discriminate between slight changes in the formulation, providing better discriminatory potential.

1.4 Dissolution Testing of PLGA-based Micro-/Nanoparticles

PLGA is a synthetic aliphatic copolymer of poly(lactic acid) (PLA) and poly(glycolic acid) (PGA). PLGA is available in a wide range of lactic acid-to-glycolic acid ratio (LA:GA) and molecular weights (Mw) of 10 – 200 kDa [68]. PLGA is a very versatile, biodegradable and biocompatible polymer approved by the FDA for human use. The versatility of PLGA stems from the ease with which properties of the polymer can be manipulated to obtain a suitable release profile. Some of the properties that affect the release of drugs from PLGA-based systems are listed below:

A. **LA:GA Ratio**: PLA contains a methyl group in its structure, making it more hydrophobic in nature as opposed to PGA, which lacks the methyl group. Thus, increase in LA:GA ratio of PLGA increases the hydrophobicity of the polymer and results in lower rate of drug release [69].

B. **Polymer Crystallinity and Glass Transition**: In general, when drug release is controlled by diffusion, increase in polymer crystallinity and glass transition temperature (Tg) decreases the rate of drug release [70]. PLGA can be prepared using poly(L-lactic acid) (PLLA), which is highly crystalline or poly(D-lactic acid) (PDLA), which is
completely amorphous or a mixture of the two forms. Although GA is more crystalline in nature, it reduces the overall crystallinity of PLGA. Thus, higher LA content causes an increase in crystallinity of the final PLGA, leading to decrease in rate of drug release [71]. Higher LA content, in addition to its effect on hydrophobicity, also increases the Tg of the polymer, reducing polymer chain motility and rate of drug release [70].

C. *Molecular weight of PLGA:* Increase in Mw of PLGA causes increase in Tg and hydrophobicity. Thus, higher Mw PLGA releases drug more slowly than lower Mw PLGA [72, 73]. It is important to note that higher Mw PLGA degrades at a faster rate than lower Mw PLGA due to more sites of hydrolysis. However, polymer degradation affects drug release at a later stage than diffusion. Thus, the effect of faster polymer erosion in case of higher Mw PLGA is countered by more drug diffusing out of the particles during the initial phase in case of lower Mw PLGA [69].

D. *Size of Matrix:* Autocatalysis of PLGA is one of the major mechanisms of drug release from PLGA systems. There are two processes countering each other during PLGA hydrolysis. First is the formation of acidic components which causes further hydrolysis of PLGA. Second in the influx of relatively basic release media, which neutralizes the acidic moieties [71]. Microparticles with a larger particle size have a longer diffusion path which reduces the rate of influx of basic moieties. Thus, larger microparticles are expected to increase the rate of drug release from PLGA microparticles.

E. *Type of Drug:* Hydrophilic drugs diffuse through water-filled pores and hydrophobic drugs diffuse through the polymer. Diffusion through the polymer is usually slower than diffusion through water [74] and it has been shown previously that hydrophobic drugs show lower rates of drug release than hydrophilic drugs [75]. Weakly acidic and
basic drugs can also impact drug release from PLGA systems. Acidic drugs decrease the pH inside particles, increasing the PLGA hydrolysis. Basic drugs can either increase PLGA hydrolysis increasing the rate of drug release or neutralize the hydrolysis products and reduce drug release rate [71]. Further, time-dependent changes in pH within PLGA microparticles can affect drug ionization and charge-based interaction with PLGA. Drugs molecules are also known to interact with PLGA, altering the rates of drug release in these special cases. For example, Klose and coworkers [76] showed that ibuprofen (slightly acidic) has a much faster rate of drug release than lidocaine (slightly basic). This was attributed to the interaction of positively charged lidocaine to the negatively charged PLGA end groups.

F. Drug Loading: Generally, microparticles which contain higher amount of drug show greater drug release. This is because the ratio of oligomer-to-drug is lower in particles with higher drug loading [72]. However, when a basic drug is encapsulated in PLGA systems, the Tg of the product increases with increase in drug loading. This is because basic drugs interact with PLGA, increasing matrix rigidity. Thus, the rate of drug release reduces with an increase in drug loading [77].

G. Fabrication Technique and Sterilization: Various formulation and process parameters can significantly affect the characteristics of the PLGA particles [78]. Porosity of the particle is highly influenced by the manufacturing technique [79]. Increase in porosity increases the mobility of drug molecules leading to an increase in drug release. Increase in porosity can also change drug release mechanisms. In comparison to large porous particles, smaller porous particles display increased rate of drug release [80]. This is because drug molecules in smaller porous particles have access to more media and
surface area for drug release as compared to non-porous molecules, which require the polymer to degrade to release the drug.

1.4.1 Mechanisms of Release from PLGA-based Systems

Although there are various factors that affect the release of drug from PLGA matrices, the actual mechanisms of drug release are limited. The “true” mechanisms of drug release, as summarized by Fredenberg, S. and colleagues [74] include:

A. *Diffusion through water filled pores* is most common during the initial phase of drug release. Molecules diffuse through the water present in the pores in the matrix through random movements. This transport is driven by concentration gradient and is dependent on the porosity of the matrix.

B. *Osmotic pumping* occurs usually in a non-swellable matrix. This phenomenon is a result of water absorption, which increases the osmotic pressure inside the particle. In this case, the drug releases out of the particle due to convection and not diffusion. Osmotic pumping is not a very commonly-observed mechanism of drug release in PLGA-based systems as PLGA has mobile chains which allow for swelling. However, it has been reported in literature in cases of hydrophobic (LA:GA ratio of 85:15) and high molecular weight (> 300 kDa) systems [81].

C. *Diffusion through polymer* occurs in cases of very small hydrophobic molecules. The molecules partition into the polymer and travel through the polymer chains to the surface of the matrix. From there, the molecules need to dissolve in water before they can be released. Diffusion through polymer chains is not dependent on the porosity of
the matrix. However, increased porosity affects the overall release rate by increasing the rate of drug dissolution in water.

D. Polymer erosion is the main mechanism of drug release for low Mw PLGA systems. Polymer erosion is the hydrolytic cleavage of polymer chains leading to production of smaller oligomers. Once the polymer matrix erodes, the encapsulated drug is exposed to dissolution fluid, causing release of drug. This mechanism is especially important for high Mw drugs that cannot diffuse through the polymer or water to get released.

1.4.2 Methods to Determine Drug-Release from PLGA-based Micro-/Nanoparticles

Since there are a number of PLGA-based products in the market, the polymer is popular among researchers for investigation. This has driven the development of a number of approaches to test the drug-release from PLGA-based systems. The methods used to evaluate drug-release from PLGA micro- and nanoparticles can be categorized into sample-and-separate, dialysis-based and continuous flow methods. Most methods used for PLGA-based systems can be used for drug-release testing of other microparticles and are summarized in the previous section. The development of accelerated drug-release testing methods is popular amongst PLGA microparticles as well. The USP IV apparatus is being increasingly used and explored for determining drug-release from PLGA-based particulate systems. Particles are either dispersed with glass beads in dissolution cells [56] or placed in a dialysis adapter [57]. Dissolution tests are run either under “real-time” (37°C) or accelerated conditions, such as increased temperature [56, 61].

The dialysis adapter combines the advantages of dialysis and continuous flow methods. It helps in separating particles from the release media, reducing problems of filter blockage. The USP IV method is preferred over other methods as it is a standardized equipment, enabling the
lab-to-lab transferability [56]. The USP IV method has been reported to simulate the subcutaneous environment present in clinical settings as the media in the environment is continuously replenished [82]. Thus, the USP IV apparatus is very suitable for drug release testing of parenteral PLGA particles which are administered subcutaneously.

However, despite all these efforts, there is a lack of biorelevant dissolution testing methods for microparticles that are intended to be administered in extremely small cavities, such as the periodontal and ocular pockets. There is thus a need for development of a small volume apparatus (SVA) that can be reliably used for drug release testing of particulate formulations intended to be delivered in small cavities.
2.0 Project Goal and Approach

2.1 Project Goal

Based on the current need for development of small volume dissolution apparatus, the goal of my project was to develop a biorelevant dissolution method for microparticulate systems intended to be delivered in small cavities. A rapid screening method utilizing the USP IV apparatus was also developed. The specific aims of my project were:

A) To evaluate the discriminatory potential of the rapid screening and biorelevant dissolution methods

B) To assess the reproducibility of the developed methods
2.2 Approach

![Figure 4. Overview of Approach Taken to Achieve Project Goal](image)

2.2.1 Model Drug Product

The overall approach undertaken in order to achieve the project goal is summarized in Figure 4. In order to develop and demonstrate the applicability of a small volume dissolution apparatus, microparticles applied in the periodontal pocket were selected. Specifically, minocycline hydrochloride (MIN), an antibiotic, loaded PLGA-based microparticles were evaluated. These microparticles are used in periodontitis. Periodontitis affected about 64.7 million people (46 % of the population) between 2009-2012 in United States alone [83]. It is a chronic...
bacterial inflammation of the epithelial and connective tissues that surround and support the teeth [84]. In healthy adults, the gap between these periodontal tissues and the tooth is 1-3 mm [85]. One of the hallmarks of periodontal disease is increase in pocket depth and formation of a periodontal pocket [86]. Figure 5 illustrates the differences between a healthy and inflamed periodontal pocket.

![Figure 5. Differences between a healthy and inflamed periodontal pocket. Reproduced with permission [84].](image)

Various studies show a positive correlation between the volume of the periodontal pocket and the stage of periodontal disease [87-89]. The pocket volume varies from 0.4 µL to 1.5 µL depending on stage of disease [90]. The periodontal pocket is filled with a physiological and inflammatory exudate, called the gingival crevicular fluid (GCF). The composition of GCF is
similar to that of any inflammatory exudate with lower protein content [91]. The flow of GCF is highly variable and ranges between 0.33 – 0.73 µL/min [90].

The most common and effective treatment of periodontitis is scaling and root planing (SRP). It is often accompanied with administration of antibiotics either locally or systemically. Tetracyclines, doxycycline, metronidazole, amoxicillin, ciprofloxacin and macrolide antibiotics are reported to be useful as systemic antibiotics in periodontitis [92]. To avoid problems of systemic antimicrobial resistance and side effects, focus was shifted to locally administered antimicrobial therapy [93]. The first locally acting system for periodontitis was Actisite™, which was comprised of tetracycline-loaded fibers that were wound around the affected teeth. Since then, various films, gels, chips, cubes and micro- or nanoparticles have been studied for management of periodontitis [94].

In the current study, we used Arestin® as a model drug product for the development of dissolution methods. Arestin® is manufactured by OraPharma and is composed of PLGA microspheres encapsulating 1 mg of MIN, a bacteriostatic agent. Arestin® is shown to maintain therapeutic levels in the periodontal pocket for a period of 14 days [95]. Clinical studies have shown Arestin® in conjunction to SRP is more effective than SRP alone [96]. Another study compared the effectiveness of Arestin® and Elyzol (25 % metronidazole gel) as adjunct treatments in periodontitis. At 3 months, only the Arestin®+SRP group showed a statistically significant increase in clinical attachment level and reduction in pocket probing depth [97]. Thus, Arestin® has been shown to be an effective strategy for management of periodontitis and has been available in the market for almost two decades. The physicochemical properties of the two main components of Arestin®, MIN, the active agent and PLGA, the release controlling polymer are listed below.
2.2.1.1 Minocycline Hydrochloride – Active Pharmaceutical Ingredient

![Figure 6. Structure of Minocycline Hydrochloride](image)

**Table 2. Properties of Minocycline Hydrochloride [98]**

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>493.941 g/mol</td>
</tr>
<tr>
<td>Molecular formula</td>
<td>C_{23}H_{27}N_{3}O_{7}.HCl</td>
</tr>
<tr>
<td>Class</td>
<td>Tetracyclines (semi-synthetic)</td>
</tr>
<tr>
<td>Mechanism of action</td>
<td>Inhibition of protein translation</td>
</tr>
<tr>
<td>Water solubility</td>
<td>52,000 mg/L at 25°C</td>
</tr>
<tr>
<td>Log $K_{ow}$</td>
<td>0.05</td>
</tr>
<tr>
<td>pKa</td>
<td>2.8, 5.0, 7.5, 9.6 [99]</td>
</tr>
<tr>
<td>Stability</td>
<td>Stable in air; sensitive to heat, light, and oxidation. Degrades in acidic and basic conditions (pH 0.38 – 9.35) [100]</td>
</tr>
</tbody>
</table>

2.2.1.2 Poly (lactic-co-glycolic acid) – Release Controlling Polymer

As reviewed earlier, PLGA is a versatile polymer that has been used in multiple products which are currently in the market. Being FDA approved, the polymer finds applications in a wide variety of fields, from drug delivery to bone regeneration. Like in other PLGA-based systems, the role of PLGA in Arestin® is to control the rate of MIN release. Based on the results of reverse engineering of Arestin® (unpublished data), Arestin® is composed of PLGA with LA:GA ratio of
50:50. The polymer has acid-end groups and molecular weight of about 24 kDa. Arestin® microparticles encapsulate 25 % w/w of MIN. The slow degradation of PLGA inside the periodontal pocket helps in maintaining the concentration of MIN above therapeutic levels in the pocket for at least 14 days [101].

2.2.2 Development of Comparators to Challenge Developed Methods

The discriminatory ability of dissolution methods is generally assessed by testing the effects of deliberate changes in manufacturing of the products on dissolution characteristics [102]. Thus, a panel of microparticles with deliberate changes in composition were prepared (unpublished data). These microparticles were prepared by varying the LA:GA ratios, $M_w$ of PLGA and MIN drug loading and were expected to show differences in drug release.

Additionally, a panel of microparticles with similar composition to Arestin® but prepared using different manufacturing conditions, specifically stir rate and solvent ratio were prepared. These microparticles were tested on the USP IV method to evaluate if the method can detect differences based on manufacturing differences. All microparticles were designed, fabricated and characterized for particle size by Ms. Ashlee C. Greene in Dr. Steven R. Little’s research group (Swanson School of Engineering, University of Pittsburgh).

2.2.3 Development of Dissolution Methods

Currently, the USP method for dissolution testing of periodontal systems containing MIN utilizes a tube rotator equipment. Briefly, one dose of the periodontal system is dispensed in a 15 cm long glass tube between two 25 µm screens. 10 mL of phosphate buffer (pH 4.2) is added to
the tubes and they are sealed with a snap-seal closure made of Teflon. The tubes are rotated in the tube rotator and the entire assembly is maintained at 37°C. Samples are taken at 4 h, 24 h, 48 h and 72 h by total medium replacement. The amount of MIN in dissolution samples is calculated using a liquid chromatography method at 280 nm [103]. The acceptance criteria as given in the USP monograph is as follows:

Table 3. Acceptance Criteria as per USP Monograph

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Release Rate (µg/h)</th>
<th>Calculated Amount to be Released (µg)</th>
<th>Cumulative Amount to be Released (µg)</th>
<th>Calculated % Released (Ideal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 – 4</td>
<td>NLT 25</td>
<td>NLT 100</td>
<td>NLT 100</td>
<td>NLT 10</td>
</tr>
<tr>
<td>4 – 24</td>
<td>NLT 1</td>
<td>NLT 20</td>
<td>NLT 120</td>
<td>NLT 12</td>
</tr>
<tr>
<td>24 – 48</td>
<td>NLT 0.2</td>
<td>NLT 4.8</td>
<td>NLT 124.8</td>
<td>NLT 12.48</td>
</tr>
<tr>
<td>48 – 72</td>
<td>NLT 0.05</td>
<td>NLT 1.2</td>
<td>NLT 126</td>
<td>NLT 12.6</td>
</tr>
</tbody>
</table>

*Note: NLT = Not less than

Tariq M. and colleagues reviewed the numerous methods that have been used for drug release testing of periodontal systems in vitro [104]. A variety of dissolution media, from distilled water to simulated salivary fluid, human serum and hydroalcoholic solutions have been reported in literature. The media volumes also vary greatly, from 1 mL to 500 mL. Continuous flow methods with flow rates ranging from 0.65 mL/min to 10 mL/min have been used for testing. However, none of the methods simulated the extremely low fluid volumes and flow rates present in the periodontal pockets. The current work was focused on bridging this gap between in vitro evaluation settings and in vivo release conditions.

First, a rapid screening method based on a previously reported USP IV method [57] was developed. This method was used to test drug release from the prepared microparticles and to ascertain that the microparticles produced broad differences in dissolution profiles. After
confirming that the microparticles showed differences using the USP IV method, the novel small volume method (SVM) was evaluated using these microparticles.

The SVM was developed on a small volume apparatus (SVA) which was designed previously in our lab (Invention Disclosure Number 04686). The apparatus (Figure 7) consists of an inner slotted chamber and outer closed chamber. The inlet and outlet caps are designed to allow unidirectional flow of media, allowing the assembly to function like a flow-through cell. The assembly is connected to a syringe pump which allows for continuous media flow in an open-loop configuration. These flow through cells can be placed in an oven to regulate the temperature.

![Figure 7. Images of the Small Volume Apparatus. (A) Inlet cap, (B) Outlet cap, (C) Outer chamber, (D) Inner chamber with dialysis membrane, (E) Assembled device](image)

A GCF simulant (sGCF) was also developed in the laboratory. The sGCF was used as a biorelevant media for the SVM. Table 4 lists the composition of the sGCF and Table 5 compares the composition of sGCF and human GCF.
Table 4. Composition of GCF Simulant

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (g/L)</th>
<th>Amount (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric acid monohydrate</td>
<td>0.338</td>
<td>1.608</td>
</tr>
<tr>
<td>Trisodium citrate dihydrate</td>
<td>5.411</td>
<td>20.968</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>6.740</td>
<td>115.332</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.719</td>
<td>9.644</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>0.056</td>
<td>0.001</td>
</tr>
<tr>
<td>Calcium chloride dihydrate</td>
<td>0.490</td>
<td>3.333</td>
</tr>
<tr>
<td>MilliQ water</td>
<td>1 L</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5. Comparison between sGCF and human GCF [105]

<table>
<thead>
<tr>
<th>Component</th>
<th>Human GCF</th>
<th>sGCF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium (mEq/L)</td>
<td>174.7 ± 18</td>
<td>174.5</td>
</tr>
<tr>
<td>Potassium (mEq/L)</td>
<td>9.54 ± 2.4</td>
<td>9.52</td>
</tr>
<tr>
<td>Calcium (mEq/L)</td>
<td>5.41 ± 0.37</td>
<td>5.44</td>
</tr>
<tr>
<td>pH</td>
<td>6.8-8.7</td>
<td>7.2 [106]</td>
</tr>
</tbody>
</table>

2.2.4 Development of Analytical Methods

MIN is sensitive to heat, light and oxidation [107]. About 10 % MIN degrades within an hour in phosphate buffered saline (PBS) at 37°C [108]. The degradation of MIN poses a challenge in the development of UV assay methods for quantitation of MIN in dissolution samples. It has been reported that for unstable drugs, UV analysis can be carried out at isosbestic point [109]. The isosbestic point is the wavelength at which the total absorbance of the sample does not change during a chemical reaction or when the sample undergoes a physical change [110]. Simply put, at
the isosbestic point, the total absorbance of the sample depends on the total molar concentration of two or more species in equilibrium.

Isosbestic point analysis has been used in the compendial dissolution method for Aspirin tablets [111]. Previous reports have demonstrated that tetracycline hydrochloride also displays an isosbestic point and that analysis of tetracycline and its degradants can be carried out at the isosbestic point without separation of the components [112]. Yang, Z. and coworkers reported to have analyzed MIN dissolution samples using UV-Visible spectroscopy without chromatographic separation of the degradants [113]. This indicates that MIN may exhibit an isosbestic point, which can be used for UV analysis of MIN samples. Thus, an online UV-based analytical method was developed for analysis of MIN samples using the USP IV method. The dissolution samples obtained using the biorelevant dissolution method were evaluated using a liquid chromatography based-assay.

A brief account of the development of analytical methods is presented in chapter 4. Chapters 4 and 5 also demonstrate the development and testing of the USP IV method and SVA using the developed microparticles as well as Arestin®. Specifically, Arestin® was utilized during method development and to test the reproducibility of the methods. The discriminatory ability of the method was evaluated using the developed microparticle panel(s).
3.0 Materials and Methods

3.1 Materials

Minocycline hydrochloride was purchased from Sigma Aldrich. Citric acid monohydrate, sodium hydroxide, sodium chloride, sodium citrate, bovine serum albumin, calcium chloride dihydrate, sodium azide and dimethyl sulfoxide were purchased from Spectrum Chemical. Potassium phosphate monobasic, potassium chloride, acetonitrile, ethanol and phosphate buffered saline (10x) were procured from Fisher Scientific. Tetra butyl ammonium hydrogen sulphate (98%) was obtained from J. T. Baker®. Float-A-Lyzer® G2 devices of 50 kDa MWCO and dialysis tubing (1 cm flat length, 50 kDa MWCO) were obtained from Spectrum Laboratories. Tygon tubing of 5/32” and 1/8” outer diameter was obtained from Fisher Scientific while tubing of 0.09” outer diameter was obtained from Cole-Parmer. All small volume chambers were manufactured by Mr. James Scott Macpherson at Swanson School of Engineering, University of Pittsburgh.

3.2 Fabrication of MIN loaded PLGA Microparticles

A panel of microparticles (Panel A), prepared using different process parameters, were prepared using the single emulsion method. Another panel of microparticles having differences in formulation (Panel B) were also prepared. The microparticles in Panel A were prepared using PLGA of Mw 24 – 38 kDa. The ratio of PLGA:dichloromethane (DCM) and the stir rate used to
prepare the microparticles was varied, as given in Table 6. Panel B (Table 7) was prepared by varying the MIN drug loading, Mw and LA:GA ratio of PLGA. All microparticles were prepared using the single emulsion technique and were designed and fabricated by Ms. Ashlee C. Greene in Dr. Steven R. Little’s Laboratory (Swanson School of Engineering, University of Pittsburgh).

Table 6. Microparticles Differing in Process Parameters (Panel A)

<table>
<thead>
<tr>
<th>Microparticle Name</th>
<th>Stir Rate</th>
<th>Ratio of PLGA:DCM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.025H</td>
<td>1500</td>
<td>0.025</td>
</tr>
<tr>
<td>0.05H</td>
<td>1500</td>
<td>0.05</td>
</tr>
<tr>
<td>0.0625M</td>
<td>1000</td>
<td>0.0625</td>
</tr>
<tr>
<td>0.1L</td>
<td>500</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Table 7. Microparticles Differing in Formulation (Panel B)

<table>
<thead>
<tr>
<th>Microparticle Name</th>
<th>LA:GA</th>
<th>Molecular Weight (kDa)</th>
<th>Theoretical Drug Loading (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50L3</td>
<td>50:50</td>
<td>15.4</td>
<td>50</td>
</tr>
<tr>
<td>50H1</td>
<td>50:50</td>
<td>64.14</td>
<td>25</td>
</tr>
<tr>
<td>75L2</td>
<td>75:25</td>
<td>14.2</td>
<td>37.5</td>
</tr>
<tr>
<td>85L1</td>
<td>85:15</td>
<td>21.8</td>
<td>25</td>
</tr>
<tr>
<td>85H3</td>
<td>85:15</td>
<td>43.3</td>
<td>50</td>
</tr>
</tbody>
</table>

3.3 Characterization of Prepared Comparators

All microparticles were characterized for particle size, surface morphology, drug content and polymer Tg. Particle size and surface morphology were determined by Ms. Ashlee C. Greene. Measurements were performed by volume impedance using Coulter Counter (Multisizer 3, Beckman Coulter). Surface morphology was tested using scanning electron microscopy (SEM).
The microparticles were sputter coated with gold/palladium (Au/Pd) using the Denton Sputter Coater and then imaged using the JEOL JSM-6510LV/LGS (JEOL Inc., USA). Drug content was measured by dissolving around 10 mg particles (or 2 units of Arestin®) in 100 mL of 20 % acetonitrile in MilliQ water. The particles were vortexed and stirred in the dark for 1 hour. The solution was then filtered using 0.22 µm teflon syringe filters (Restek™) and analyzed using the ultra-high performance liquid chromatography (UPLC) method described below. The Tg of PLGA in microparticles was characterized using differential scanning calorimetry (DSC). 2-5 mg microparticles were weighed in aluminum pans and sealed with a sealing press (METTLER TOLEDO). The lids were punctured to allow for escape of water. Tg was calculated in a cyclic run on DSC 2 STAR™ System (METTLER TOLEDO). First, the microparticles were heated at 10°C/min from 25 – 125°C. This was done to remove moisture from the sample. The samples were then cooled to 0°C at -10°C/min and heated again to 250°C at 10°C/min. All runs were carried out under nitrogen gas at a flow rate of 50 mL/min.

3.4 Determination of Isosbestic Point

To determine the isosbestic point of MIN, 25 µg/mL samples of MIN in 0.1 % sodium azide in PBS were kept in amber colored vials at 37°C for 5 days. Every day, one vial was removed and stored at -80°C. UV absorption spectra of all samples were taken using VISIONpro software with a UV spectrophotometer (Evolution 300, Thermo Scientific).
3.5 Dissolution of Microparticles by USP IV Method

A USP IV method was developed based on a previously published method [57]. The media pumps were calibrated using water at 10 mL/min for 2 minutes. A % error of less than 5 % was accepted. Float-A-Lyzer® G2 devices of 50 kDa molecular weight cut-off (Spectrum Laboratories) were prepared by rinsing them first with 10 % ethanol for 10 minutes, then with purified water for 30 minutes and finally with 0.1 % sodium azide in PBS (dissolution media) for another 30 minutes. About 10 mg microparticles (or two Arestin® cartridges) were added to the prepared devices. 1.8 mL of dissolution media was added to the devices before capping them. The prepared devices were placed in 22.6 mm flow through cells and dissolution was carried out using USP IV apparatus (SOTAX CP7 manual closed loop system). 80 mL of dissolution media served as the reservoir and was made to recirculate at 10 mL/min flow rate. The test was carried out at 37°C for 3 days. At predetermined intervals, the cumulative amount of drug released was measured by an online UV spectrophotometer (Evolution 300, Thermo Scientific) at 292 nm. At regular intervals, a calibration curve of minocycline hydrochloride in dissolution media was prepared and analyzed at 292 nm. The readings were used to determine the E11 value, utilized by the software to measure % dissolved values during on-line UV analysis. Figure 8 shows a schematic representation of the USP IV-based dissolution method.
3.6 Dissolution of Microparticles Using Small Volume Apparatus

Syringe pumps (KD Scientific) were filled with sGCF and calibrated at 0.5 µL/min. An error of <10 % was accepted. The inner chamber of the SVA was wrapped with a dialysis membrane of 50 kDa MWCO (Biotech CE, 1 cm flat width, Spectrum Laboratories) using an adhesive (Liquid Nails All Purpose Adhesive) and teflon tape. About 10 mg microparticles (or 1 cartridge of Arestin®) were added to the chamber followed by 250 µL of sGCF. The inner chamber was inserted into the outer chamber and sGCF was made to flow through the assembled cell at 100 µL/min to fill the chamber entirely. Once filled, the flow rate was reduced to 0.5 µL/min. Samples were collected daily for at least 14 days and diluted to a final of 20 % of acetonitrile in sGCF. The diluted samples were analyzed by UPLC. Figure 9 illustrates the small volume dissolution method.
3.7 UPLC Analysis of Minocycline Hydrochloride

A UPLC-based assay for MIN was developed in-house. A UPLC system (Acquity, Waters) with a TUV detector was used for analysis. An Acquity UPLC BEH C18 column (1.7 µm, 2.1 mm × 50 mm) maintained at 35°C was used. The mobile phase composed of 76 % of citrate-phosphate buffer at pH 7.0 and 24 % of acetonitrile, added gravimetrically. A flow rate of 0.2 mL/min was used for the isocratic method. Samples were stored at 7°C throughout the run. Samples were run for 10 minutes and calibration standards were run for 7 minutes in the respective matrix. Analysis was carried out at 277 nm.
3.8 Statistical analysis

Wherever necessary, the dissolution profiles were compared for similarity using the $f_1$ difference factor (Equation 7) and $f_2$ similarity factor (Equation 8).
4.0 Results

Microparticles of both panels were characterized for particle size, surface morphology, drug loading and Tg. The particles were evaluated for drug release using the rapid screening USP IV method. Microparticles of Panel B were evaluated using the SVM as well. Both dissolution methods exhibited discriminatory ability.

4.1 Characterization of MIN loaded PLGA Microparticles

All microparticles that were manufactured to challenge the developed methods were spherical and had a smooth surface. Figure 10 shows a representative SEM image of Arestin®. The characteristics of microparticles in Panel A are summarized in Table 8. The particle size of all microparticles of Panel A was found to be 32 µm and 44 µm. The MIN content of these microparticles ranged between 9 – 22 % w/w. Figure 11 is a typical curve that was obtained using DSC. All microparticles had a Tg between 48 – 55°C, characteristic of the PLGA used. Table 9 summarizes the properties of Panel B microparticles. All microparticles of Panel B and Arestin® had a particle size between 28 µm to 40 µm and drug loading between 9 – 21 % w/w. Overall, all microparticles that were prepared had a drug loading between 9 – 22 % w/w, which was found to be lower than Arestin® (25 % w/w).
Figure 10. SEM Image of Arestin® Microparticles

Table 8. Characteristics of Panel A Microparticles

<table>
<thead>
<tr>
<th>Microparticle</th>
<th>Solvent Ratio</th>
<th>Stir Rate</th>
<th>Particle Size (µm)</th>
<th>Drug Loading (% w/w)</th>
<th>Tg (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Onset</td>
</tr>
<tr>
<td>0.025H</td>
<td>0.025</td>
<td>1500</td>
<td>32.3±10.3</td>
<td>12.24</td>
<td>50.6</td>
</tr>
<tr>
<td>0.05H</td>
<td>0.05</td>
<td>1500</td>
<td>38.8±9.9</td>
<td>9.2</td>
<td>47.77</td>
</tr>
<tr>
<td>0.0625M</td>
<td>0.0625</td>
<td>1000</td>
<td>43.2±12.1</td>
<td>12.43</td>
<td>47.28</td>
</tr>
<tr>
<td>0.1L</td>
<td>0.1</td>
<td>500</td>
<td>39.6±14.9</td>
<td>21.67</td>
<td>46.33</td>
</tr>
</tbody>
</table>

*Particle size data generated by Ms. Ashlee Greene, Dr. Steven R. Little Lab
Table 9. Characteristics of Panel B Microparticles

<table>
<thead>
<tr>
<th>Microparticle</th>
<th>LA:GA Ratio</th>
<th>PLGA Mw (kDa)</th>
<th>Particle Size (µm)</th>
<th>Drug Loading (% w/w)</th>
<th>Tg (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Onset</td>
</tr>
<tr>
<td>85H</td>
<td>85:15</td>
<td>43.3</td>
<td>33.9 ± 11.9</td>
<td>15.50</td>
<td>51.62</td>
</tr>
<tr>
<td>85L</td>
<td>85:15</td>
<td>21.8</td>
<td>39.2 ± 11.3</td>
<td>9.90</td>
<td>51.93</td>
</tr>
<tr>
<td>75L</td>
<td>75:25</td>
<td>14.2</td>
<td>36.9 ± 12.8</td>
<td>11.42</td>
<td>45.83</td>
</tr>
<tr>
<td>50H</td>
<td>50:50</td>
<td>64.14</td>
<td>32.9 ± 10.1</td>
<td>9.10</td>
<td>47.49</td>
</tr>
<tr>
<td>50L</td>
<td>50:50</td>
<td>15.4</td>
<td>28.7 ± 9.6</td>
<td>21.05</td>
<td>49.05</td>
</tr>
<tr>
<td>Arestin®</td>
<td>50:50</td>
<td>~24</td>
<td>28.6 ± 12.3 (Lot H)</td>
<td>25.13 ± 0.4 (Lot F)</td>
<td>45.87</td>
</tr>
</tbody>
</table>

*Particle size data generated by Ms. Ashlee Greene, Dr. Steven R. Little Lab
Figure 11. DSC Curve of Arestin® Microparticles. The light blue region represents the curve obtained during the first and second heat-cool cycle. The dark blue curve was obtained during the second heating cycle and was used for calculating Tg. The peaks at the end of the chromatogram indicate drug degradation.

In order to quantify the released MIN from microparticles during dissolution using USP IV apparatus, an online UV-Vis spectrophotometer was utilized. However, rapid degradation of MIN precluded use of UV spectrophotometer for drug quantitation. Moreover, chromatographic methods were not preferred because the total degradation products cannot be accounted given their varied absorptivity. Therefore, to account for the degradation, isosbestic point was determined for MIN in the dissolution media.
4.2 Determination of Isosbestic Point of MIN

Figure 12 shows an overlay of the UV spectra of MIN samples (25 µg/mL in 0.1% sodium azide in PBS) maintained at 37 °C over a period of 5 days under mild shaking. As can be observed from the graphs, the absorption maxima of MIN is 246 nm. The absorbance at this wavelength is influenced mainly by the amount of MIN in the sample. Thus, as MIN degrades with time, the absorbance at 246 nm decreases. However, the wavelength region from 276 – 312 nm showed overlapping UV spectra indicating no or minimal changes in absorbance for samples stored for different lengths of time. This region is termed as the isosbestic region. The absorbance of the sample in this region is proportional to the total concentration of MIN and its degradants in the sample. A wavelength of 292 nm, which lies in the middle of the isosbestic region, was chosen for online UV analysis. Absorbances were found to be linear from 0.5 µg/mL – 50 µg/mL at 292 nm. This range was used to calculate the E11 value, necessary for on-line UV analysis with the USP IV apparatus. E11 is a composite value relating absorbance, path length and concentration of the samples. Figure 13 shows an overlay of four calibration curves obtained using UV spectrophotometer at 292 nm. The average E11 value obtained was 258.62, with a relative standard deviation of 4.05%.
**Figure 12. Determination of Isosbestic Region.** MIN samples in 0.1% sodium azide in PBS (25 µg/mL) were kept at 37°C under mild shaking for a period of 5 days. Samples were collected daily and their UV spectra were obtained. All UV spectra showed almost constant absorbance in the region between 276 – 312 nm. This region is called the isosbestic region. A wavelength of 292 nm, which lies in the middle of this range, was chosen for online UV analysis.
Figure 13. Overlay of Calibration Curves of MIN (0.5 – 50 µg/mL in 0.1% sodium azide in PBS) used for E11 Calculation at 292 nm. The E11 values were calculated on different days and using different lots of dissolution media. The average E11 values was found to be 258.62, with a relative standard deviation of 4.05 %.

4.3 Analysis of Samples using the USP IV Apparatus

USP IV dissolution apparatus was utilized to assess dissolution of MIN solution, Arestin®, and the prepared microparticle panels to test the reproducibility and discriminatory ability of the method.
4.3.1 MIN Solution

Figure 14 shows the release profile of MIN solution (1 mg/mL) using the USP IV method. Complete release (100%) of MIN was observed within 4 hours and the drug release remains almost constant up to 3 days. The release reached a plateau in 6 hours, indicating that the release of MIN is not affected by the dialysis membrane. The release remained constant over a period of 3 days, suggesting that the isosbestic point can be reliably used to test MIN release from microparticles. The greater than 100% release observed is attributed to possible interference from unknown degradants formed during degradation affecting the sensitivity of detection. This result further supports the use of USP IV method for a semi-quantitative comparison of dissolution profiles.

![Graph showing the release profile of MIN solution using the USP IV method.](image)

**Figure 14. Release of Minocycline Solution in USP IV Method.** MIN solution (1 mg/mL) was added to Float-A-Lyzer® dialysis devices of 50 kDa MWCO. The dialysis devices were inserted in USP IV flow-through cells and incubated at 37°C. Dissolution cells were subjected to a continuous flow of 0.1% sodium azide in PBS at 10 mL/min for a period of 3 days. At predetermined intervals, samples were analyzed by an online UV-Vis spectrophotometer at
isosbestic point. Results are presented as Mean ± SD of n=5 samples. Complete release of MIN was observed within 6 hours and the release profile remained constant over a period of 3 days.

4.3.2 Arestin®

Figure 15 shows the dissolution profile of Arestin® obtained using the USP IV method. The release profile resembled zero-order release kinetics for the first 13 hours (70 % drug released), after which the release approached a plateau. About 85 % of MIN releases within 1 day and complete release is observed within 3 days.

![Figure 15. Drug Release of Arestin® Microparticles from USP IV Method.](image)

Two units of Arestin® were added to Float-A-Lyzer® dialysis devices of 50 kDa MWCO. The dialysis devices were inserted in USP IV flow-through cells and incubated at 37°C. Dissolution cells were subjected to a continuous flow of 0.1% sodium azide in PBS at 10 mL/min for a period of 3 days. At predetermined intervals, samples were analyzed by an online UV-Vis spectrophotometer at isosbestic point. Results are presented as Mean ± SD of n=3 samples. About 85 % MIN releases within 1 day and complete release is observed at the end of 3 days.
4.3.3 Panel A Microparticles (Process Changes)

The dissolution profiles of Panel A microparticles (process changes) are shown in Figure 16. Both, 0.05H and 0.1L microparticles release about 70% of the drug over 3 days but demonstrate differences in release profiles. Microparticles 0.05H exhibit a faster release while 0.1L microparticles release in a more sustained fashion. Microparticles 0.0625M release about 60% MIN over a period of 3 days. Microparticles 0.025H release the least cumulative amount of MIN, with about 15% of MIN released over 3 days. Table 10 lists the $f_1$ (difference) and $f_2$ (similarity) values of the dissolution profiles of Panel A microparticles. $f_1$ and $f_2$ values are calculated to statistically evaluate the similarities and differences between dissolution profiles. $f_1$ values above 15 and $f_2$ values below 50 indicate that the dissolution profiles are not similar to each other. As seen from Table 10, none of the dissolution curves were found to be $f_1$-$f_2$ similar to each other, indicating that the USP IV method could discriminate between microparticles produced using different process parameters.
Figure 16. Drug Release Profiles of Panel A Microparticles (Process Changes) Obtained Using USP IV Method.

About 10 mg of microparticles were added to Float-A-Lyzer® dialysis devices of 50 kDa MWCO. The dialysis devices were inserted in USP IV flow-through cells and incubated at 37°C. Dissolution cells were subjected to a continuous flow of 0.1% sodium azide in PBS at 10 mL/min for a period of 3 days. At predetermined intervals, samples were analyzed by an online UV-Vis spectrophotometer at isosbestic point. Results are presented as Mean ± SD of n=3 samples. The USP IV method could successfully discriminate between microparticles of Panel A.

Table 10. Statistical Analysis of Dissolution Profiles of Panel A Microparticles. Numbers in bold denote values which are within limits (f1 < 15, f2 > 50).

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Difference Factor (f1)</th>
<th>Similarity Factor (f2)</th>
<th>Similar?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.025H vs. 0.0625M</td>
<td>233.51</td>
<td>23.97</td>
<td>N</td>
</tr>
<tr>
<td>0.0625M vs. 0.1L</td>
<td>17.40</td>
<td><strong>54.23</strong></td>
<td>N</td>
</tr>
<tr>
<td>0.1L vs. 0.025H</td>
<td>72.50</td>
<td>20.54</td>
<td>N</td>
</tr>
<tr>
<td>0.025H vs. 0.05H</td>
<td>322.88</td>
<td>17.92</td>
<td>N</td>
</tr>
<tr>
<td>0.0625M vs. 0.05H</td>
<td>26.80</td>
<td>45.74</td>
<td>N</td>
</tr>
<tr>
<td>0.1L vs. 0.05H</td>
<td>21.05</td>
<td>43.10</td>
<td>N</td>
</tr>
</tbody>
</table>
Previous studies have shown that drug release profiles can be normalized to the total amount of drug released in order to compare the mechanism of drug release from dosage forms [114-116]. Thus, in order to compare the way in which MIN releases from Panel A microparticles, the dissolution profiles obtained using the USP IV method were normalized to the total amount of MIN released. Figure 17 shows these normalized dissolution profiles of Panel A microparticles. Note that the normalized drug release profile of 0.025H microparticles in very uneven. This can be attributed to the low amounts of drug released from the microparticles, which may have fallen below the sensitivity of the method. A crude comparison of dissolution profiles indicates that increase in stir rate increases the burst release properties of microparticles (Figure 17), while the solvent ratio may affect the total amount of MIN that is released from the microparticles (Figure 16). However, there are multiple variables that can affect the drug release profiles of microparticles. Thus, additional information is needed before any conclusions about the effect of microparticle properties on drug release profiles can be made. Nevertheless, normalization of drugs release profiles removes bias caused due to differences in cumulative amount of drug released. Thus, normalization facilitates a fair comparison of drug release profiles of different microparticles.
Figure 17. Drug Release Profiles of Panel A Microparticles Normalized to Total Drug Released. Drug release profiles of Panel A microparticles (process changes) that were obtained using the USP IV apparatus were normalized to the total amount of MIN released over a period of 3 days. Normalization was carried out to understand the way in which MIN releases from the microparticles. Results are reported as Mean ± SD of n=3 samples. A basic comparison of dissolution profiles suggests that increase in stir rate causes an increase in burst release from microparticles (as for microparticles 0.025H and 0.05H) while reducing stir rate causes a more sustained release (0.0625M and 0.1L).

4.3.4 Panel B Microparticles (Formulation Changes)

Figure 18 shows the dissolution profiles of microparticles differing in composition. Microparticles 85H released the highest amount of MIN, about 60 %, whereas 85L released the lowest amount of MIN. Given the sensitivity limitations of this method, such low drug release cannot be quantified. Microparticles 50H, 75L and 50L released about 40 %, 25 % and 10 % of
MIN over the test duration. Overall, The USP IV method could successfully discriminated between the microparticles of Panel B.

![Figure 18. Dissolution Profiles of Panel B Microparticles (Formulation Changes) Obtained Using USP IV Method.](image)

About 10 mg of microparticles were added to Float-A-Lyzer® dialysis devices of 50 kDa MWCO. The dialysis devices were inserted in USP IV flow-through cells and incubated at 37°C. Dissolution cells were subjected to a continuous flow of 0.1% sodium azide in PBS at 10 mL/min for a period of 3 days. At predetermined intervals, samples were analyzed by an online UV-Vis spectrophotometer at isosbestic point. Results are presented as Mean ± SD of n=3 samples. The USP IV method could successfully discriminate between microparticles of Panel B.

Different batches of 85H microparticles were prepared and tested using the developed USP IV method to assess if the method could identify batch-to-batch differences between microparticles. Table 11 shows the characteristics of the multiple 85H batches. Microparticles 85H_1, 85H_2 and 85H_3 were three different batches of 85H that were produced. “Batch 0” is
the original batch that was produced and is included for comparison. Slight differences in drug loading (10-16% w/w) was observed. Two of the three microparticle samples (Batches 1 and 3) showed similar dissolution profiles (Figure 19, Table 12), releasing about 80 % MIN over a period of 3 days. One batch (Batch 2) showed a different drug release profile, releasing a cumulative of 60 % MIN over 3 days, similar to the original batch (Batch 0).

Table 11. Characteristics of 85H Reproducibility Batches

<table>
<thead>
<tr>
<th>Microparticle</th>
<th>Particle Size (µm)</th>
<th>Drug Loading (%)</th>
<th>Tg (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Onset</td>
</tr>
<tr>
<td>85H_1</td>
<td>40.96±11.24</td>
<td>13.34</td>
<td>52.09</td>
</tr>
<tr>
<td>85H_2</td>
<td>43.51±12.00</td>
<td>11.07</td>
<td>52.15</td>
</tr>
<tr>
<td>85H_3</td>
<td>43.25±9.87</td>
<td>10.44</td>
<td>51.56</td>
</tr>
<tr>
<td>85H_Batch0</td>
<td>33.9±11.19</td>
<td>15.50</td>
<td>51.62</td>
</tr>
</tbody>
</table>
Figure 19. Dissolution Profiles of Multiple Batches of 85H. About 10 mg of microparticles were added to Float-A-Lyzer® dialysis devices of 50 kDa MWCO. The dialysis devices were inserted in USP IV flow-through cells and incubated at 37°C. Dissolution cells were subjected to a continuous flow of 0.1% sodium azide in PBS at 10 mL/min for a period of 3 days. At predetermined intervals, samples were analyzed by an online UV-Vis spectrophotometer at isosbestic point. Results are presented as Mean ± SD of n=3 samples. The microparticles showed lot-to-lot differences, which could be identified using the USP IV method.
Table 12. Statistical Analysis of Dissolution Profiles of Multiple 85H Batches. Numbers in bold denote values which are within limits \((f_1 < 15, f_2 > 50)\).

<table>
<thead>
<tr>
<th></th>
<th>Difference Factor ((f_1))</th>
<th>Similarity Factor ((f_2))</th>
<th>Similar?</th>
</tr>
</thead>
<tbody>
<tr>
<td>85H_1 vs. 85H_2</td>
<td>19.84</td>
<td>42.51</td>
<td>N</td>
</tr>
<tr>
<td>85H_2 vs. 85H_3</td>
<td>24.37</td>
<td>41.23</td>
<td>N</td>
</tr>
<tr>
<td>85H_1 vs. 85H_3</td>
<td><strong>3.86</strong></td>
<td><strong>74.92</strong></td>
<td>Y</td>
</tr>
<tr>
<td>85H_1 vs. 85H_Batch0</td>
<td>27.67</td>
<td>37.89</td>
<td>N</td>
</tr>
<tr>
<td>85H_2 vs. 85H_Batch0</td>
<td><strong>11.25</strong></td>
<td><strong>61.51</strong></td>
<td>Y</td>
</tr>
<tr>
<td>85H_3 vs. 85H_Batch0</td>
<td>26.81</td>
<td>37.94</td>
<td>N</td>
</tr>
</tbody>
</table>

4.3.5 Method Reproducibility

Arestin® dissolution was performed at three different times by two analysts using the USP IV method to check for reproducibility of the method. As seen in Figure 20, all three Arestin® runs were found to be \(f_1- f_2\) similar to each other. The \(f_1\) and \(f_2\) comparisons of the obtained dissolution profiles are listed in Table 13. All values fall within the range, confirming the repeatability of the method.
Figure 20. Overlay of Dissolution Profiles of Three Arestin® Runs. Two units of Arestin® were added to Float-A-Lyzer® dialysis devices of 50 kDa MWCO. The dialysis devices were inserted in USP IV flow-through cells and incubated at 37°C. Dissolution cells were subjected to a continuous flow of 0.1% sodium azide in PBS at 10 mL/min for a period of 3 days. At predetermined intervals, samples were analyzed by an online UV-Vis spectrophotometer at isosbestic point. Results are presented as Mean ± SD of n=3 samples of 3 individual runs of Arestin®. All three runs of Arestin® were similar to each other, indicating that the method is reproducible.

Table 13. Statistical Analysis of Dissolution Profiles of Multiple Runs of Arestin®. Numbers in bold denote values which are within limits ($f_1 < 15, f_2 > 50$).

<table>
<thead>
<tr>
<th></th>
<th>Difference Factor ($f_1$)</th>
<th>Similarity Factor ($f_2$)</th>
<th>Similar?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run 1 vs. Run 2</td>
<td>3.48</td>
<td>77.05</td>
<td>Y</td>
</tr>
<tr>
<td>Run 2 vs. Run 3</td>
<td>6.96</td>
<td>64.74</td>
<td>Y</td>
</tr>
<tr>
<td>Run 3 vs. Run 1</td>
<td>10.55</td>
<td>56.55</td>
<td>Y</td>
</tr>
</tbody>
</table>
4.4 Analysis of Microparticles using the Small Volume Method

The SVM was evaluated using microparticles of Panel B to assess its discriminatory potential and reproducibility. The effect of flow rate on drug release of MIN solution and Arestin® was also assessed. The discriminatory ability was assessed by performing dissolution of Panel B microparticles (compositional changes). The reproducibility of the method was determined by performing the dissolution of Arestin® microparticles multiple times.

4.4.1 Analysis of MIN by UPLC

A stability-indicating UPLC assay was developed and qualified for use in-house. The method was used for analyzing drug content of microparticles and dissolution samples. Figure 21 shows a representative chromatogram. The method was linear in the concentration range of 0.125 – 50 µg/mL (Figure 22). Dissolution samples were collected during the biorelevant test and stored at -80°C prior to analysis. The stability of samples (20 and 50 µg/mL) was measured in 20 % acetonitrile in sGCF over a period of 7 months. A percent recovery of 97 – 101 % was obtained, which suggested that the dissolution samples were stable during the time frame. Stability of the samples on tray by measuring % recovery of QC samples (20 µg/mL) over 53 hours. More than 95 % recovery was obtained over the time frame, suggesting that the samples were stable on tray.
<table>
<thead>
<tr>
<th>Degradant</th>
<th>Retention Time (min)</th>
<th>Relative Retention Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epimer</td>
<td>1.394</td>
<td>0.425</td>
</tr>
<tr>
<td>Minocycline HCl</td>
<td>3.278</td>
<td>1.000</td>
</tr>
</tbody>
</table>

**Figure 21. Representative Chromatogram of Arestin® Dissolution Sample**

\[
y = 18805x - 1175.9 \\
R^2 = 1
\]

**Figure 22. Overaly of Three Calibration Curves of MIN in 20 % acetonitrile in sGCF**
4.4.2 MIN solution

Figure 23 shows the dissolution profiles of MIN solution (2 mg/mL) performed using flow rates of 0.5 µL/min, 2 µL/min and 10 µL/min. As expected, release of MIN solution was dependent on the flow rate used. The time to reach plateau decreased from 4 days for 0.5 µL/min to 22 hours for 2 µL/min and 9 hours for 10 µL/min. This result suggests that the release of MIN is dependent on the concentration flux created inside the dissolution cells and is not a function of membrane permeability.

![Graph showing the dissolution profiles of MIN solution](image)

**Figure 23. Effect of Flow Rate on Release of Minocycline Solution from Small Volume Method.** Two-hundred and fifty microliters of MIN solution (2 mg/mL in sGCF) was added to dialysis enclosures of 50 kDa MWCO. The dialysis enclosure was incubated at 37°C and subjected to continuous flow of sGCF at 0.5, 2 or 10 µL/min. Samples were collected at regular intervals and analyzed for MIN content by a UPLC-based assay. Results are presented as Mean ± SD of n=4 samples. Increase in flow rate increases the rate of release of MIN from the dialysis enclosure, indicating that the release of MIN depends on the concentration gradient and is not affected by the dialysis membrane.
4.4.3 Arestin®

Arestin showed a cumulative release of about 65 % over 2 weeks (Figure 24) in the biorelevant method. A fairly constant release was observed until a period of 5 days, after which the release plateaued.

![Graph showing drug release from Arestin®](image)

**Figure 24. Drug Release from Arestin® Obtained by Small Volume Method.** One unit of Arestin® was dispersed in 250 µL of sGCF in dialysis enclosures of 50 kDa MWCO. The dialysis enclosure was incubated at 37°C and subjected to continuous flow of sGCF at 0.5 µL/min. Samples were collected daily and analyzed for MIN content by a UPLC-based assay. Results are presented as Mean ± SD of n=4 samples. A fairly constant release is observed during the initial 5 days, after which the release plateaus.

In order to assess the effect of flow rate on dissolution of Arestin®, experiments were performed at 0.5 and 2 µL/min flow rates. Dissolution profiles of Arestin® at 0.5 and 2 µL/min are shown in Figure 24. Because the sampling time points did not match between these runs, the
dissolution data was fitted with a polynomial function (Figure 26) to obtain predicted % dissolved values from the trendlines. The release profiles were analyzed for similarity (f2) and difference (f1) factors. As seen in Table 14, flow rate did not have a significant impact on drug release from Arestin®, although an increased dissolution trend was observed with higher flow rate. It has been reported that the flow rates in the gingival pocket range from 0.33 – 2.24 µL/min [90]. These results indicate that the release of MIN from Arestin® microparticles is independent of the flow rates present clinically. However, the dissolution may be affected with further increase (or decrease) in flow rates, which was not evaluated in this study.

![Figure 25. Drug Release from Arestin® in Small Volume Method at Different Flow Rates.](image)

One unit of Arestin® was dispersed in 250 µL of sGCF in dialysis enclosures of 50 kDa MWCO. The dialysis enclosure was incubated at 37°C and subjected to continuous flow of sGCF at 0.5 or 2 µL/min. Samples were collected daily and analyzed for MIN content by a UPLC-based assay. Results are presented as Mean ± SD of n=4 samples. The release of Arestin® is fairly constant under the clinical relevant flow rates of 0.5 and 2 µL/min.
Figure 26. Fitting of Polynomial Trendline to Obtained Drug Release Profiles of Arestin®. The dissolution profiles of Arestin® obtained using flow rates of 0.5 and 2 µL/min were acquired at different timepoints. Similar timepoints are required in order to calculate $f_1$ and $f_2$ values to compare the dissolution profiles. Thus, the profiles were fitted onto polynomial curves and % dissolved values at pre-determined timepoints were estimated. These estimated values were used to calculate the $f_1$ and $f_2$ values for comparing the two profiles.

Table 14. Statistical Analysis of Drug Release Profiles of Arestin® at 0.5 and 2 µL/min Flow Rate. Numbers in bold denote values which are within limits ($f_1 < 15$, $f_2 > 50$).

<table>
<thead>
<tr>
<th>Difference Factor ($f_1$)</th>
<th>Similarity Factor ($f_2$)</th>
<th>Similar?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arestin® at 0.5 µL/min vs. Arestin® at 2 µL/min</td>
<td>13.64</td>
<td>55.40</td>
</tr>
</tbody>
</table>

4.4.4 Panel B Microparticles (Formulation Changes)

The small volume dissolution profiles of Panel B microparticles (differing in composition) are shown in Figure 27. The rank order of microparticles (85H > 50H > 75L > 50L > 85L) is same as that observed with the USP IV method. However, the cumulative amount of MIN released within 14 days is less than the MIN amount released over 3 days in the USP IV method. As seen
in Figure 27, the small volume dissolution method could effectively discriminate between the prepared microparticles that are compositionally different.

**Figure 27. Drug Release Profiles of Panel B Microparticles (Formulation Changes) Obtained Using Small Volume Method.** Around 10 mg of microparticles were dispersed in 250 µL of sGCF in dialysis enclosures of 50 kDa MWCO. The dialysis enclosure was incubated at 37°C and subjected to continuous flow of sGCF at 0.5 µL/min. Samples were collected daily and analyzed for MIN content by a UPLC-based assay. Results are presented as Mean ± SD of n=4 samples. The biorelevant method could successfully discriminate between microparticles of Panel B. The rank order of release from microparticles is constant between the USP IV and biorelevant methods.

4.4.5 Method Reproducibility

Dissolution of Arestin® was performed three times using the SVM to check for reproducibility of the method. Two different batches of Arestin® were run at different times. As seen in Figure 28, two of the three Arestin® runs were $f_1$-$f_2$ similar to each other and different
than the first run (original run). The $f_1$ and $f_2$ comparisons of the obtained dissolution profiles are listed in Table 15. These results can indicate differences between Arestin® batches, as the runs performed using the same batch (Run #2 and Run #3) are shown to be $f_1$- $f_2$ similar.

**Figure 28. Dissolution Profiles of three Arestin® Runs Obtained Using Small Volume Method.** One unit of Arestin® was dispersed in 250 µL of sGCF in dialysis enclosures of 50 kDa MWCO. The dialysis enclosure was incubated at 37°C and subjected to continuous flow of sGCF at 0.5 µL/min. Samples were collected daily and analyzed for MIN content by a UPLC-based assay. Results are presented as Mean ± SD of n=4 samples of 3 individual runs of Arestin® microparticles. Runs #2 and #3 were similar to each other and different to run #1. The differences between runs can be attributed to differences between lots of Arestin®.
Table 15. Statistical Analysis of Dissolution Profiles of Multiple Runs of Arestin®. Numbers in bold denote values which are within limits ($f_1 < 15, f_2 > 50$).

<table>
<thead>
<tr>
<th></th>
<th>Difference Factor ($f_1$)</th>
<th>Similarity Factor ($f_2$)</th>
<th>Similar?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run 1 vs. Run 2</td>
<td>23.91</td>
<td>43.78</td>
<td>N</td>
</tr>
<tr>
<td>Run 2 vs. Run 3</td>
<td>5.85</td>
<td>78.33</td>
<td>Y</td>
</tr>
<tr>
<td>Run 3 vs. Run 1</td>
<td>39.27</td>
<td>40.26</td>
<td>N</td>
</tr>
</tbody>
</table>
5.0 Discussion

There are several methods reported in the literature that can be used to evaluate drug release from PLGA microparticles [17, 44-46, 55, 61, 117, 118]. Many of these tests are conducted for microparticles that are developed for parenteral administration. For microparticles intended to be administered in the periodontal pocket (such as Arestin®), the current USP method utilizes a sample-and-separate type approach. This method utilizes agitation forces during incubation and is not biorelevant. The discriminatory potential of the method has also not been reported. Given these limitations, the current work attempts to address some of the limitations of the currently used methods by developing a novel, more biorelevant dissolution method. Further, attempts were made to develop a rapid screening method utilizing a compendial-level standardized USP IV apparatus. This method can be used during routine quality control analysis. Both the developed methods were shown to be discriminatory and reproducible.

5.1 Selection of Test Conditions for Dissolution Methods

As discussed previously, there are multiple variables that need to be determined while developing a dissolution method. In general, the dissolution media, test temperature, type and rate of agitation and method of analysis need to be defined. Type of pump and flow rate are important variables that need to be determined for continuous flow methods. For dialysis-based methods, the MWCO of the membrane and the volume-ratio of inner chamber to outer chamber are essential.
The USP IV method was designed to be a quick method for screening of microparticles. An easy-to-prepare and widely used dissolution media was selected for rapid analysis. The USP IV method was operated under closed-loop configuration at a flow rate of 10 mL/min. An internal volume of 1.8 mL and a reservoir volume of 80 mL was used for the method. The test was carried out at a physiological temperature of 37°C. Selection of MWCO of the dialysis membrane was based on the molecular size of MIN (~ 494 Da). It is reported that the MWCO of dialysis membranes should be around 100 times more than the molecular size [119]. Hence, a Float-A-Lyzer® device of MWCO of 50,000 Da (50 kDa) was used for the method.

It has been previously reported that MIN undergoes extensive degradation in PBS [108]. About 10 % MIN degrades within an hour at 37°C. PLGA microparticles have been reported to release drug in multiple phases. The first “burst release” phase is usually controlled by diffusion. The second phase is usually a “lag phase” which is the phase of lower drug release. This phase is followed by the third phase of “second burst release” which is controlled by polymer erosion [63]. During the “lag” phase, the amount of drug released will be lower than the amount of drug being degraded in the release medium. Thus, the apparent MIN concentration in the release medium will continuously reduce over 3 days due to degradation instead of remaining constant, posing a challenge for development of an online-UV assay. This problem was partially solved by measuring drug release at isosbestic point. As seen in Figure 12, MIN displays near constant absorbance in the isosbestic region between 276 – 312 nm, where the absorbance of the sample is proportional to the total concentration of MIN and its degradants. A wavelength of 292 nm, which lies approximately in the middle of this range was selected for online UV analysis. The choice of wavelength was confirmed by evaluating drug release from MIN solution in the USP IV method. As seen from Figure 14, the drug release from MIN solution plateaus in about 6 hours and remains
constant (instead of reducing) over a period of 3 days, justifying the choice of wavelength for online-UV analysis.

For the biorelevant SVM, sGCF was developed and used as the biorelevant release media. The sGCF simulates the ionic strength of the major ions present in GCF, pH, and to some extent the protein content. The actual protein content of GCF is significantly high and poses challenges to simulate \textit{in vitro}. The test was run at a physiological temperature of 37°C. Arestin® microparticles adhere to the gums immediately after insertion [120]. Thus, the use of any type of agitation to decrease the presence of unstirred layers surrounding the dialysis tubes is not biorelevant and was avoided. A dialysis-based method was selected as it serves a dual purpose. First, the dialysis enclosure contains microparticles and separates them from the dissolution media. Secondly, they more closely mimic \textit{in vivo} settings where microparticles are deposited in the periodontal pocket and become immobilized, surrounded by a layer of unstirred fluid (GCF) [121]. The SVM was run at the biorelevant flow rate of 0.5 µL/min in an open loop configuration, to mimic the continuous flow and replenishment of GCF as experienced \textit{in vivo}.

A media volume of 250 µL in the inner chamber was utilized in the SVM. Though a volume of 250 µL is not biorelevant, it is more practical to use in an \textit{in vitro} setting. In order to maintain an appropriate concentration gradient between the inside and outside of a dialysis membrane, the volume outside the dialysis enclosure should be 6-10 times more than the volume inside [44]. However, this requirement holds for pure dialysis methods that are not under continuous flow of media. In our setting, at any given point of time, the media in the outer chamber is about 3 times the media in the inner chamber. However, media is continuously flowing through the dissolution cells, replenishing the dissolution media present outside the dialysis membrane. This continuous media flow should help in maintaining the concentration gradient. Moreover, since the SVM is a
biorelevant method, it is not subject to the requirements of general dialysis-based methods, which do not mimic *in vivo* conditions. Of note, the requirement for sink may be met in the dialysis enclosure. However, the rate of media replenishment outside the dialysis membrane affects drug diffusion and may impact release from the microparticles.

In order to quantify the amount of MIN released during the biorelevant dissolution run, a stability-indicating UPLC-based assay was utilized. Only the MIN peak, and not the epimer or other degradant peaks, was considered to account for the percent drug released. Thus, the SVM is a quantitative method that can be utilized to measure the absolute amount of MIN released over the test period.

### 5.2 Effect of Flow Rate on Drug Release

The effect of flow rate on release of MIN solution and Arestin® was assessed using the SVM. MIN solution (2 mg/mL) was subjected to dissolution using SVA at flow rates of 0.5, 2 and 10 µL/min. Since MIN solution had drug in dissolved state, the amount of MIN released will depend on two factors: a) rate of re-establishment of concentration gradient between the sGCF in the inner and outer chambers and b) the diffusibility of MIN across the dialysis membrane. When the flow rate is increased, media in the outer chamber is replenished at a faster rate, which increases the concentration gradient between the inner and outer chambers. Increased flow rate also reduces the layer of unstirred water molecules on the boundary of the dialysis membrane [44]. Thus, higher flow rate is expected to increase the rate of drug release, which was observed in case of MIN solution (Figure 23). Greater than 90 % MIN solution is released within 24 hours at 2 and 10 µL/min flow rate, suggesting that the low release observed in the 0.5 µL/min run is due to a low
concentration flux and not because of the barrier properties of dialysis membrane. Interestingly, when MIN solution was evaluated for drug release at a flow rate of 0.5 µL/min, the release approached a plateau at ~ 80% release (~ 5 days). A complete release was not observed. This can be attributed to the degradation of MIN in the dissolution cells and samples as the solutions were exposed to 37°C for prolonged periods of time.

In case of Arestin®, increase in flow rate from 0.5 to 2 µL/min did not significantly increase the release of MIN (Figure 25, Table 14). A maximum difference of ~ 10% in the two dissolution profiles was observed at Day 1. This difference was maintained almost constant throughout the run. This can be due to the extended release behavior of Arestin® microparticles. Arestin® releases ~20% of MIN within one day under biorelevant conditions. This amounts to 200 µg of MIN, released in 250 µL of sGCF (inner chamber volume). The final concentration of MIN in the inner chamber is 800 µg/mL, which is significantly less than the saturation solubility of MIN in sGCF (> 2 mg/mL). Under higher flow rate, MIN is released faster due to faster re-establishment of concentration gradient between the inner and outer chambers. On the contrary, under lower flow rate, the concentration flux will not be re-established that fast, hindering drug diffusion from inside the inner chamber to outside. This explains the 10% difference in MIN release observed between 0.5 and 2 µL/min flow rates. Interestingly, the flow rate in the periodontal pocket has been reported to be anywhere between 0.33 – 2.28 µL/min [90]. As seen from Table 14, the release of MIN from Arestin® microparticles does not vary significantly in this clinically relevant range.

It is worthy to note that flow rate is an important parameter when using a purely continuous-flow method, where the media comes in direct contact with the test product. In this case, a higher flow rate not only determines the concentration gradient but also interaction between media and
test sample. Higher flow rate leads to better hydration of the particles [122], and induce shear depending on the flow regime (laminar vs. turbulent), resulting in higher drug release. However, when the particles are enclosed in a dialysis-sac, flow rate can be expected to have a lesser effect on drug release.

5.3 Assessing the Discriminatory Potential of Developed Methods

A good dissolution method for quality control analysis of finished drug products should be able to discriminate between microparticles that are chemically equivalent but may not have similar physical properties. For example, physical properties of microparticles can change due to differences in processing parameters such as stir rate and ratio of solvent [123, 124]. To replicate these differences, microparticles were fabricated using the same raw materials but by deliberately changing the process parameters (stir speed and solvent ratio, see Panel A). The goal of the experiment was to assess if the USP IV method, with potential application in routine quality control analysis, could identify differences between particles that were chemically equivalent but had different physical properties. The prepared microparticles showed differences in particle size, as summarized in Tables 8 and 9. The USP IV method was able to differentiate between microparticles of Panel A (Figure 16, 17, Table 10). Since differences in manufacturing during production usually arise due to deviations in process parameters, the ability of a dissolution method to identify such dissimilarities become crucial.

In order to assess bioequivalence of generic products, the FDA requires generic products to be Q1/Q2 similar to the RLD. Accordingly, a dissolution method intended for bioequivalence testing should be able to discriminate between microparticles that are Q1/Q2 different than the
RLD. Thus, a panel of microparticles that were compositionally different to Arestin® were fabricated. These microparticles had different LA:GA ratios, Mw and drug loading than Arestin® (see Panel B). As seen in Figures 18 and 27, both the developed methods could effectively discriminate between microparticles of Panel B.

In general, the USP IV method operates under conditions that can be considered as ‘accelerated’ compared to the biorelevant method. The method utilizes a very high flow rate of 10 mL/min, internal volume of 1.8 mL and media reservoir of 80 mL. Although the method utilizes physiological temperature of 37°C, the larger amount of media in the dialysis tube, nature of media and high flow rate can accelerate drug release from microparticles. As reported earlier, increased flow rate causes a greater diffusion flux, resulting in higher diffusion of the drug from inside the dialysis bag to outside [56]. Zolnik, B. S. and colleagues reported that increasing flow rate increases drug release from low Mw PLGA microparticles, with diffusion-controlled release kinetics. However, flow rate did not have any effect on drug release from high Mw PLGA microparticles, which were believed to show erosion-controlled release kinetics [56]. The differences in media flow rate between USP IV method and SVM are significantly higher than reported in the literature, which in combination with the dialysis enclosure volume could impact drug release. The initial burst phase, which is usually diffusion-controlled, is the region that defines the discriminatory potential of a dissolution method. Thus, a slower burst phase, as observed in the SVM, can potentially enable the biorelevant dissolution method to have a better discriminatory potential than the USP IV method.
5.4 Dependence of Rate of Release on Microparticle Properties

In general, it was observed that drug release from microparticles composed of a particular LA:GA ratio depended on the Mw of PLGA. Microparticles composed of higher Mw PLGA released higher amount of MIN than those composed of lower Mw PLGA (Figures 18, 27). As previously mentioned, usually Mw and drug release have an inverse relationship. The opposite effect seen in our studies can be attributed to the following two reasons. First, MIN is a tetracycline analogue with multiple sites of ionization. MIN has four pKas, as depicted in Figure 29A [99, 125]. During dissolution, MIN is already positively charged, which can interact with the acid end groups of PLGA. The effect of ionic interaction between positively charged proteins and uncapped (acid-end) PLGAs on drug release has already been studied by Balmert, S. C. and coworkers [126]. The group showed that positively charged peptides show lesser drug release than neutral peptides. Moreover, drug release of positively charged peptides from higher Mw PLGA was higher than that from lower Mw PLGA. This effect is similar to what was observed in our studies.
Figure 29. (A) Sites of Interaction between MIN and PLGA and (B) Flow Chart Explaining the Relation Between Mw and Drug Release

Another reason for the positive correlation of drug release and Mw can be attributed to faster decrease in intraparticular pH of lower Mw PLGA microparticles. Balmert, S. C. and group reported that the intraparticular pH decreases much faster in case of lower Mw PLGA as compared to higher Mw PLGA [126]. The group found that the intraparticular pH was below 4.5 for lower Mw PLGA (7 and 15 kDa) within 1 hour of incubation in PBS solution. The pH reduced to 3.3 within 3 days and as low as 2.2 within 12 days. In contrast, the pH of higher Mw PLGA (43 kDa), was 6 after 1 hour of incubation in PBS and reduced gradually to 3.2 over 21 days. Thus, the interaction of MIN in microparticles composed of lower Mw PLGA would be stronger than that in higher Mw PLGA, reducing the drug release from microparticles fabricated with lower Mw PLGA (Figure 29B).

It should be noted that, drug release during the initial timepoints can be attributed to un-encapsulated drug present at the surface of the microparticles. During this initial phase, drug
release will not be dependent on the fraction of MIN that is bound to PLGA within the microparticles. However, once the un-encapsulated drug is released from the microparticles, the effect of interaction of MIN with PLGA becomes more prominent. During this stage, release of MIN is diffusion controlled. Thus, the higher amount of unbound MIN present in microparticles composed of high Mw PLGA would be released faster (and to a greater extent) than from microparticles composed of low Mw PLGA. However, drug-polymer interaction may not significantly affect drug release during the later stages, where drug release is controlled by polymer erosion. Polymer erosion can explain the almost zero-order release of MIN from 50L microparticles (Figure 27). Microparticles 50L are composed of low Mw PLGA (50:50 LA:GA) and can be expected to have the fastest rate of polymer erosion [72].

Other reasons that may affect the release of MIN from microparticles include the internal structure of microparticles, porosity, particle size, drug loading and distribution of drug within the microparticles. There may be differences in the amount of free drug vs. bound drug inside the microparticle, which may affect drug release but has not been accounted for in this work. It is important to note that although attempts have been made to explain the order of drug release from different microparticles using the designed methods, the goal of the study was to assess the discriminatory potential of the method and not study the effects of microparticle properties on release behavior of MIN.

5.5 Assessing the Reproducibility of the Methods

It is important for any dissolution method to produce reproducible and reliable results. The reproducibility of the methods was assessed by comparing multiple runs of Arestin®. The same
batch of Arestin® microparticles were used to assess repeatability of the USP IV method. Using the same batch assured that the results were not affected by differences in microparticles that may exist between different batches. One of the runs was performed by a second analyst to account for variations between analysts. As seen in Figure 20, the three Arestin® dissolution profiles were very similar. Statistical analysis of the dissolution profiles (Table 13) confirm that the dissolution profiles are f1-f2 similar to each other.

Once the reproducibility of the USP IV method was determined, different batches of 85H microparticles were prepared and tested for drug release using the method. As seen in Figure 19 and Table 12, only two of the four batches produced showed f1-f2 similarity in the dissolution profiles. Differences in dissolution profiles may be attributed to the following three factors: variation in the method, batch-to-batch differences between microparticles and differences in sampling. Since the reproducibility of the USP IV method has already been established by comparing multiple runs of Arestin®, differences in dissolution profiles of 85H reproducibility batches can be attributed to batch-to-batch differences or differences in sampling. It has been previously reported that the effect of lot-to-lot differences during dissolution method development can be eliminated by normalizing release profiles to the total amount of drug released [127]. Thus, the obtained drug release profiles of 85H repeatability batches were normalized to the total amount of drug released over 3 days to account for batch-to-batch differences between microparticles. Figure 30 shows the dissolution profiles of 85H repeatability batches upon normalization. When the dissolution profiles were normalized, the profiles were found to be f1/f2 similar (Table 16).
Figure 30. Normalized Dissolution Profiles of 85H Repeatability Batches. Drug release profiles of various batches of 85H microparticles were normalized to the total amount release in order to eliminate batch-to-batch differences between the microparticles. After normalization, the release profiles were found to be $f_1/f_2$ similar to each other, indicating that the differences between microparticles are not indicative of method variability.

Table 16. Statistical Analysis of Normalized Dissolution Profiles of Multiple 85H Batches. Numbers in bold denote values which are within limits ($f_1 < 15$, $f_2 > 50$).

<table>
<thead>
<tr>
<th></th>
<th>Difference Factor ($f_1$)</th>
<th>Similarity Factor ($f_2$)</th>
<th>Similar?</th>
</tr>
</thead>
<tbody>
<tr>
<td>85H_1 vs. 85H_2</td>
<td>4.79</td>
<td>65.28</td>
<td>Y</td>
</tr>
<tr>
<td>85H_2 vs. 85H_3</td>
<td>8.87</td>
<td>51.86</td>
<td>Y</td>
</tr>
<tr>
<td>85H_1 vs. 85H_3</td>
<td>4.81</td>
<td>65.75</td>
<td>Y</td>
</tr>
<tr>
<td>85H_1 vs. 85H_Batch0</td>
<td>5.18</td>
<td>66.99</td>
<td>Y</td>
</tr>
<tr>
<td>85H_2 vs. 85H_Batch0</td>
<td>4.73</td>
<td>64.71</td>
<td>Y</td>
</tr>
</tbody>
</table>
The differences in the total amount of MIN released from 85H microparticles can stem from differences in the internal structure of microparticles, the distribution of MIN within the microparticles, porosity and overall size differences. Moreover, there can be differences in the amount of bound vs. free MIN inside the particles. Free drug diffuses faster than the bound drug, increasing the total amount of MIN released. Thus, to completely understand the drug release behavior of the produced microparticles, a thorough microparticle characterization will have to be performed, which is beyond the scope of this work. Moreover, the clinical relevance of these differences in drug release is not known and will have to be studied to assess the applicability of the method.

For the SVM, two batches of Arestin® were used due to unavailability of the same lot. Runs 2 and 3 were similar to each other and different from the first run (Figure 28 and Table 15). The differences in release profiles could be attributed to differences between the lots of Arestin®. Runs 2 and 3 were carried out using the same lot of Arestin®, while run 1 was carried out on a different lot. To account for batch-to-batch differences between Arestin® runs, the release profiles were normalized to the total amount of drug released. Because the sampling time points did not match between the runs, the dissolution data was fitted with a polynomial function to obtain predicted % dissolved values from the trendlines (data not shown). Similarity ($f_2$) and difference ($f_1$) factors were calculated using these values. After normalization, the dissolution profiles of all Arestin® runs were $f_1$-$f_2$ similar, as seen in Figure 31 and Table 17. This suggests that the individual runs differ in absolute amount of MIN released but not in the way in which they release MIN, which may reflect differences between individual lots of Arestin®. Although the different
batches of Arestin® show different release profiles in SVM, they may have passed the dissolution test as per USP criteria for periodontal systems containing MIN (Table 3), where a minimum of 12.6 % of MIN should be released at the end of 3 days. This argument seems plausible as the lots release at least 32 % MIN over 3 days even in the SVM, under extremely small volumes and flow rates. However, in order to be confident about repeatability of the method, another SVM run of Arestin® using the same lot will have to be performed.

![Figure 31. Normalized Dissolution Profiles of the Three Arestin® Small Volume Runs.](image)

Drug release profiles of three individual Arestin® runs carried out using the biorelevant method were normalized to the total amount released. Normalization was done in order to eliminate batch-to-batch differences between the microparticles. After normalization, the release profiles were found to be f1/f2 similar to each other, indicating that the differences between microparticles stem from differences between lots of Arestin® and are not indicative of method variability.
Table 17. Statistical Analysis of Normalized Dissolution Profiles of Multiple Arestin® Batches. Numbers in bold denote values which are within limits ($f_1 < 15, f_2 > 50$).

<table>
<thead>
<tr>
<th></th>
<th>Difference Factor ($f_1$)</th>
<th>Similarity Factor ($f_2$)</th>
<th>Similar?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run 1 vs. Run 2</td>
<td>1.36</td>
<td>89.47</td>
<td>Y</td>
</tr>
<tr>
<td>Run 2 vs. Run 3</td>
<td>2.17</td>
<td>79.32</td>
<td>Y</td>
</tr>
<tr>
<td>Run 3 vs. Run 1</td>
<td>3.44</td>
<td>72.67</td>
<td>Y</td>
</tr>
</tbody>
</table>

5.6 Impact of the Work and Potential Applications

Two reliable, discriminatory and reproducible dissolution methods were developed that have distinct advantages over each other. The USP IV method utilizes a compendial dissolution apparatus to test drug release from PLGA microparticles under real-time conditions (37°C). A complete release from Arestin® is achieved within 3 days without employing elevated temperatures. The method is carried out using an online UV-Vis spectrophotometer, which avoids the need to analyze the samples after completion of the run. Data is obtained reasonably fast, reducing the number of person-hours required to carry out the test. Using the USP IV apparatus comes with the inherent advantages of lab-to-lab uniformity, reliability and easy transferability. The method is simple and can be employed during drug development for determining ideal process and formulation parameters. The method can also be used for routine quality control analysis to check for batch-to-batch variability.

The SVM is a novel and more sensitive dissolution method. It provides the most practical representation of the in vivo environment for periodontal systems. Currently, there are more than 15 PLGA-based FDA approved products in the market. However, no generic PLGA-based product has received FDA approval as yet. The SVM can potentially be used as an in vitro alternative to
test generic versions of Arestin® for bioequivalence. It can also be used in lieu of clinical testing during scale-up of batches and to prove bioequivalence during post-approval changes. Further, given the similarity of test conditions to in vivo environment, the antimicrobial activity of the samples collected can be tested and the results can serve as proof of efficacy of the product over a period of 14 days (or longer, unpublished results). SVM can also be used to validate the manufacturing processes on an annual basis during large-scale production of periodontal microparticles. The use of SVM can be extended to test other dosage forms intended for localized delivery in the periodontal pocket.

In fact, the pumps and flow rates can be adjusted to simulate fluid-flow rates present in different parts of the body. The dissolution cells can be agitated to simulate the blinking of eyelids and turnover rate of lacrimal fluid. Thus, the use of the SVA shows promise to test drug release from other micro- and nanoparticulate systems, powders, solutions, suspensions, sprays and other formulations intended to be administered in small cavities such as the nasal, otic and ocular pockets. The small volume cell can also be modified to test drug release from vaginal products. For instance, a miniaturized USP I-type basket can be used as the sample holder. Clips can be utilized to secure the dosage form in the basket. The mesh size of the basket will have to be optimized in order to separate undissolved material from the dissolution media while preventing problems of back-pressure and clogging. The inner chamber can be designed to have a volume of 1 mL and flow rate of 4.2 µL/min to simulate conditions present in the vagina [41, 128]. Glass beads and agitation can be introduced to simulate mechanical forces normally encountered. Flow rate and agitation can be modified during the run to simulate changes relating to the menstrual cycle.
Overall, the SVA is a novel biorelevant apparatus that has potential for adaptation towards a variety of applications. The SVM described here is the first application and proof-of-concept of the apparatus. The current work focusses on assessing the method’s discriminatory ability and reproducibility in an effort to justify the SVA’s use for drug release testing of MIN-loaded PLGA microparticles intended for application in the periodontal pocket.

5.7 Limitations of the Developed Methods

Both the developed methods were reproducible and exhibited a good discriminatory potential. Despite this, the developed methods possess certain limitations. Some advantages of the USP IV method include that it quick, it is based on a compendial system and it has an on-line UV system that reports the results without having the need to collect and analyze samples separately. However, since MIN is highly sensitive to hydrolytic degradation, the UV analysis needs to be carried out at isosbestic point. Because the online-UV system measures the total amount (drug + degradants) of MIN released, it does not give the absolute amount of MIN released. Moreover, the method may overestimate the amount of MIN released by up to 20% for MIN solution. Thus, the drug release profiles may need to be normalized with respect to MIN solution during practical applications. A simple way to improve the sensitivity of the method is to collect samples at predetermined timepoints and analyze them to give the drug release profile. Although more cumbersome, this technique may be more reliable and quantitative, especially for the first 24 hours. However, it should be noted that MIN may degrade into several degradants that do not appear on the chromatogram, making quantitation challenging.
The SVM is a miniaturized version of the USP IV method with modifications that make the method more biorelevant. The method simulates more closely the flow rates, media and volumes that are experienced by periodontal microparticles \textit{in vivo}. Since the length of dissolution study is 2 weeks, its application during formulation development may be met with practical limitations of length timelines. However, SVM may be more applicable to determine bioequivalence of generic and brand name products. Another major limitation of the SVM is that it requires special dissolution cells which are not yet commercially available. The method may also show extremely small differences between microparticles, which may not be clinically relevant. Thus, more work needs to be done to determine the criteria for acceptance of products tested using the SVM.
6.0 Conclusion and Future Directions

Two reliable and reproducible dissolution methods were developed which could successfully discriminate between the prepared comparators. The USP IV method is a rapid dissolution test that can be used in drug development for determining optimum process and formulation parameters. It is semi-quantitative in nature as it provides the overall percentage of MIN released but cannot be used to quantify the absolute amount of MIN released. The method is reproducible and can be reliably used during product development for rapid screening of prototype drug formulations. The SVM has potential to exhibit a better discriminatory power than the USP IV method, as the SVM extends the ‘burst phase’ drug release from microparticles to ~ 5 days. The SVM can be used to calculate the amount of MIN released over time and is thus quantitative in nature. With certain modifications, the SVA can be used to test other dosage forms intended to be delivered in extremely small cavities, such as the nasal, otic and ocular sacs. The SVM can also be potentially used to test the bioequivalence of different MIN-loaded PLGA microparticles used in periodontitis.

More studies comparing the \textit{in vitro} release data to \textit{in vivo} dissolution profiles will need to be performed in order to determine the clinical relevance of the SVM. The acceptance criteria for products tested with both the methods needs to be established. Future work will also focus on testing microparticles of Panel A (process changes) using the SVM to demonstrate that the method can discriminate between them. The main goal of future experiments would be to compare the \textit{in vitro} and \textit{in vivo} release profiles in order to develop and validate a robust IVIVC.
Appendix A: Abbreviations Used

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BA</td>
<td>Bioavailability</td>
</tr>
<tr>
<td>BCS</td>
<td>Biopharmaceuticals classification system</td>
</tr>
<tr>
<td>BE</td>
<td>Bioequivalent</td>
</tr>
<tr>
<td>CFR</td>
<td>Code of Federal Regulations</td>
</tr>
<tr>
<td>$C_{\text{max}}$</td>
<td>Maximum plasma concentration</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>ER</td>
<td>Extended-release</td>
</tr>
<tr>
<td>$f_1$</td>
<td>Difference factor</td>
</tr>
<tr>
<td>$f_2$</td>
<td>Similarity factor</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GCF</td>
<td>Gingival crevicular fluid</td>
</tr>
<tr>
<td>GI</td>
<td>Gastro-intestinal</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>IUPAC</td>
<td>International Union of Pure and Applied Chemistry</td>
</tr>
<tr>
<td>IR</td>
<td>Immediate-release</td>
</tr>
<tr>
<td>IVIVC</td>
<td>In vitro-in vivo correlation</td>
</tr>
<tr>
<td>LA:GA</td>
<td>Lactic acid-to-glycolic acid ratio</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
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<tr>
<td>MIN</td>
<td>Minocycline hydrochloride</td>
</tr>
<tr>
<td>MR</td>
<td>Modified-release</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut-off</td>
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<tr>
<td>NF</td>
<td>National Formulary</td>
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<tr>
<td>NLT</td>
<td>Not less than</td>
</tr>
<tr>
<td>PBPK</td>
<td>Physiologically-based pharmacokinetic model</td>
</tr>
<tr>
<td>PDLA</td>
<td>Poly(D-lactic acid)</td>
</tr>
<tr>
<td>PGA</td>
<td>Poly(glycolic acid)</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly(lactic acid)</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PLLA</td>
<td>Poly(L-lactic acid)</td>
</tr>
<tr>
<td>QbD</td>
<td>Quality by design</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>sGCF</td>
<td>Simulated gingival crevicular fluid</td>
</tr>
<tr>
<td>SOP</td>
<td>Standard operating procedure</td>
</tr>
<tr>
<td>SRP</td>
<td>Scaling and root planing</td>
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<td>SVA</td>
<td>Small volume apparatus</td>
</tr>
<tr>
<td>SVM</td>
<td>Small volume method</td>
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<tr>
<td>Tg</td>
<td>Glass transition temperature</td>
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<tr>
<td>UPLC</td>
<td>Ultra-high performance liquid chromatography</td>
</tr>
<tr>
<td>USP</td>
<td>United States Pharmacopoeia</td>
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