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## A BIORELEVANT IN VITRO MODEL TO CHARACTERIZE IN VIVO RELEASE OF BONE MORPHOGENETIC PROTEIN-2 (rhBMP-2)

DEBLINA BISWAS

*Virginia Commonwealth University*

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**A BIORELEVANT *IN VITRO* MODEL TO CHARACTERIZE *IN VIVO*  
RELEASE OF BONE MORPHOGENETIC PROTEIN-2 (rhBMP-2)**

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor  
of Philosophy at Virginia Commonwealth University.

by

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July 2017

## **Dedication**

*This thesis is dedicated*

*To all my family members for their love and support*

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## List of Abbreviations

%CV	Percent coefficient of variation
%DFN	Percent deviation from nominal
%PE	Percent prediction error
%RSD	Percent relative standard deviation
°C	Degree celsius
®	Registered trademark
ACS	Absorbable Collagen Sponge
ANOVA	Analysis of Variance
API	Active Pharmaceutical Ingredient
AUC	Area under the curve
BA	Bioavailability
BCS	Biopharmaceutics Classification System
BIVDR	Biorelevant <i>In Vitro</i> Drug Release
BPC	Bone packing chamber
BRC	Bone regenerating chamber
CA	Cellulose acetate
CE	Collision energy

CV	Coefficient of variation
DAD	Diode array detector
DNA	Deoxy ribonucleic acid
DP	Declustering potential
dF	Degrees of freedom
ELISA	Enzyme Linked Immunosorbent Assay
ESI	Electrospray ionization
f1	Difference factor
f2	Similarity factor
FA	Formic acid
FaSSIF	Fasted State Simulated Intestinal Fluid
FDA	Food and Drug Administration
FeSSIF	Fed State Simulated Intestinal Fluid
FIP/AAPS	Federation Internationale Pharmaceutique/American Association of Pharmaceutical Scientists
FTC	Flow Through Cell
GRAVY	Grand average of hydropathy
HBSS	Hanks' balanced salt solution
HEPES	N-(2-Hydroxyethyl)piperazine-N-(2-ethanesulfonic acid)
HPLC	High Performance Liquid Chromatography
IR	Immediate Release
IVIVC/IVIVR	<i>In Vitro In Vivo</i> Correlation/ <i>In Vitro In Vivo</i> Relationship
IVR	<i>In vitro</i> relationship

IVR	<i>In vitro</i> relationship
kDa	Kilo dalton
LC-MS	Liquid chromatography – mass spectrometry
LLOQ	Lower limit of quantitation
LOD	Limit of detection
LOQ	Limit of quantitation
MDT	Mean dissolution time
mg	Milligram
mL	Milliliter
MRT	Mean residence time
PBS	Phosphate buffered saline
PC	Polycarbonate
PDA	Photo diode array
PEEK	Poly ether ether ketone
PES	Poly ether sulfone
PLGA	Poly (lactic-co-glycolic) acid
PTFE	Poly tetra fluoro ethylene
QC	Quality control
R <sup>2</sup>	Coefficient of determination
RC	Regenerated cellulose
rhBMP-2	recombinant human Bone Morphogenetic Protein -2
RPLC	Reverse phase liquid chromatography
rpm	Rotations per minute

SC	Sinus chamber
SD	Standard deviation
SE	Standard error
SUPAC	Scale up and post approval changes
$t_{1/2}$	Half life
TFA	Trifluoro acetic acid
TGF- $\beta$	Transforming growth factor – $\beta$
USP	United States Pharmacopeia
USP-IV	USP- IV flow through cell
UV/Vis	Ultraviolet/visible
$\alpha$	Alpha
$\mu\text{L}$	Microliter

# **ABSTRACT**

## **A BIORELEVANT *IN VITRO* MODEL TO CHARACTERIZE *IN VIVO* RELEASE OF BONE MORPHOGENETIC PROTEIN-2 (BMP-2)**

By Deblina Biswas, MS

A dissertation submitted in partial fulfillment of the requirement for Doctor of  
Philosophy degree at Virginia Commonwealth University.

Virginia Commonwealth University, 2017.

Major Director: Matthew Sean Halquist, Ph.D.  
Assistant Professor, Department of Pharmaceutics

Biorelevant *in vitro* release/dissolution tests are designed to predict the *in vivo* behavior of a drug and are crucial in understanding its *in vivo* performance. Currently, there is no standardized compendial *in vitro* release testing methods or regulatory guidance's for release/dissolution testing of implants due to their complex physiological locations.

Furthermore, existing compendial methods do not capture the local release profile of 'novel' parenterals in physiological low fluid volume surrounding areas.

Long acting and in situ forming implants with orthobiologic proteins and peptides have increased over the past few decades due to a better understanding of genetic engineering. One of these products, INFUSE<sup>®</sup> Bone Graft (Medtronic, MN, USA), is an implant which helps in bone regeneration at the trauma site and is comprised of a) an absorbable collagen sponge (ACS) and b) recombinant human bone morphogenetic protein-2 (rhBMP-2). INFUSE<sup>®</sup> Bone Graft is an FDA approved product for acute, open shaft tibial fractures, lumbar spinal fusions and sinus or ridge augmentations in the jaws. The evaluation of implant products such as INFUSE<sup>®</sup> Bone Graft requires a good understanding of local and systemic release *in vivo* in order to ensure safe, effective, and predictable product performance.

The primary goal of this study is to develop a predictive '*biorelevant*' release model, which factors in clinically relevant physiological parameters suitable for studying and effectively predicting extended release of implants, using INFUSE Bone Graft<sup>®</sup> as our model implant. A novel biorelevant *in vitro* model was designed and tested. The model was observed to be discriminatory between two different carrier formulations of rhBMP-2 using a model independent approach - similarity factor ( $f_2$ ). Additionally, a high throughput assay to quantify rhBMP-2 release using high performance liquid chromatography with UV/VIS detection was also developed and validated. Successful completion of this study facilitated an *in vitro* release study design that incorporated the

complex biorelevant parameters of implant dosage forms, the model will offer crucial insights into biological performance, and aid in developing methods to characterize release of other similar dosage forms.

# CHAPTER 1

## 1 INTRODUCTION

*Parts of this chapter have been drawn from an editorial published in J Pharmacovigilance, (Biswas and Halquist, 2016)*

### 1.1 BACKGROUND

#### 1.1.1 Dissolution testing and its significance

Dissolution testing is the utilization of surrogate *in vitro* conditions to mimic *in vivo* release conditions of a dosage form (Azarmi et al., 2007). This is achieved with the help of dissolution medium, dissolution apparatus and a robust study design (US Pharmacopeia, 2011b). The aim of dissolution testing is to develop a test that represents the drugs *in vivo* dissolution profile but is reproducible and easy to perform in the lab. It is a performance verification test for a dosage form and helps in being an evaluative quality control tool in the commercial manufacturing process (Dressman and Krämer, 2005; Siewert et al., 2003). A dissolution test can be developed early on during the pre-clinical stages of a drug when formulation design changes are made to the dosage form such as incorporation of excipients or other components (Lipka and

Amidon, 1999). Effects of inclusion or exclusion of such components in the formulation can then be studied with the help of a simple in-house dissolution test instead of using *in vivo* animal models at each step. A dissolution test for oral dosage forms ensures minimal batch-to-batch variability and hence works as a check that the dosage form meets the requisite regulatory and quality standards. It can also be applied in examining methodically the stability of a dosage form (Nazzal and Khan, 2006). The stability of dosage forms can be affected by changes in storage temperatures, shelf time duration, minor changes in pH, moisture content at the location of storage and even light conditions (Carstensen, 1974; Yoshioka and Stella, 2000). The US Pharmacopeia provides chapters <711> and <724> as test chapters for methods to perform dissolution and drug release respectively (Formulary, 2012; US Pharmacopeia, 2011a). These tests are therefore part of a series of acceptance criteria tests required by regulatory agencies like US Food and Drug Administration (FDA) and required to be included in the public specification repertoire of tests (US Pharmacopeia, 2011b).

Dissolution tests are also an essential requirement for establishing *bioequivalence* and *biowaivers* for generic drug products (Chen et al., 2001; US Pharmacopeia, 2011b). Bioequivalence as defined by US FDA, is the establishment of equivalence of drug bioavailability (BA) between two different formulations (Chen et al., 2001). Biowaivers are waivers for *in vivo* bioavailability and bioequivalence studies by substituting *in vitro* data such as *in vitro* dissolution studies (Löbenberg and Amidon, 2000; Yu et al., 2002). Biowaivers are mostly granted to generic drugs based on their Biopharmaceutics Classification System (BCS) (Yu et al., 2002). Highly soluble and highly permeable

Class I drugs, such as immediate release (IR) oral dosage forms often fall into the category where a request for biowaivers can be made based on rapid *in vitro* dissolution data (Yu et al., 2002). Dissolution tests are therefore used in establishing bioequivalence between formulations, during scale up and post approval changes (SUPAC) or minor formulation changes (Rudman et al., 1996; Yu, 2008). An essential requirement for bioequivalence is for the dissolution test to be discriminatory between different formulations (such as tablets and capsules) and different strengths (Qureshi, 2006). The dissolution method is thus selected based on its ability to discriminate between different formulations (Anand et al., 2011). At least 12 units of each type of formulation needs to be tested to establish bioequivalence between the test and reference product (Anand et al., 2011). United States Pharmacopeia (USP) suggests a list of seven apparatuses for dissolution testing in its chapter <711> on dissolution (US Pharmacopeia, 2011a). The general guidance provided by the FDA for establishing bioequivalence of a generic drug product is to perform a dissolution test by initially selecting one of the seven available USP methods (USP apparatus I to VII). If the USP methods are not predictive of the *in vivo* profile or not discriminatory between the formulations of the dosage form the next approach should be to select a test method suggested by FDA. In case the FDA method is not available or it is found that the results of the test method are not representative of the *in vivo* profile or/and is not discriminatory a dissolution method should be developed (Anand et al., 2011).

Chapter <1092> of the USP describes in detail the guidelines for 'the dissolution procedure' (US Pharmacopeia, 2011b). A primary requirement for a dissolution study

design is its ease of transfer between laboratories and reproducibility. Once an *in vitro* dissolution test has been successfully established, variability of *in vitro* and *in vivo* data is particularly significant in analyzing batch variability, process variables, formulation variables and even analytical variability (Qureshi and McGilveray, 1999). Dissolution method development is thus a delicate balance between the individual components of medium, apparatus and a robust, predictive and applicable study design (Biswas and Halquist, 2016).

## **1.1.2 Components of a dissolution / release test study design**

### *Dissolution medium:*

Selection of the dissolution medium is one of the most significant steps in development of a dissolution method (Galia et al., 1998). The dissolution media would serve the study design in a better way if it were representative of the fluid conditions surrounding the dosage form *in vivo*. Hence, for 'special' dosage forms such as implants and parenteral it becomes critical to select a biorelevant dissolution/release medium. Biorelevance is the simple representation of the *in vivo* physiological conditions in the study design. This helps in establishing clinical relevance for the test procedure and in evaluating further how a change in behavior of the dosage form actually is affected by the *in vivo* factors.

Common factors to consider while selecting the medium are its pH and buffering capacity, osmolality, and changes in pH with changes in temperature conditions (Iyer et

al., 2007c). The dissolution medium also needs to provide sink conditions to the dosage form. Sink conditions can be defined as the volume of media three times the volume in which the dosage form is in saturated solution (US Pharmacopeia, 2011b). Use of surfactants is permitted if it improves the solubility of the drug substance in the media. USP generally discourages using organic mixtures as a dissolution medium unless justifications are provided (US Pharmacopeia, 2011b). The medium in some cases also needs to be de-aerated to remove any bubbles, which might interfere with the diffusion and exchange of drug substance with the media.

For IR solid oral dosage forms the volume recommendation are 500- 1000mL in basket or paddle apparatus(US Pharmacopeia, 2011a). The volume can also be raised to 2-4L if the media needs to meet the sink conditions for the drug substance such as in poorly soluble drugs Class II BCS drugs. In such cases addition of surfactants such as polysorbate 80 for solubilization of the drug substance can be justified (US Pharmacopeia, 2011a). Recommended pH requirements for the media in case of solid oral dosage forms are between pH 1.2 – 7.5 to simulate gastric and intestinal fluids (US Pharmacopeia, 2011a). The general choice of dilute hydrochloric acid, and various simulated fluids such as Fasted State Simulated Intestinal Fluid (FaSSIF- for fasted state intestinal media) and Fed State Simulated Intestinal Fluid (FeSSIF - for fed state intestinal media) with incorporation of components such as enzymes, bile salts to make the media more biorelevant has been observed so far (Galia et al., 1998; Jantratid et al., 2008; Marques, 2004). To summarize dissolution media needs to provide sink conditions and solubility to the dosage form, stability during analytical method validation with minimum interferences to the quantitation of the drug substance, ruggedness and

should not interfere with the study design of the dissolution method being discriminatory between formulations. Added to this if a dissolution medium is biorelevant in its composition such as osmolality, inorganic ion composition and pH it helps in establishing clinical relevance. Factors such as changes in pH of the media, with the release of drug substance and with changes in temperature, especially around the physiological range of 37 °C needs to be evaluated.

*Dissolution apparatus:*

The choice of the dissolution apparatus is generally based on the type of the dosage form. The design of formulation, its unique release, diffusion and disintegration mechanism also affect the selection of the model apparatus. For solid oral dosage forms the most recommended apparatus are USP apparatus 1 and 2 (Brown et al., 2009; Klancke, 2003). The basket apparatus also called as USP apparatus 1, with sinkers is recommended for formulations that float such as capsules. It has nominal volumes of 1 – 2L (US Pharmacopeia, 2011a). The paddle apparatus or USP apparatus 2 is recommended for tablets. Sinkers may also be used with this model if required. Apparatus 3 is the reciprocating cylinder and apparatus 4 is the flow through cell respectively. They are often also used for testing modified or delayed release oral dosage forms. Apparatus 3 allows the flexibility of using different types of media in series for monitoring drug release studies. Apparatus 4 is generally used for drug substances with low solubility. The various *agitation or stirring factors* that are of significance in the different apparatuses are: rotation speed for apparatus 1 and 2, dip rate for reciprocating cylinder/apparatus 3 and flow rates for flow through cell/apparatus 4. These factors ensure proper mixing, release and dissolution of the drug substance in

the dissolution media. Generally in all the apparatuses the recommended temperature for the dissolution media is the physiological temperature of 37°C (US Pharmacopeia, 2011a).

*Dissolution study design:*

The final element, which ties together the choice of dissolution medium and the apparatus selection, is the study design. Dissolution study design requires a comprehensive understanding of the formulation design; the drug substance or active pharmaceutical ingredient (API); release mechanism of the API from the formulation; the rate limiting step: i.e. dissolution, release or permeability and finally the scope of the dissolution method to solve the specific problem in question. The US Pharmacopeia has set required acceptance criteria for dissolution tests which need to be followed especially in cases of establishing *bioequivalence* of generics (US Pharmacopeia, 2011a). Generally the testing is done through three stages: S1, S2 and S3 and the percentage of drug substance dissolved or released is a critical parameter. At stage S1 at least 6 units of the dosage forms are tested and none of the units should have the total amount dissolved less than 5% of the label claim of the API. At stage S2, 6 units are tested again however; at this stage comprising S1+S2 (i.e 12 samples) none of the units should have less than 15% of the API label claim dissolved. Stage S3 is the last stage and a total of 12 units are tested. So by stage S3 a total of 24 units of each formulation are required to be tested. For a successful dissolution test not more than 2 units can be less than 15% of the mentioned label claim of the API and in the whole batch of 24 units no unit can be less than 25% (US Pharmacopeia, 2011b).

Apart from the number of samples tested another key factor in the study design is the sampling method and the selection of sampling time points. Sampling can be performed manually or automated sampling can be used. For automated sampling requisite validation is required for use of the method in conjunction with the apparatus since automated sampling often requires the inclusion of an additional sampling probe component into the dissolution experimental set up. For IR solid oral dosage forms time points of 15, 20, 30, 45 and 60 min are recommended by USP in chapter <1092>, since in most of the cases 85 -100% of API is dissolved in 30- 45min (US Pharmacopeia, 2011b). For extended release and novel dosage forms such as parenteral, it is crucial the study design includes sampling time points such that they bracket the different phases of release: especially in cases of dosage forms with '*multiphasic*' release. This is essential so that the *in vitro* dissolution or release method might adequately represent the *in vivo* release profile of the drug.

Consideration for the analytical test methodology is the last and significant factor in the design of a successful dissolution test (Ermer and Miller, 2006; Wang et al., 2006). Generally UV spectrophotometric methods are recommended for sample analysis over HPLC methods because of their ease of use and time efficiency (US Pharmacopeia, 2011b). HPLC methods though high-throughput require an initial time investment for method development and validation as compared to UV spectrophotometric methods. However, HPLC methods offer certain advantages over simple UV methods primarily when the formulation contains interfering excipients at the same wavelength as the drug substance. A well-developed HPLC method could allow for separation of the excipient

peak from the drug substance peak and help in precise and accurate quantitation of the API. In addition a stability indicating HPLC method also allows the detection of degradants in the dissolution or release media. Detection of appearance of degradants would indicate to researcher either to replace the dissolution media with fresh media in a timely fashion or re-evaluate the dissolution conditions to encourage stability of the released drug substance (Iyer et al., 2007c). Other physical components that a dissolution and analytical scientist should be aware of are the use of filtration and centrifugation if required, as essential tools for sample analysis. The general USP guidelines suggest avoiding the use of centrifugation (since it establishes a concentration gradient in the supernatant) as a means of particulate material separation in the dissolution or release media, and instead suggest the use of in line or off line filters before sample analysis (US Pharmacopeia, 2011b).

### **1.1.3 ‘Release Testing’: Application of dissolution testing to complex non-oral dosage forms**

Non-oral dosage forms often encompass complex dosage forms such as parenterals, implants, drug eluting stents, transdermal patches, liposomes, microspheres and nano particles as injectables. These dosage forms are frequently characterized by complex delivery systems and release mechanisms of the active drug substance (Gregoriadis and Florence, 1993; Widder et al., 1978). With the rapid advances in genetic engineering and recombinant DNA technology in the past few decades the active drug moiety can also be a sensitive and labile biotherapeutic protein, which adds an

additional layer of complexity to the drug product. In the case of non-oral dosage forms dissolution/release tests that ensure performance verification of the drug are called “*in vitro release tests*” rather than dissolution tests (Burgess et al., 2002a). The rationale being often non-oral dosage forms are placed at diverse locations in the body e.g. ocular implants in the chambers of eye, transdermal patches on skin, drug-eluting stents in coronary arteries, suppositories in rectum or urethra, subcutaneous or intramuscular implants all of which have dramatically different physiological milieu (Seidlitz et al., 2011b). As a result the action of the drug depends to a large extent on its *release* or *retention* from the dosage form. For example in the bone regenerating indication INFUSE® Bone Graft the local retention and in turn controlled release of the bone morphogenetic protein from the collagen carrier scaffold is directly related to bone growth and bone density scores (Yasko et al., 1992).

Frequently in ‘novel’ dosage forms such as liposomes, implants, drug eluting stents the drug substance is carried in complex delivery systems e.g. carrier based biopolymer scaffolds such as collagen matrix, injectable biodegradable polymer based matrix which solidifies at the site of action e.g. Atrigel®, in a lipid based system such as liposomes (Malik et al., 2010). These complex delivery systems help in ascertaining drug release in a controlled manner and in some cases for extended periods of time. The release mechanism in each of the above cases is unique. It significantly affects the maintenance of the therapeutic levels of the drug substance in the blood/systemic circulation or in some models e.g. INFUSE® Bone Graft specifically at the local site of action. In drug modalities like transdermal patches the release needs to occur through

multiple membranes and needs to penetrate through the skin and layers of external barriers before reaching the site of action (Barry, 2001). In subcutaneous and intramuscular implants the blood flow conditions might affect the time taken by the drug to reach the targeted site. Therefore, to list a few factors the release mechanism, rate of release, and the time required by the drug to reach the site of action varies notably in each unique case. Hence the *in vivo* performance of these complex (non-oral) dosage forms can be characterized more precisely by carefully designed *in vitro release tests*, with design space criteria that are 'biorelevant'. Although initially dissolution tests were designed as a tool for oral immediate release products they have also been used as a means of ensuring biotherapeutic performance or quality characterization within a defined design space criteria for implant like products. Similar to oral IR dosage forms non-oral/parenteral dosage forms utilize release tests as a means of quality control and testing variation in formulations.

#### **1.1.4 Significance of biorelevance while designing release study designs in 'novel' dosage forms:**

The significance of biorelevance for novel dosage forms such as implants increases manifold due to their complex release and pharmacokinetic tissue distribution of the drug substance e.g. varying viscous ocular compartments in case of ocular implants. Biorelevant parameters, which are often of significant influence, are criteria pertaining to the physiological positioning of the dosage form and site of action of the drug. Incorporation of biorelevance in an *in vitro* release test would involve a) recognition of the 'crucial' *in vivo* parameters that significantly affect release of the drug from the

dosage form and b) selection/inclusion of these parameters in the *in vitro* design space. These *in vivo* factors are frequently temperature, blood flow rates, tissue barriers, and acidity of the microenvironment, osmolarity and pH. These parameters can considerably influence the release of the active drug substance from the dosage forms as well as influence their therapeutic effect at the site of action. To make the test more predictable and clinically relevant it is essential to incorporate at least some or most of these factors into an *in vitro* release test. Inclusion of these parameters can also help the researcher in studying how a minor change in one of the parameters affects a) the other parameters and b) the overall release of the drug from the dosage form for future formulation changes.

Similar to IR oral dosage forms application of biorelevance in release testing of non-oral dosage forms would ensure that the release test data is clinically meaningful and also predictive for detection of changes in the post approval drug product. For complex non-oral dosage forms designing of the *in vitro* release test and selection/modification of the appropriate apparatus requires careful research to prevent addition of unrequired layers of complexity to the design space. However, the long-term predictive capabilities of such “*in vitro* release test” far outweigh the initial design inconvenience. Dissolution/release is a test model where a delicate balance needs to be established between the lure of simulation of physiological variables as opposed to making the test simple and more reproducible. Introduction of too many design variables in the design space with the intention of focusing the test towards biorelevance can also cause the introduction of overly complex and non-predictive factors. A very simple biorelevant test

maybe rejected on the premise, that it does not provide sufficient discriminatory capability towards crucial process parameters. The paradox thus is, although addition of more biorelevant parameters makes the test clinically meaningful; at the same time addition of complex physiological parameters and their interplay might make the release test less reproducible. It is essential that ultimately the *in vitro* release test is an optimal predictor of the beginning phase (e.g. burst release phase), middle and end phase of the *in vivo* release profile.

However, unlike oral dosage forms where a single standardized USP method or apparatus can be used for dissolution testing of a class of compounds, for complex non-oral dosage forms the apparatus and the method used for testing release often are required to be adapted. Both the release testing apparatus and method parameters have to be selected or modified according to crucial *in vivo* parameters to design a biorelevant reproducible and predictable *in vitro* release test.

### **1.1.5 Currently available *in vitro* models for release testing of implants**

The current standardized tests offered by the United States Pharmacopeia (USP) though suitable for oral immediate release (IR) products do not address the specific needs of designing a biorelevant or biomimetic study design for 'novel' dosage forms. Having a biorelevant model to predict or relate to the pharmacokinetics of locally and systemically delivered controlled release biotherapeutic would help establish clinical pertinence. Depending upon the development level of the model it could possibly also

offer valuable insights towards the discriminatory capability of various process variables within specific design space. Currently, there are no regulatory standards for the release testing of implants. The United States Pharmacopeia (USP) currently offers *in vitro* release testing methods (seven apparatuses - USP I to VII) that may not be suitable for implant products e.g. INFUSE<sup>®</sup> Bone Graft. These apparatuses are not standardized for parenterals such as implants, microspheres. The tests do not allow simulation of the unique physiological environment(s) to which the product is exposed and hence may not be good predictors of the *in vivo* performance (Bhardwaj and Burgess, 2010; Siewert et al., 2003). It is beneficial to the researcher to ascertain the applicability of the USP model before applying to the release tests. The complex physiological positioning of implant based dosage forms causes a lack of information regarding the consistency of the design space criteria and variables. This has prompted the FDA to exercise caution in establishing regulatory guidelines for the dissolution/release testing of such “novel” dosage forms. Currently, the most common approaches for *in vitro* release testing of parenterals and implants are: sample and separate methodology, flow-through cell (USP-IV), and modified USP-IV such as dialysis methods (Bhardwaj and Burgess, 2010). Hybrid methods of flow-through and dialysis are also in use by some labs; however, none of these methods have been standardized for parenteral products (Xu et al., 2012).

#### **1.1.5.1 Sample and separate methodology**

Sample and separate is currently the most popular method for *in vitro* release testing of implants due to its ease of use (D'Souza and DeLuca, 2006). The method entails a

simple suspension of the dosage form in a certain amount of media to allow the maintenance of sink conditions. Sampling occurs at different time intervals to best capture the release profile. Generally, the burst release phase (specially applicable in case of polymer based scaffolds where release of drugs due to diffusion might occur), the middle and the end phase (>80% release) needs to be captured. Samples collected at each time point are analyzed to determine the amount of drug released. This is followed by either entire media replacement or the amount sampled at each time point. Sample and separate experiments should be adjusted according to the volume of media required for sink conditions especially in case of parenteral products like implant and microspheres and are not required to be performed in large volumes of media as in USP apparatus 2.

*Advantages of sample and separate methodology:* Agitation or stirring can also be included as a parameter in sample and separate experiments to increase release and affect the *in vitro* release profile and achieve a good *In Vitro In Vivo* Correlation (IVIVC). Sample and separate experiments entail an adjustment of the various factors such as agitation, sink conditions, sampling volume and sampling frequency. The method is useful in establishing experimental baselines such as checking the performance of the product for inter lab variability, in determining the most optimized handling conditions for complex dosage forms such as microspheres, liposomes or protein based therapeutics. The method also helps in investigation of the interaction of the dosage form with the release/dissolution media and its stability in the media. Once the baseline and boundary conditions for the release test are known to the researcher, (media selection, drug stability boundaries of time and solubility, sampling volumes and media

replacement with sampling at each time point) a more complex, predictable and physiologically biorelevant release or dissolution apparatus can be selected. Changes can then be made to the flow rate, container material, and incorporation of physiological vital parameters that are associated with release.

*Disadvantages* of this method are: the simulation of crucial *in vivo* parameters affecting the dosage form is very limited with this technique. It lacks the vessel hydrodynamics and functional modules to simulate biorelevant parameters affecting the dosage form. The lack of defined hydrodynamics might also cause uneven distribution of the drug molecule in the media, thereby giving rise to variability between experiments. Dosage form aggregation might also be observed due to the lack of appropriate media flow. In addition there is no physical separation of the dosage form from the media which maybe required for certain special carrier scaffolds like collagen, hydroxyapatite particles, microspheres and liposomes. This makes sampling at each time point difficult.

#### **1.1.5.2 USP-IV Flow through cell methodology**

Continuous flow release methods such as USP-IV flow through cell have been used as an industry standard for *in vitro* release testing of extended release dosage forms (Fotaki et al., 2009; Thomas, 2016; Xu et al., 2012; Zolnik et al., 2005). A USP-IV flow through experimental set up comprises of various components: glass cells constituting the USP-IV apparatus, small glass beads to position the dosage form, membranes or a large glass bead of diameter 5mm to prevent the flow of the glass beads and dosage form positioned on top of the cell inlet tubing, a pump for driving the flow of the media,

tubings, a reservoir to maintain sink conditions, and finally an incubator or water bath which generally maintains the reservoir and its fluid at the required temperature (USP, 2012).

Traditional USP-IV flow through cells as described by US Pharmacopeial Convention in Chapter <711> on dissolution are generally of two types: a) 26mm i.d. cell and b) 12mm i.d. cell. They are called as the large cell (fluid volume 19mL) and small cell (fluid volume 8mL) respectively (USP, 2012). Each has a tablet holder for tablet and capsules. The tubing connecting the cell to the pump driving the media should be as small as possible in length. Care should also be taken to select tubing of inert material so that the drug substance e.g. protein has minimal adsorption and chemical interaction with the material of the tubing. Media is generally forced up the inlet and into the flow through cell. The dosage form e.g. tablet or capsule in case of immediate release dosage form and implants in case of non-oral dosage forms is placed perpendicular to the path of the flow. The addition of small glass beads helps in making the flow laminar and not turbulent (Burgess and Wright, 2012). Either half of the cells are held together by two O-rings and a steel clamp. An attempt must be made to physically position the pump driving the media on a different surface from where the USP-IV apparatus is placed. This would protect the cell from any unplanned agitations (such as vibrations due to the pump).

*Closed or Open Loop flow of media:* A flow through cell set up can be configured in a *closed or open loop* system. In an open loop system fresh dissolution or release media

is continuously pumped into the flow through cell via the reservoir. In a closed loop system the media volume is kept constant and the same media is circulated in a loop through the dosage form. The volume of media to be circulated is calculated based on the net diameter of the flow through cell chosen, the tubing connections and the sink conditions for the drug substance. Generally sink conditions are chosen as three times the solubility of the drug substance in the media as defined by the USP (Burgess and Wright, 2012; US Pharmacopeia, 2011b). An open loop configuration is generally selected for dissolution studies involving poorly soluble drugs whereas closed loop is selected for drugs with good solubility.

*Advantages of using the USP-IV apparatus* are evaporative losses are minimal with this apparatus and the hydrodynamics of the apparatus have been well studied and defined. Adjustments to sample immobilization (i.e. glass beads for positioning), flow rate, and media recycling can also be made as necessary.

*Disadvantages* of this method are filter blocking and clogging due to disintegration of the dosage form caused by flow. This is observed particularly when used with polymer materials such as PLGA and collagen carrier scaffolds, which lose material from the surface over an extended period of time. The blocking of the filter material by damaged formulation components causes excess backpressure since the flow of the media in USP-IV is through the filter membrane. This leads to disruption of the experimental set up for extended release dosage form studies and greater variability in measurements. Drug or protein adsorption on tubing or glass beads and specifically on the glass flow through cell (for protein biotherapeutics) leads to loss of the measured drug substance or API and again enhanced variability. Another crucial factor is the minimal volume of

the flow through cell cannot be decreased beyond a certain value. This is important in case of implants and some parenteral which require minimal media volume to mimic physiological biorelevant conditions.

### **1.1.5.3 Dialysis methodology**

*Dialysis* is an important modification often incorporated in many release test study designs (Bhardwaj and Burgess, 2010). Dialysis methods are also used where the sample is required to be segregated from the bulk media by placement in a dialysis sac (D'Souza and DeLuca, 2005). This helps simulate a permeation barrier. The dialysis sac can be an artificially available applied membrane with requisite molecular weight cut off (MWCO). The sac allows the drug to diffuse out and into the media for sampling. Dialysis techniques can be performed where the drug is placed in the sac and sampling takes place in the media or reverse dialysis where the drug is placed outside the sac and sampling takes place inside the sac. Many a times *in vitro* release profiles obtained in these cases do not correlate with the *in vivo* performance, which may be derived from lack of agitation and subsequent aggregation of dosage forms like microspheres inside the dialysis sacs (Burgess et al., 2002b). Frequently, one or a combination of these methods (i.e. sample and separate and dialysis or USP-IV modified with dialysis adapters) are used by researchers in designing biorelevant release tests for unique dosage forms depending on their needs (Bhardwaj and Burgess, 2010). It is therefore critical to choose the most relevant apparatus for release testing of complex dosage forms. Efforts have been underway to develop modified, biorelevant *in vitro* release

testing approaches for accurate release characterization of novel products (Delvadia et al., 2012; Iyer et al., 2007a; Iyer et al., 2006).

### **1.1.6 Evaluation of *in vitro in vivo* relationships**

Biorelevant 'real time release tests' can be of particular significance during scale up and post approval stages of a drug (SUPAC) : a) for monitoring and predicting the outcome during minor formulation changes, b) as a quality control test during the commercial manufacturing process for monitoring of 'batch to batch variability' (Brown et al., 2011; Siewert et al., 2003) and c) can essentially function as a discriminatory dissolution/release model between different formulation variants. The primary expectation from a well-developed and validated biorelevant test method would be the ability to predict in terms of release or dissolution, how any intrinsic variation in any of the process variables of commercial manufacturing conditions would affect changes in the release profile of the drug. At the same time the principal impetus behind developing such tests are also to: 1) minimize time 2) cost and 3) the significant reduction of animal clinical trials. Over the past few decades, applying biorelevance in release and dissolution testing has proven to be a powerful tool towards providing *In Vitro In Vivo* Relationships/Correlation (IVIVR/IVIVC). Such predictive relationships obtained from IVIVR/IVIVC can be effectively translated to clinically meaningful specifications and contribute towards relevant information pertaining to the performance of the drug. However, often times since such complex dosage form might have only one kind of formulation and reaching a Level A correlation or meeting the levels of correlation as that for an immediate release dosage form might be difficult. Due to the complexity in

physiological positioning of parenterals, implants, injectable liposomes based dosage forms there is lack of information regarding the consistency of the design space criteria and variables. This has prompted the FDA to exercise caution in establishing regulatory guidelines for the dissolution/release testing of such complex non-oral dosage forms. There is sufficient evidence for a significant need to develop adaptable *in vitro* model systems that can be predictive of *in vivo* formulation release of complex non- oral dosage forms.

## **1.1.7 IVIVC/IVIVR/IVR**

### **1.1.7.1 IVIVC for Parenteral/ IVIVR**

*In Vitro In Vivo* Correlations serve as essential tools in guiding and speeding up the process of drug development by providing a scalable and predictive relationship. A successful IVIVC helps in predicting the *in vivo* performance of the drug in a time efficient way with the help of *in vitro* dissolution profiles. For obtaining a predictive IVIVC it is essential that a clinically biorelevant predictive release method be developed. Since dissolution or release tests are predictive, repeatable and set up in a controlled environment in the lab they can be easily used to save time and predict *in vivo* release of either different formulations or minor changes to a formulation.

There are four levels of IVIVC: Level A, B, C and D as described by the FDA guidelines for extended release dosage forms (FDA Rockville, 1997). A level A correlation is a point to point correlation between *in vitro* dissolution data and *in vivo* absorption data. It

represents a relationship between *in vivo* fraction of drug absorbed and *in vitro* fraction of drug dissolved. The fraction of drug absorbed *in vivo* is obtained from the *in vivo* pharmacokinetic profile using deconvolution and transformation of data on drug plasma profiles. The transformation of data is carried out using methods such as Wagner Nelson or Loo Riegelmann. Generally a linear relationship is expected of a Level A correlation and is most commonly used to establish bioequivalence and biowaivers. A level B correlation demonstrates a relationship between mean *in vitro* dissolution time (MDT *in vitro*) and mean *in vivo* residence time (MRT *in vivo*). Since a MRT *in vivo* and MDT *in vitro* is based on the principle of statistical moment analysis and can be obtained from a number of plasma *in vivo* and *in vitro* dissolution/release profiles, it is not a point to point correlation. Level C correlation specifies a relationship between a dissolution profile parameter and a *in vivo* pharmacokinetic parameter such as AUC, C<sub>max</sub> or T<sub>max</sub>. The *in vitro* dissolution parameter can be a parameter such as time taken for 50% of the dissolution to occur T<sub>50%</sub> or percent dissolved in 4hrs (FDA Rockville, 1997). As is evident it is a single point correlation and not as predictive as a level A point to point correlation. A level D correlation is the final stage of correlation and is a rank order correlation. Since level A IVIVC is the highest degree of correlation it is often the targeted standard. An IVIVC is validated by measuring the percentage prediction error (%PE) for its ability in predicting AUC, C<sub>max</sub> and other relevant pharmacokinetic factors. The industry standard is for the % PE to be within ± 10% (FDA Rockville, 1997).

In many cases such as immediate release dosage forms where dissolution is rapid it is observed that the *in vitro* dissolution progresses at a much rapid rate than *in vivo*

absorption. In such release/dissolution profiles the *in vivo* absorption lags behind the *in vitro* dissolution and makes achieving a linear correlation difficult. In such studies either scaling can be used and a predictive 'relationship' can be determined between the *in vitro* and *in vivo* profile (Polli, 2000). The primary aim of developing such an *in vitro in vivo* relationship (IVIVR) like IVIVC, is to determine a predictive or/and scalable *relationship* between *in vivo* absorption profile and *in vitro* dissolution profile.

Immediate release dosage forms require much shorter time *in vivo* for their release. However, in case of parenteral or extended release parenteral like INFUSE<sup>®</sup> Bone Graft this time is further extended. Thus, an *in vitro* dissolution or release test, which, is accelerated, can be very helpful in speeding up the drug development process. In case of parenteral an IVIVR might not be always possible. Achieving particularly a Level A, Level B, Level C correlation might not be possible. In such a case scenario an IVIVR can act as a good predictor of the *in vivo* profile.

### **1.1.7.2 Challenges with developing IVIVC/IVIVR for complex dosage forms**

Although many studies and IVIVC have been established in the past for immediate release dosage forms and extended release oral dosage forms, application of IVIVC/IVIVR to complex dosage forms such as parenterals (liposomes, nanoparticles, carrier based biodegradable implants, ocular inserts and drug eluting stents) have been limited in literature (Shen and Burgess, 2015). Complex dosage forms give rise to multiple factors that offer hindrance to a conventional IVIVC or IVIVR.

First, complex or special dosage forms such as INFUSE® Bone graft, ocular implants are placed in complex *physiological environment* such as maxillary sinus cavity and ophthalmic chambers of the eye. These regions generally vary greatly in their vascularity, fluid viscosity, fluid pressure, and organic and inorganic composition of fluids. This gives rise to major changes in the drug release, diffusion, distribution and clearance processes. In many situations such as INFUSE® Bone graft the systemic clearance of the drug substance rhBMP-2 protein is high. The  $t_{1/2}$  of rhBMP-2 in rats and non human primates is 16 minutes and 6.7 minutes respectively (FDA Rockville, 2014). In such scenarios getting precise plasma concentration pharmacokinetic profile is difficult because of rapid systemic clearance and difficulty *in vivo* in accessing the complex tissue chambers. In case of INFUSE® Bone graft the performance efficiency of the dosage form has been measured by the retention of rhBMP-2 in the carrier scaffold at the *local* site rather than in the systemic circulation. Local retention has been in turn correlated in multiple studies with bone growth in terms of height and density and compared to autologous bones (Boyne et al., 2005; McKay et al., 2007). Therefore, in such carrier based complex dosage forms retention in the scaffold can be the primary indicator of bone healing and regeneration. Development of an IVIVR or establishing an *In Vitro Relationship* (IVR) in such a case would be more clinically relevant, if it would enable to establish a predictive relationship between *in vitro* release and *in vivo* retention. Such a relationship then would be an indicator of how minor changes in formulation, stability, manufacturing would affect the local retention and hence performance of the dosage forms.

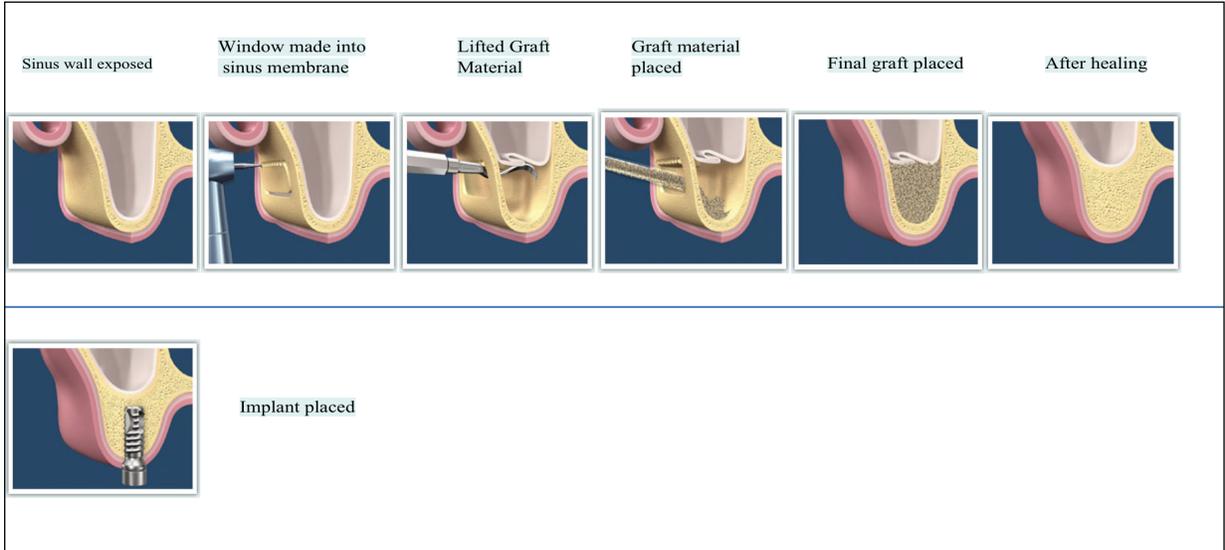
Secondly, especially in case of controlled release dosage forms a *multiphasic* release is observed. Dosage forms such as liposomes, carrier based scaffolds (INFUSE<sup>®</sup> Bone graft) show an initial phase of burst release *in vivo* followed by an exponential steady release and finally a lag phase in release (Monkhouse et al., 2003). The initial burst release phase can be due to diffusion controlled processes or presence of enzymes and proteins in the plasma such as collagenases which speed up the initial release. Also an externally placed implant in the physiological environment maybe subjected to local effects of cells as a part of the inflammatory stage, which might attack and partly clear off the carrier scaffold or polymer resulting in a *burst* release phase. Many times degradation products of the polymer such as PLGA, or scaffold such as collagen are known to accumulate in the tissue healing microenvironment and may increase or decrease the pH of the surrounding tissue fluid thereby affecting release.

Although biorelevant media can be best picked for biorelevant release studies depending upon its inorganic ion concentration, pH and osmolality mimicking exact variability of the complex plasma protein concentrations is difficult. In addition to the variability, introduction of serum or plasma proteins into the *in vitro* release testing methodology gives rise to problem with the analytical methodology due to interference of these components of the plasma, and makes such release tests less predictable. Hence, it is better to use biorelevant media components, which have minimal to no interference in the quantitation and analytical reproducibility of the active pharmaceutical ingredient (API).

Lastly, the biorelevant apparatus and the analytical methodology can themselves pose difficulties for characterizing release and developing a predictive release method. To develop a clinically relevant release/dissolution method it is essential to add parameters to the release test that mimic the actual physiological environment without making the test too complex to be performed in a lab setting or compromising the ability of the test to be reproducible. Addition of such parameters help in predicting changes in the behavior of the dosage form, with, changes in these biorelevant parameters. The analytical test methodology can also have an impact on the development of the release test. A stability indicating analytical method capable of detecting degradation products in the *in vivo* physiological environment and also for release media should be used to calculate of the amount of drug released.

## **1.2 DESCRIPTION OF THE MODEL DOSAGE FORM: INFUSE<sup>®</sup> BONE GRAFT**

INFUSE<sup>®</sup> Bone Graft is an osteoinductive and osteointegrating implant based dosage form for *de novo* regeneration of biologic bone. It has been used in many parallel indications such as spinal fusion in patients with degenerative disc disease, healing of tibial shaft fractures and in sinus lift/sinus augmentation surgeries (FDA Rockville, 2001, 2014; Medtronic, 2011)(FDA Rockville, 2001; Medtronic, 2011). Figure 1-1 is a pictorial representation of INFUSE<sup>®</sup> Bone Graft being used in sinus lift surgery (Medtronic, 2011).



**Figure 1-1.** A pictorial representation of application of Infuse Bone Graft during sinus lift / augmentation surgery

(Medtronic, 2011)

INFUSE<sup>®</sup> is composed of two major components: 1) recombinant human Bone Morphogenetic Protein-2 (rhBMP-2) which is the functional bone regenerating protein at a concentration of 1.5mg/mL and 2) Absorbable Collagen Sponge (ACS), the carrier scaffold for the protein. ACS is a bovine Type I collagen cross-linked by formaldehyde and sterilized by ethylene oxide to increase its retention capability for rhBMP-2. ACS serves two primary functions: 1) It acts as a carrier for the rhBMP-2 to enhance its retention at the local targeted trauma/healing site and 2) It acts as an adjuvant scaffold for the invading osteoprogenitor cells to act as a supportive matrix for initiating the process of bone growth.

## **1.2.1 Structural and Physicochemical properties of rhBMP-2**

### **1.2.1.1 Structural properties of rhBMP-2:**

Bone Morphogenetic Protein-2 (BMP-2) is a member of the Transforming Growth Factor- $\beta$  (TGF- $\beta$ ) family of signaling molecules (Clark and Coker, 1998). TGF- $\beta$  are multifunctional cytokines that regulate inflammation and immunity. They are also vital to growth, cellular proliferation and differentiation during both the adult and embryonic phases (Clark and Coker, 1998). BMP-2 is initially synthesized as a 453 amino acid proprotein. The signal peptide and the propeptide regions of this proprotein are then cleaved to give a 114-residue monomer (Porter et al., 2004; Scheufler et al., 1999). The monomers further form dimers by a disulfide linkage. Since rhBMP-2 in INFUSE<sup>®</sup> Bone graft is synthesized in the Chinese Hamster Ovary (CHO) mammalian cell line, it is also glycosylated and folded into a homodimer within the cell itself. Unlike general globular

proteins the TGF-  $\beta$  family lacks a stabilizing hydrophobic core. As a result the protein acquires stability by a distinct cysteine knot structure and by dimerization. The cysteine knots are formed within a monomer between 6 cysteine residues: Cys296->Cys361; Cys325->Cys393; Cys329->Cys395. The dimerization occurs by a covalently linked disulfide bond formed between Cys360 residues of the two single units. This results in a hydrophobic like core and provides the required stability to the protein. Although as a monomer the hydrophobicity of BMP-2 is extremely low (calculated hydropathy score: -.56 (Gasteiger E., 2005)) the dimerization renders BMP-2 with a) sufficient hydrophobicity to be eluted by reverse phase chromatography column and b) also leads to its aggregation under unfavorable conditions of pH, temperature and storage (Scheufler et al., 1999)]. The net dimensions of the homodimer are 70 Å × 35 Å × 30 Å (Scheufler et al., 1999).

The actual processed peptide is from Threonine266 or Glutamine283- 396 arginine residues depending on the N terminal cellular processing. The protein has a 'NST' is the single N-glycosylation site of the protein beginning at Asparagine338 (Bernstein et al., 1977; Kouranov et al., 2006)

#### **1.2.1.2 Physicochemical properties of rhBMP-2:**

The physicochemical properties of rhBMP-2 were calculated using ExPASy- ProtParam tool available online (Gasteiger E., 2005). This step helps establish a basic idea about the hydrophobicity and theoretical calculated pI of the protein (Scheufler et al., 1999).

ProtParam helps in computing various physicochemical parameters for proteins (Gasteiger E., 2005). These are deduced based on the protein sequence and modeling of this sequence in three dimension. The software generates a Grand Average of Hydropathy (GRAVY) value for a protein or peptide. The hydropathy scores give a basic idea about the hydrophobicity of the protein. The GRAVY value for a protein is calculated by summing the hydropathy values of each amino acid divided by the net total number of residues in the molecule. Since chromatographic methods basically work on the mechanism of partitioning of the analyte between the mobile and stationary phases knowledge about hydrophobicity of the protein molecule can be useful especially in RPLC in providing ideas about the kind of column that must be selected, for the development of an RPLC method. The calculated hydropathy score/GRAVY for rhBMP-2 was: - 0.56. This meant that the protein had comparatively low hydrophobicity (hence the C3 column selected in the development of the method). The theoretical pI calculated by the software was 8.92. Knowledge of the pI of the protein helps in determining what net charge the protein will carry at the sample injection pH and the mobile phase pH. This helps in approximately estimating the degree of hydrophobicity of the analyte molecule. In conclusion, all these factors added together such as: a) the dimensions of the protein (essential for determining the pore size of the column); b) hydropathy scores (for selecting the type of column e.g.C3-C18 or column functional group extensions); c) protein pI (for determining the pH of the eluting mobile phase and sample injection pH at which the protein moiety will be most stable) help in getting an optimal analyte response during chromatographic method development and validation.

## 1.2.2 Factors affecting rhBMP-2 release from its carrier biopolymer

### 1.2.2.1 rhBMP-2 release from ACS:

Recombinant human Bone Morphogenetic Protein-2 (rhBMP-2) is a growth factor that helps in bone growth and differentiation. The delivery of rhBMP-2 for osteoinduction requires a *prolonged retention*, at supraphysiological doses, at the site of action. For this purpose the best strategy for rhBMP-2 delivery is using a carrier scaffold that can *retain* the protein at the implant site. A distinct positive correlation has been found between the retention of rhBMP-2 at the local site of action and osteoinduction (Uludag et al., 2001). An ideal carrier for rhBMP-2 needs to strike a delicate balance between: biodegradability and controlled *in vivo* degradation. The 3D porous structure for cellular adherence and penetration by osteoprogenitor cells, needs to be *malleable* enough to be packaged into wound healing sites. At the same time, it has to be *tensile* enough to withstand a) the force of packaging b) not collapse and c) retain the incorporated rhBMP-2 under masticatory pressure for controlled release over several days. A few of the required attributes in a carrier suited for rhBMP-2 delivery have been listed in Table 1-1. Collagen scaffolds i.e Absorbable Collagen Sponge (ACS) used in INFUSE<sup>®</sup> Bone graft meets almost all the requirements mentioned in Table 1-1 to a considerable extent (Friess, 1998). Collagen sponges have been used widely in clinical settings as hemostatic agents. The mechanism of *release* of rhBMP-2 from ACS is thus a complex balance of two principal characteristics: a) *binding or retention* of rhBMP-2 in ACS and b) ACS biodegradation at the requisite rate.

### 1.2.2.2 Mechanism of rhBMP-2 release from ACS:

The mechanism of rhBMP-2 retention and release from the ACS was primarily elucidated by Friess et al. (Uludag et al., 1999b). They studied the effects of *in vitro* sponge characteristics, protein pI and retention on *in vivo* rhBMP-2 pharmacokinetics (Friess et al., 1999a; Uludag et al., 1999b). rhBMP-2 has an isoelectrical point of ~8.5. Thus, it carries a net positive charge at physiological pH. The binding of rhBMP-2 on the collagen scaffold is mostly by non-covalent interactions like *electrostatic and hydrophobic interactions* between the rhBMP-2 protein and collagen residues. However, changes in various physicochemical and biological properties of the collagen sponge and the rhBMP-2 solution can influence the above interactions. Collagen scaffolds generally show a biphasic release profile- a) the initial burst release phase and b) sustained release for days to a month (Friess et al., 1999b; Geiger et al., 2003). The various significant factors that had a major effect on the retention and release of rhBMP-2 from ACS were: pH, ACS mass, anion concentration and crosslinking and sterilization of the ACS. Their effects on the mechanism of rhBMP-2 release from and retention in ACS have been discussed in detail below:

*Effect of pH changes:* Equilibrium binding studies of rhBMP-2 and ACS were conducted (Friess et al., 1999b). The ACS was initially soaked in excess rhBMP-2 solutions at varying pH and incubated. After 24-48 hr the amount of rhBMP-2 bound to the ACS was found as the percentage incorporated (%Inc). It was observed that by increasing the pH

**Table 1-1.** Critical attributes of an ideal rhBMP-2 carrier

(This table has been modified from references (Burg et al., 2000; Geiger et al., 2003b))

- 
1. Permits cellular infiltration, cellular adherence and vascularization – Acts as a supportive matrix.
  2. Sufficient binding affinity to rhBMP-2.
  3. Degrades into biocompatible components – biodegradable.
  4. Low immunogenicity and – biocompatibility
  5. Can be packaged into a physiological trauma site – malleable.
  6. Can withstand compression without structural collapse- tensile.
  7. Controlled release of rhBMP-2 during the period of bone healing.
-

of the rhBMP-2 solution from pH of 3 to a pH 6.5 the mg/mg of rhBMP-2 binding to ACS equilibrium binding increased from less than 0.01 mg/mg to 0.18 mg/mg. This binding and retention mechanism of rhBMP-2 with ACS was explained by the difference in their isoelectrical points. The alkali treated collagen has cleaved negatively charged asparagine and glutamine residues, which imparts a net pI of 5.1 to collagen. The pI of rhBMP-2 is between 8~9. Thus, rhBMP-2 carries a net positive charge at the above-mentioned pH of 3-6.5. At the same time with an increase in pH beyond the pI of 5.1 for collagen the negative charge on the molecule increases. The result is a net increase in binding between the two protein molecules as we move from a pH of 3-6.5.

ACS mass was also found to have a significant effect on rhBMP-2 protein binding (Friess et al., 1999b). It was observed that with an increase in ACS mass the reduction in protein loss due to mechanical pressure during implantation could be minimized. This was due to the maximization of rhBMP-2 incorporated in ACS at a higher mass. The increase in ACS mass facilitated greater availability of rhBMP-2 binding sites and hence enhanced retention of the rhBMP-2 in the scaffold.

Anion concentration was also found to have an effect on rhBMP-2 retention and release mechanism from the ACS (Friess et al., 1999b). When ACS is equilibrated with increasing concentrations of chloride ion ranging from 5mM to 15mM enhanced retention occurs from 50% at pH 5.1 to approx. 80%. However, an anionic increase above 20mM for sodium chloride ions leads to rhBMP-2 precipitation.

Crosslinking and Sterilization also affected the rhBMP-2 incorporation and release from ACS (Friess et al., 1999a). Crosslinking was carried out with *formaldehyde* and sterilization of the sponge with *ethylene oxide*. Crosslinking led to a stronger interaction within the collagen mesh and made it more tensile. Sterilization by ethylene oxide caused a reduction in the denaturation temperature of ACS and made it more sensitive to collagenase (which at a controlled rate is required for biodegradation of the scaffold) (Uludag et al., 2001). The non-crosslinked/non-sterile, crosslinked/non-sterile and crosslinked/sterile ACS were implanted in a rat ectopic model with radiolabelled  $^{125}\text{I}$ -rhBMP-2. The *in vivo* release kinetics was studied by sacrificing two positive controls (4 implants) and two negative controls at each time point. The implants were excised and the total radioactive counts were calculated as the percentage of rhBMP-2 retained. The *in-vivo* release kinetics was elucidated non-compartmentally and by a bi-exponential model. The  $t_{1/2a}$  and the  $t_{1/2b}$  of the two phases of the model were 10min and 89hr. It was observed that crosslinked/sterile sponges gave the highest mean residence time for  $t_{1/2b}$  *in vivo* of 89hr (Uludag et al., 1999b).

### **1.2.3 Mechanism of action of INFUSE<sup>®</sup> Bone Graft**

rhBMP-2 acts as a chemotactic agent for attracting mesenchymal stem cells and osteoblasts, to the local application site by initiating chemotactic migration of cells (Ebara and Nakayama, 2002; Li and Wozney, 2001) . Once the required pluripotent cells have migrated to the site rhBMP-2 binds to the receptors on their surface and causes their differentiation into bone generating cells-osteoblasts. The advantage of using rhBMP-2 on a biodegradable scaffold in comparison to a patient's autologous

bone is there is no inflammation and secondary complications like pain and healing delays associated with the bone harvested *secondary* site. INFUSE<sup>®</sup> Bone Graft induced bone when compared to autologous bone showed that the density of the newly formed bone was higher than that of autologous bone and it functioned and responded normally. Revascularization and consolidation of the new bone occurred as would occur for autologous bone which, is currently the gold standard for bone regeneration.

However, rhBMP-2 has some side effects, since it is a member of the TGF- $\beta$  family of inflammatory molecules it cannot be used in patients with tumor/malignancy or those suffering from active inflammation at the trauma site. INFUSE<sup>®</sup> Bone Graft shows a distinct burst release phase during which about 25% of the molecule is released from the scaffold which might adversely affect the trauma site that might already be experiencing inflammation. Nor can rhBMP-2 be used in pregnant women since BMP-2 is actively involved during the stages of fetal cell differentiation (Chen et al., 1997). This limits the use of rhBMP-2 due to potential risks and hence the need for a better understanding of the controlled release of rhBMP-2 from its scaffold and exploration of its elusive local pharmacokinetics.

#### **1.2.4 INFUSE<sup>®</sup> Bone Graft as a model test implant for biorelevance in vitro release studies**

While designing our biorelevant *in vitro* system we decided to select INFUSE<sup>®</sup> Bone Graft as a model implant as it would be a classic test-bed considering 1) it's wide yet

novel usage in orthopedic trauma and healing surgeries for the regeneration of bone and 2) In spite of the predictable clinical performance of INFUSE<sup>®</sup> Bone graft rhBMP-2 has been associated with many safety and efficacy issues due to its less studied local release pharmacokinetics from the ACS, which might have unprecedented effects in patients. There is a tremendous scope to study how different formulations involving different carrier matrices might affect the release kinetics of rhBMP-2. Controlling the initial burst release phase would significantly affect the safety and efficacy of the formulation. This model biorelevant *in vitro* system when developed can be translatable and applied to study the local and systemic release of other protein formulations. Although the release of rhBMP-2 from ACS using radiolabelled <sup>125</sup>I has been studied (Uludag et al., 1999a), this research will provide a means of studying and quantifying the release *in vitro* in a release study system using a label free methodology of rhBMP-2 detection. Application of our developed and validated High Performance Liquid Chromatography (HPLC)-UV method would limit destructive sampling of the implant at each sampling time point. This biorelevant *in vitro* model will help in predicting and relating *in vivo* orthobiologic delivery (IVR) of the only marketed bone tissue regenerating molecule rhBMP-2.

### **1.2.5 Model Design and innovation**

The engineered biorelevant *in vitro* model will be a novel approach towards testing an intra-osseous implant at a complex physiological position involving low vascularity and a distinct microenvironment. In addition to the model system having multiple chambers for testing local release, the model also facilitates physical separation of the carrier scaffold

from the release media if required. The novel biorelevant in vitro model design helped in studying the effects of flow directionality on the release of rhBMP-2 from ACS.

According to the guidelines and specifications provided by the USP chapter <1092> the release test design was performed in a step-by-step modular approach for this research investigation. Chapter 3 of the thesis describes the development and validation of a stability indicating HPLC-UV method for the quantitation of rhBMP-2 (the drug substance) in the release medium. Chapter 4 of the thesis introduces the use of conventional methodologies such as sample and separate followed by a USP method-flow through cell and finally leads to the development of an in-house release model called Biorelevant *In Vitro* Drug Release (BIVDR) model and its study for discriminating between formulations. The *in vitro in vivo* relationship was evaluated with the help of Sprague Dawley rat cumulative release profiles and a predictive relationship was established. The difference (f1) and similarity (f2) factors were calculated to investigate the discriminatory capability of the developed release study design.

## CHAPTER 2

### 2 DISSERTATION OBJECTIVES

*In vitro* dissolution/release testing is an essential tool for performance verification of a dosage form in pre-clinical and clinical stages. These tests help us in development and evaluation of an *in vitro in vivo* relationship/correlation (IVIVR/IVIVC). IVIVRs help establish a predictive clinical relevance of the *in vitro* release test. With the development in the fields of biotherapeutic modalities the number and diversity of ‘novel’ non-oral dosage forms placed in complex physiological tissues have also taken a leap forward e.g. ocular implants, drug eluting stents, sinus augmentation implants, spinal implants (McKay et al., 2007; Seidlitz et al., 2011a; Wong et al., 2001). The primary objective of this research is to develop a biorelevant *in vitro* release test model for a ‘novel’ non-oral dosage form INFUSE<sup>®</sup> Bone Graft. The model dosage form is unique and challenging in its study because of three central aspects:

- i) The API is a recombinant biotherapeutic bone regenerating protein modality (rhBMP-2) of molecular weight 32kDa, thereby posing challenges in its stability in dissolution/release media and analytical method development.

- ii) The drug substance is carried in a biodegradable carrier scaffold (absorbable collagen sponge), thereby making its release mechanism complex and 'multiphasic'.
- iii) The efficiency of the dosage form is dependent on its retention and release at the local site of action (inflammatory physiological conditions) instead of in the systemic circulation, thereby posing challenges in acquiring representative pharmacokinetic data and establishment of an *in vitro in vivo* relationship.

With these challenges in perspective, the first objective of the research was to develop a stability-indicating high performance liquid chromatography (HPLC) method. The method would support the analysis of the recombinant human bone morphogenetic protein-2 (rhBMP-2) in the selected biorelevant release media- modified Hanks' balanced salts solution (HBSS). Stability analysis of the drug substance rhBMP-2 was explored in the media over selected ranges of temperature and time. Forced degradation studies during the development of the method helped to verify if the method could detect presence of degradant peaks. This helped ascertain if the analytical test methodology could detect interferences and degradants during the *in vitro* release test study design. The method was also validated to ensure precision and accuracy of the results obtained during the *in vitro* release test.

The second objective of the study was to apply conventional *in vitro* dissolution / release methods like sample and separate and USP-IV flow through cell methodology to the

model implant. A comparative *in vitro* cumulative release profile of the dosage form was developed and compared to the *in vivo* cumulative release profile. The components of the *in vitro* release apparatus that led to incomplete and non-representative cumulative release profiles were analyzed and a biorelevant *in vitro* drug release (BIVDR) model was finally designed and developed to meet the requirements of the novel implant based dosage form.

The third objective of the research was to design and develop the chambered BIVDR model to simulate a physiological low volume fluid surrounding area meeting the requisite sink conditions for the implant. The BIVDR model was developed in a sequential modular approach to optimize the model components for low protein adsorption and maximal recovery. The model was applied to the model implant INFUSE<sup>®</sup> Bone graft and a cumulative *in vitro* release profile was obtained for >80% release of the drug substance.

The fourth and final objective of the research was to explore if the in house developed BIVDR model and release test was discriminatory between different formulations of the drug substance (rhBMP-2) by using different carrier scaffolds. Absorbable collagen sponge (INFUSE<sup>®</sup> Bone graft) and hydroxyapatite (Osteograft N-300) were used as the carriers. A predictive *in vitro in vivo* relationship was established by using time scaling and levy plots. Similarity (f2) and difference (f1) factors were established for the release profiles of the two different formulations to establish if the release method was discriminatory between formulations.

## CHAPTER 3

### **3 3 Development & Validation of a Stability-Indicating HPLC Method for rhBMP-2 Protein Formulation in a Biorelevant Release Media and Characterization of its Intact Glycoforms using Mass Spectrometric Detection**

*Drawn from a manuscript submitted to J. Pharm Analysis*

#### **3.1 INTRODUCTION**

Bone morphogenetic protein-2 (BMP-2) is an osteoinductive and osteointegrating signaling molecule (Carragee et al., 2011). It is secreted in the human body as a glycoprotein and the molecular weight of its dimer isoforms are between 26-30kDa (Carragee et al., 2011; Friess et al., 1999b; Urist, 1965). It belongs to the Transforming Growth Factor- $\beta$  family of cytokines which are critical in cellular formation and differentiation, predominantly during the embryonic stages (Clark and Coker, 1998). Recombinant BMP-2 in the past decade has largely been cloned in Chinese Hamster Ovary (CHO) cell lines. When applied as a formulation with a biodegradable scaffold at the trauma site, it helps in bone regeneration and healing. It has been used as an

alternative to autograft bone for spinal fusions, healing of tibial fractures and sinus lift augmentation surgeries (Geiger et al., 2003; McKay et al., 2007; Urist, 1965). Currently, one such FDA approved indication of recombinant human BMP-2 is INFUSE<sup>®</sup> Bone Graft (Medtronic, Minneapolis, MN, USA). The formulation is marketed as a combination of lyophilized powder of rhBMP-2, which has to be reconstituted with sterilized distilled water and an absorbable collagen sponge (ACS) (FDA Rockville, 2007b). After reconstitution at 1.5 mg/mL, the solution is distributed uniformly on the surface of the sponge. An initial period of 15-30 min is allowed for non-covalent binding of the rhBMP-2 protein to the collagen sponge. The final indication is then applied at the site of trauma or for oral surgery by the clinician (FDA Rockville, 2007b). Bone regeneration at the intended site (sinus lift, trauma or spinal fusion) occurs by *controlled* and *extended* release of the rhBMP-2 protein from the biodegradable scaffold (ACS).

'Biorelevant' *in vitro* dissolution or drug release tests act as a surrogate quality control tools for performance verification of the pharmaceutical formulation and ensures minimal inter-batch variability (Biswas and Halquist, 2016; Brown et al., 2011). It helps in establishing clinically relevant specifications. These release tests for 'novel' formulations such as implants, help in investigating the effects of physiological variables on the release mechanism of drugs at the complex site of action. To assess and compare the *in vitro* release of rhBMP-2 to the available *in vivo* data, we designed a 'biorelevant' *in vitro* drug release (BIVDR) model. Correlation of the *in vitro* data obtained using the BIVDR model, with *in vivo* data will help establish an *In Vitro In Vivo* Relationship (IVIVR). The *in vitro* studies were performed in a biorelevant release media simulating plasma, using modified Hanks' balanced salts solution (modified by removal

of phenol red and sodium bicarbonate components) (Iyer et al., 2007c). In order to analyze the rhBMP-2 release samples in biorelevant media, a reliable, precise, accurate and stability indicating high-throughput assay method was required. Previous measurements of rhBMP-2 relied upon enzyme-linked immunosorbent assays (ELISA). However, the linear range for the ELISA kits would require expansive dilutions (i.e.,  $10^4$  ~  $10^6$  dilution for release samples) not practically applicable to the release studies, due to its limited dynamic range (62.5 - 4000 pg/mL). Given that other early analytical work has shown the use of a reversed phase (RP) C4 resin for the extraction and purification of the rhBMP-2 protein from CHO cell lines, growing in cell culture media (Chen et al., 1997; Israel et al., 1992), this offers a starting point to developing an RPLC-UV method.

The objective of this chapter is to develop and validate a stability-indicating HPLC assay method which is: a) time and cost effective, b) can be reliably used for direct analysis in the range (0.5 -100  $\mu\text{g/mL}$ ) and c) the validated method must be able to quantify the release of rhBMP-2 from ACS into the biorelevant release media. Validation of the developed method focused on evaluations of linearity, intra-day and inter-day precision, intra-day and inter-day accuracy, recovery, and system suitability. Effects of forced degradation conditions have also been assessed in this work, to detect the presence of any potential chromatographic interfering compounds in the formulation such as excipients and biorelevant release media. Forced degradation studies involved studying effects of acid, base, oxidation, and temperature (thermal degradation) based stress conditions on the drug substance. Differential isoforms of rhBMP-2 in the formulation have also been investigated using mass spectrometric detection and the biotransformation of them was evaluated by inducing stress conditions.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 Chemicals and Reagents**

INFUSE<sup>®</sup> Bone Graft components were donated by Medtronic (Minneapolis, Minnesota). HPLC grade mobile phase components a) acetonitrile was purchased from VWR (Radnor, PA), b) water from Fisher Scientific (Waltham, MA USA) c) trifluoro acetic acid was purchased from EMD Millipore (Massachusetts). Modified Hanks' Balanced Salt Solution (without phenol red and sodium bicarbonate) (Iyer et al., 2007c) and 4-(2-hydroxy ethyl) piperazine-1-ethanesulfonic acid (HEPES buffer, 1 mM) were purchased from Sigma-Aldrich (St. Louis, MO). Polyethersulfone syringe filters (0.45  $\mu\text{m}$ , Whatman) that were used to filter the prepared modified HBSS were purchased from VWR (Radnor, PA). Protein biocompatible inserts for use in HPLC of rhBMP-2 samples were purchased from MicroSolv (Eatontown, NJ). ProteinLoBind tubes were purchased from Eppendorf (Hauppauge, NY). Agilent Zorbax 300-SB C3 column was donated by Agilent technologies. Phosphate buffer saline was obtained from Sigma-Aldrich (St Louis, MO).

### **3.2.2 Equipment and Software**

An Agilent 1260 Infinity HPLC was used for the method development and validation of rhBMP-2. The equipment consisted of modules of a thermostat controlled autosampler tray, a binary pump, and a photodiode array detector. Chemstation software (version: C.01.07) was used for the instrument control and data processing. The pH of the buffers and solution was measured using TruLab pH1310 (YSI, Inc., Yellow Springs, OH). The

mass spectrometric detection/characterization of the peak-trapped samples before and after forced degradation were performed on an AB Sciex 4000 Qtrap (Framingham, MA) LC-MS/MS system. The software used for control and data acquisition was Analyst software (1.5.2). For protein deconvolution algorithm, Bioanalyst™ (1.4) – Bayesian protein reconstruct tool was used.

### **3.2.3 Preparation Of Mobile Phase, Stock Solutions, Calibration Standards And Quality Controls**

The aqueous mobile phase consisted of water with 0.1% TFA and the organic mobile phase consisted of 90% acetonitrile with 0.1% TFA. Hanks Balanced Salts Solution (HBSS) is a media, which has often been used in tissue culture and has comparable ion concentration and osmolality to human plasma (Iyer et al., 2007c). Hence, it was selected to simulate biorelevant release media conditions (Hanks, 1948; Iyer et al., 2007c). As previously shown (Iyer et al., 2007c), Modified HBSS was prepared by addition of 9.8 g of HBSS powder to 975 mL of water and 25 mL of 1M HEPES (Iyer et al., 2007c). rhBMP-2 protein has been shown to be most stable at a pH of 4.5 (reduced aggregation and denaturation (Luca et al., 2010)) and hence the pH was adjusted for sample injection. After preparation, the solution was filtered through a 0.45 µm filter and stored at 4°C. Stock solutions of 200 µg/mL, 100 µg /mL, and 10 µg /mL were prepared in the biorelevant release media to cover the entire range of the calibration curve from 0.5 µg/mL -100 µg/mL. A series of calibration curves were prepared with ten calibration levels equally distributed throughout the dynamic range: 0.5, 1.25, 1.5, 5, 10, 25, 50, 75, and 100 µg/mL respectively. Quality control standard levels were selected bracketing

the expected release range at 7.0, 20 and 45 µg/mL respectively and the LLOQ at 0.5 µg /mL. No sample preparation step was required. The release samples were thawed to room temperature, vortexed for 1min, centrifuged on a tabletop centrifuge and aliquoted into biocompatible inserts for injection.

### **3.2.4 Chromatographic Method Development Conditions**

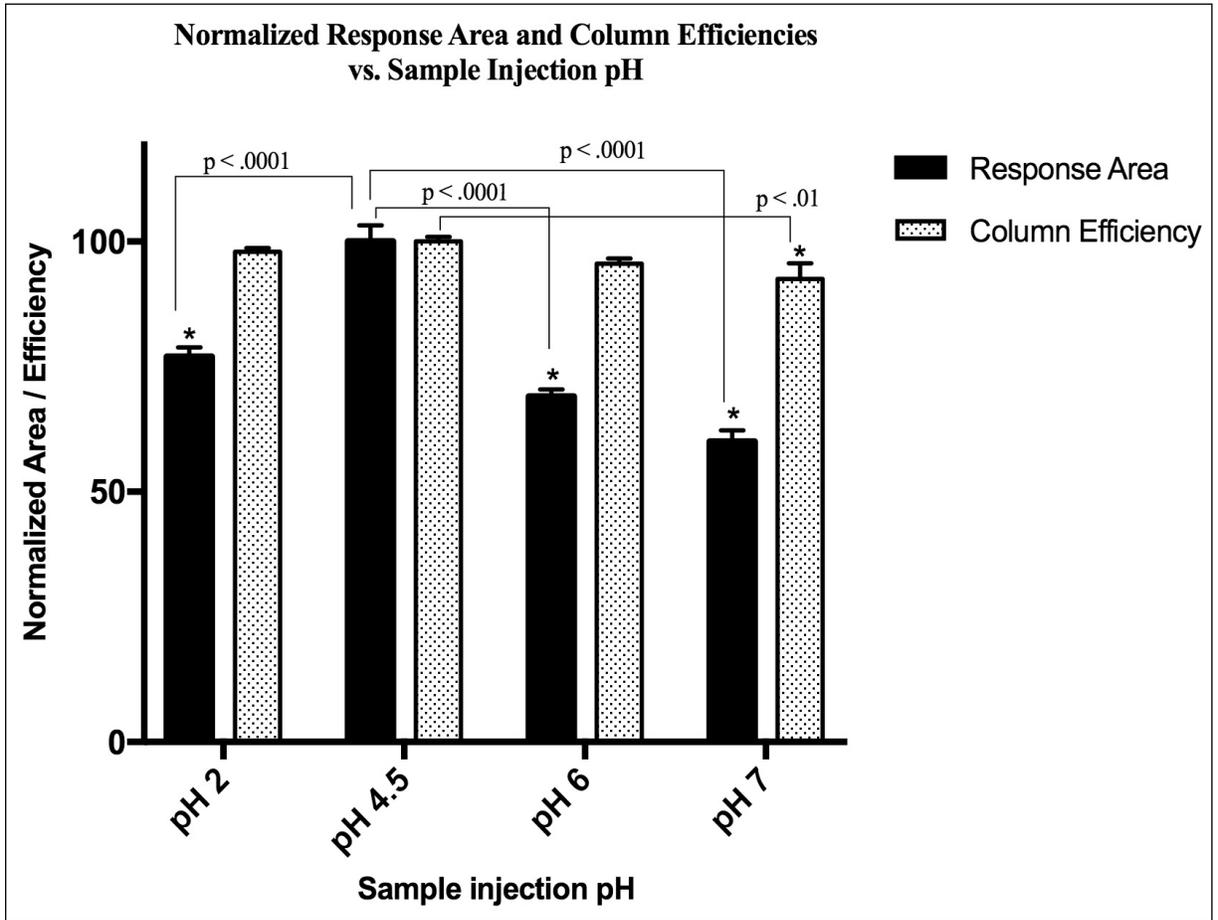
*In silico* characterization of rhBMP-2 was initiated using the sequence of the dimer for human BMP-2 from UniProt to evaluate hydrophobicity (Consortium, 2014). ExPaSy ProtParam by SwissProt was used as a software tool for a baseline theoretical prediction of the physicochemical characteristics such as isoelectric point (pI) and hydrophobicity (Consortium, 2014; Walker, 2005). Chromatographic retention of a protein can be significantly affected by its hydrophobic nature and dimension. A grand average of hydrophobicity (GRAVY) score of - 0.56 and pI of 8-9 was calculated by ProtParam (Consortium, 2014). Hence taking into consideration the hydrophobicity of the protein, the size of the dimer molecule, and pI of the protein, an HPLC column with the following parameters: C3 column, 250 mm X 2.1 mm, 5 µm, 300 Å was selected (Agilent Technologies, Santa Clara, CA).

Method development and optimization studies entailed sequentially evaluating the process variables based on the peak area response, column efficiency, peak shape, and robustness. Crucial optimization parameters that were assessed were: a) gradient profile adjustment to reduce the retention time, b) combination of optimal velocity (i.e., flow rate) to minimize band broadening c) sample injection pH d) sample volume and e) column temperature. Recombinant human BMP-2 has been found to be sensitive to pH

changes. In the body, it is released at the site of fracture healing as a chemotactic and osteoinductive agent in an acidic pH microenvironment (Hollinger and Wong, 1996; Silver et al., 1988). An in-depth pH study relative to the physical stability and conformation maintenance of rhBMP-2 in Luca et. al shows that the aggregation and denaturation behavior of rhBMP-2 depends heavily on it's media pH (Luca et al., 2010). Therefore, pH studies with respect to response area and column efficiency were critical for analysis reproducibility. Since pH 4.5 gave the best results, the response area and column efficiencies at pH 2, 6 and 7 were normalized with respect to values at pH 4.5, hence, pH 4.5 was selected as the control group. The column efficiencies in all cases were > 2000 as required by USP guidelines (US Pharmacopeia, 2012). Results of pH vs. normalized response area percentage and normalized column efficiency percentage are shown in Figure 3-1. A one-way ANOVA with Dunnett's multiple comparisons test was used for the purposes of statistical comparisons at a significance level of  $\alpha = 0.05$ . The ANOVA assumed normality and equality of variances due to limited standard deviations of each data set. Diode array detection wavelength for rhBMP-2 was selected by optimization of a combination of factors: a) wavelength (202nm-220nm), b) bandwidth (4nm-20nm) and c) reference wavelength (280nm-360nm). These parameters were finalized based on a good signal to noise ratio, rhBMP-2 peak shape, and optimized resolution between the drug product and major degradant peak during forced degradation stability analysis. Recovery studies were also performed in water and phosphate buffered saline (PBS) and compared to biorelevant media. Peak purity analysis was performed using Chemstation software (Version: C.01.07) and the threshold for passing peak purity was 990 out of 1000 for a pure peak.

### 3.2.5 Method Validation

The method was validated according to the FDA Bioanalytical Guidelines (FDA, 2001; Geenen et al., 2011) since Hanks' balanced salts solution was a cell culture medium [10]. Validation experiments consisted of selectivity, linearity, precision and accuracy, recovery, and limit of quantification. Six calibration curves were prepared and evaluated for linearity. A polynomial regression model ( $R^2 > 0.99$ ) with least squares regression was used for the calibration curve based on chromatographic peak area vs. concentrations of the drug substance. The limit of detection was calculated using three times the signal to noise. Furthermore, the limit of quantification was evaluated as ten times the signal to noise; however, a higher experimental value, which could be reliably and repeatedly quantified with an accuracy of 80-120% and a relative standard deviation (RSD) of  $< \pm 15\%$  was selected ( $0.5 \mu\text{g}/\text{mL}$ ). Intra-day and inter-day precision and accuracy were calculated for three batches ( $n=9$ ). System suitability (SST) was evaluated using ( $n=6$ ) replicate injections of rhBMP-2 at a concentration of  $20 \mu\text{g}/\text{mL}$ . Criteria for passing SST was  $\pm 2\%$  relative standard deviation percentage for precision, column efficiency ( $> 2000$ ), and a tailing factor  $< 2$ , according to USP guidelines for method validation of chromatographic methods (US Pharmacopeia, 2012).



**Figure 3-1.** Sample injection pH vs. Normalized response area/column efficiencies

A one-way ANOVA with Dunnett's multiple comparisons test was used for the purposes of statistical comparisons.

Recovery studies were performed in water and PBS and also in modified HBSS by spiking pre-analyzed QC samples with known concentrations of the drug substance.

### **3.2.6 Stability Of rhBMP-2**

Proteins tend to aggregate at low concentrations and are highly labile to minor fluctuations in temperature; therefore, evaluating the stability of rhBMP-2 was critical for the *in vitro* release experiments. Stability studies were carried out at two different concentrations (2 µg/mL and 15 µg/mL, respectively), three different temperatures (i.e., 4°C-autosampler temperature, 22°C-room temperature, and 37°C-physiological body temperature). Incubation was performed at two-time points: 12hr and 24hr, respectively. Storage stability and freeze-thaw cycle stability studies (1 and 2 cycles) were also carried out at -20°C and -70°C, respectively.

### **3.2.7 Stability Indicating Method Conditions**

Stability studies are a crucial part of method validation studies to determine the presence of interfering degradant products, which might appear in the formulation during bioprocessing, storage, handling, and/or transportation. They also shed light on the degradant detection and resolution ability of the optimized method. Therefore, it is necessary to investigate the stability of rhBMP-2 in the biorelevant media during the *in vitro* release testing process. The conformation and stability of a protein are a measure of its therapeutic value (Hermeling et al., 2004). Stress testing for the drug substance was performed using acidic (0.1M HCl), basic (0.1M NaOH), oxidative (i.e., peroxide-

30%) and temperature (70°C) at 24hr, 48hr, and 72hr stress conditions, respectively at a concentration of 45 µg /mL. Since no degradation products were observed initially with the method's 9min run time, the run time was increased to 20min to help in the detection of possible late eluting degradation products (US Pharmacopeia, 2012) .

### **3.2.8 Differential Isoforms of recombinant human Bone Morphogenetic Protein -2**

rhBMP-2 is a chemo-attractant molecule that is both osteoinductive and osteoconductive (Li and Wozney, 2001). Endogenously, it attracts the mesenchymal stem cells to the site of trauma and injury to facilitate the process of bone regeneration. rhBMP-2 is most commonly cloned in Chinese Hamster Ovary(CHO) cell lines. Since the folding and conformation of a protein is central to it being therapeutically bioactive, CHO cells are adept at post- translationally processing the BMP-2 homodimer (Kim et al., 2012). This results in six distinct isoforms of BMP-2 each of which can have varying combinations of 5-9 mannose units per monomer attached to its N-linked glycosylation site (Israel et al., 1992). Each monomer of rhBMP-2, depending upon its post-translational processing, could either begin with a glutamine at position 283 (Q283) or with an additional 17 amino acids with a threonine at position 266 (T266). Glutamine conversion to pyroglutamate at physiological pH and in solution at the N-terminal end is often observed in proteins and hence also in rhBMP-2. The glutamine undergoes cyclic conversion to pyroglutamate (Q'283) leading to a loss of -17.03Da (Reimer et al., 2011). However, since the rhBMP-2 is a dimeric molecule, about 6 different isoforms are possible. Each of these isoforms can, in turn, contain a combination of 5-9 mannose

residues on each of the monomer, giving rise to heterogeneity and complexity in the protein. All the post-translationally modified isoforms of rhBMP-2 are found in the drug formulation and have comparable bioactivity and bone induction ability as evaluated in *Porter et. al.* (Porter et al., 2004).

### **3.2.9 Qualitative characterization of rhBMP-2 glycoforms and forced degradation products using mass spectrometry**

An exploratory mass spectrometry survey was carried out on the peak-trapped samples from the HPLC, for qualitative characterization of the changes affecting the rhBMP-2 molecule. The rhBMP-2 drug product (both non-stress treated and stress treated - as mentioned in section 3.2.7) samples were injected into the Agilent 1260 Infinity HPLC at a concentration of 45 µg/mL. The rhBMP-2 peak, degraded peak 1(DP1) and degraded peak 2(DP2) (as shown in Figure 3-4A and B in results section) were peak trapped and collected in low protein bind eppendorf tubes. These were injected by direct infusion at 15 µL/min into AB Sciex 4000 Qtrap mass spectrometer. The difference in molecular weights (based on m/z) between the control samples (samples which had *not* been subjected to stress/forced degradation) and peaks trapped from acid, base, peroxide and temperature subjected stressed samples were investigated. An Enhanced Multi-Charge (EMC) scan mode was selected over a Q1 MS mode during mass spectral characterization since rhBMP-2 drug product is a relatively large protein molecule (29kDa-32kDa) which acquires multiple charges during the ionization mode and has multiple isoforms. The EMC scan mode in 4000 Qtrap facilitates the transfer of multi-

charged ions only and the low charged ions escape out of the linear ion trap (LIT) during the set delay time. Although there is some loss of multiply charged ions during this delay time; the loss of singly charged ions are much more in relative comparison. A scan range of 1200-2800 m/z was selected. Q0 trapping was switched on and LIT fill time of 10ms was applied as well as a Q3 empty time of 30ms. The MCS barrier was set at 3V; declustering potential at 57V and collision energy at 55V. Infusion rate was set at 15  $\mu$ L/min. Since the formulation is a complex mixture of isoforms of the protein, initial tuning of the method was focused on acquiring a maximum of signal/noise ratio and reproducibility for the dominant ions. The mass spectra were deconvoluted using Bioanalyst software and the Bayesian protein reconstruct algorithm. The deconvolution results were then interpreted using the GlycoMod tool of ExPASy (SIB Bioinformatics Resource Portal) to calculate the number of mannose units present on the intact protein (Cooper et al., 2003).

## **3.3 RESULTS AND DISCUSSION**

### **3.3.1 Method Development**

A stepwise gradient profile was used with the following conditions: initially at 70%- 0.1% TFA (Mobile Phase A) and 30%- 90% acetonitrile with 0.1% TFA (Mobile Phase B; followed by 0.2-1.7 min. increased to 85% Mobile phase B; 3.5 – 5 min. at 95% (Mobile phase B); and finally 7-9 min. re-equilibration at initial conditions (70% Mobile Phase A). The gradient was run in a combination with velocity between 0.4mL/min to 0.5mL/min with a total run time of 9min and an rhBMP-2 peak retention time of 3.44min. The pH of

the media is crucial to the stability of rhBMP-2, chromatographic studies were conducted for improvement of peak shape, peak area response and column efficiency with injection sample pH at 2 (pH matching with mobile phase pH 2), 4.5, 6 and 7.4. Figure 3-1 in the methods section of this chapter shows the normalized response area and column efficiency vs. sample injection pH. It was observed that the optimal peak shape, area response and column efficiency during the optimization studies were at pH of 4.5. A one-way ANOVA was performed with Dunnett's multiple comparisons test. Since pH 4.5 showed the best response area and column efficiency mean values, it was used as the control column for multiple comparisons in the Dunnett's test. The results were reported as normalized percentage for both peak area response and column efficiency.

The normalized mean peak response area for n=6 samples at pH 2.0 was  $76.80 \pm 1.83$  %, at pH 4.5-recorded mean was  $100.00 \pm 3.25$  %, at pH 6 the response area showed a considerable decrease to  $69.04 \pm 1.39$ , and with a further reduction at pH 7.4 to  $60.04 \pm 2.22$ . Results of the ANOVA multiple comparison Dunnett's test showed that response area at pH 4.5 was significantly different from each pH at 2, 6 and 7.4 at a significance level of  $\alpha=0.01$  with ( $p < .0001$ ). The normalized percent average column efficiency calculated for n=6 samples were  $97.96 \pm .70$  for pH 2;  $100 \pm .93$  for pH 4.5;  $95.58 \pm 1.07$  for pH 6 and  $92.51 \pm 3.12$  for pH 7.4. A one-way ANOVA was used with a multiple comparisons Dunnett's test to evaluate if the mean column efficiencies were significantly different from pH 4.5. It was found that the mean of pH 4.5 was significantly different from pH 7 at a significance level  $\alpha=0.01$ ( $p < .01$ ).

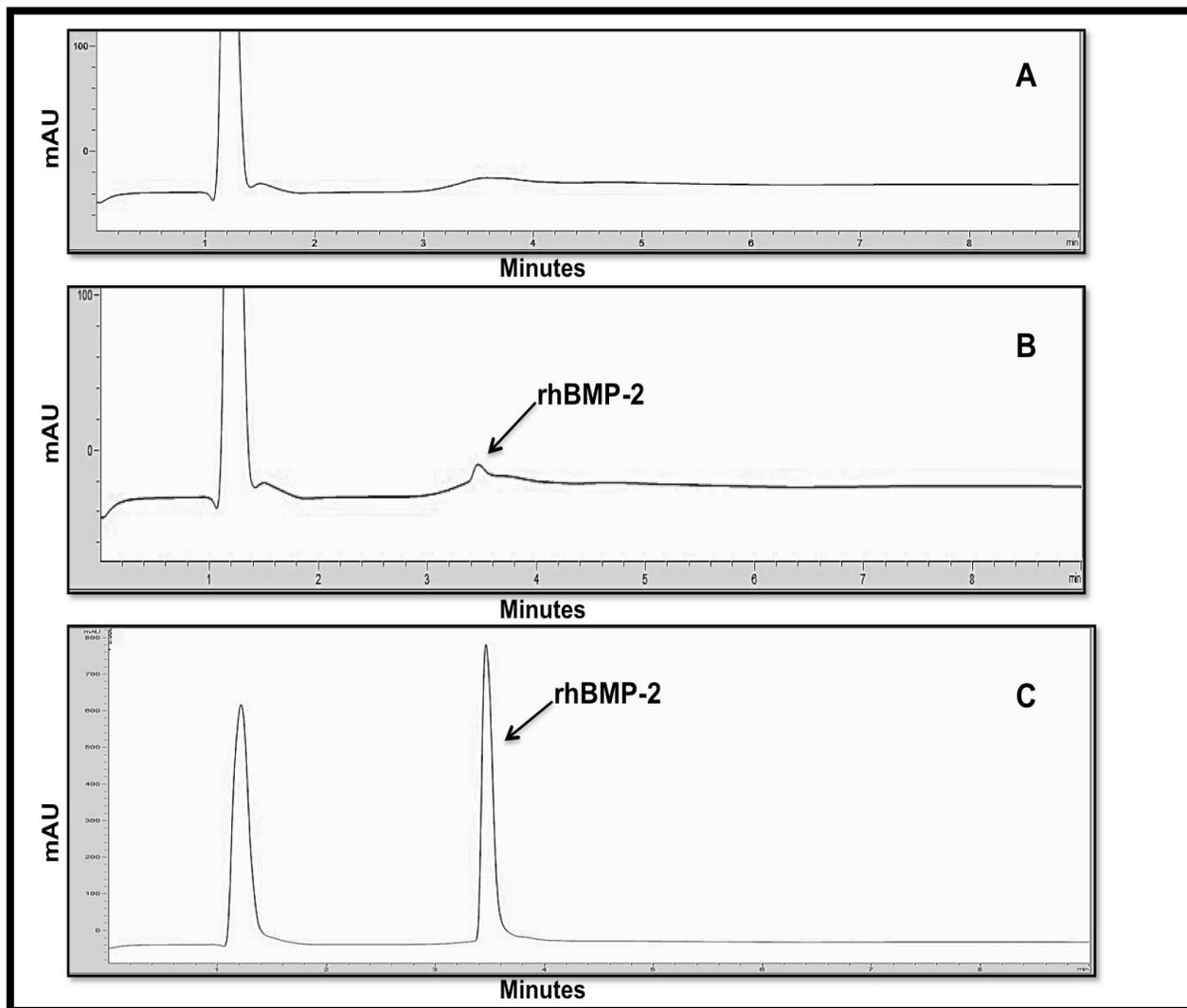
Peak shape, response and column efficiency with respect to column temperature was evaluated at 25°C, 37°C, 40°C and 50°C. Optimal peak shape and response area was observed at 40°C. Sample injection volume studies were also conducted with injection volumes of 15, 20, 25, 35, 45, 55 and 60µL in a 120 µL sample loop. Ultimately, a 60µL injection, with minimal carryover at the higher limit of quantification, was used to obtain a linear dynamic range (0.50 to 100 µg/mL). For the DAD a wavelength of 217nm, a bandwidth of 14nm and a reference wavelength of 280nm was selected to get a robust peak shape and sufficient resolution between the rhBMP-2 peak and degradant product 1(dp1).

### **3.3.2 Method Validation**

#### **3.3.2.1 Selectivity, Linearity, Limits of Detection (LOD) and Limits of Quantification**

Selectivity for the assay was determined by testing if the formulation excipients interfered or had a similar retention time as the rhBMP-2 on the HPLC system. Each 1ml of INFUSE<sup>®</sup> Bone Graft formulation consists of 1.5mg of rhBMP-2 protein; 0.1mg sodium chloride; 5.0 mg sucrose; 25mg glycine; 3.7 mg L-glutamic acid and 0.1mg polysorbate 80 (FDA Rockville, 2007b). Individual solutions of the excipients were prepared at the concentration that they are present in the original formulation as well as a mixture of all the excipients but without rhBMP-2 and injected into the column to observe the presence of interfering excipient peaks in the HPLC method. No interfering peaks were observed from any of the excipients. Peak purity evaluations with a cut off at 990 out of 1000 resulted in a peak purity similarity factor of 997 out of 1000;

therefore, the peak was pure as by spectral comparison. Figure 3-2 is a representative chromatogram of a blank sample (A), LLOQ (B), and 20µg/mL concentration sample (C). A polynomial regression model was used for three runs (n=6),  $R^2 > 0.99$ . The reverse predicted residuals from the calibration curve have been provided in Table 3-1. The concentration of the reverse predicted residuals have been reported as percent difference from nominal (%DFN) and ranged from 9.67% at the LLOQ of 0.5 µg/mL to – 6.27%. The LOD was calculated as three times the signal to noise and was found to be 0.10 µg/mL.



**Figure 3-2.** Representative chromatograms of rhBMP-2  
(a) blank medium, (b) 0.5 µg/mL (LLOQ), (c) 20 µg/mL.

### **3.3.2.2 Intra-day and Inter-Day Precision and Accuracy**

Precision and accuracy of the method are reported in Table 3-2 as %RSD and %DFN respectively. Inter-day and intra-day precision for the QC's were carried out at three different levels (7 µg/mL; 20 µg/mL and 45 µg/mL) for release concentrations which were pertinent to release from the biorelevant model. A global calculation for the three different QC levels, for three runs (n=9), was estimated. %RSD was found to be from 0.64% to 1.90%. %DFN was observed to be between -3.70% to 0.14%. Precision and accuracy of quantitation of the rhBMP-2 protein was also evaluated for the LLOQ at 0.5 µg/mL. Estimation of global calculation for the LLOQ (0.5 µg/mL) for all the three runs showed a %RSD of 3.49% and %DFN of 6.67%, respectively. Precision and accuracy for the individual inter-day runs have also been presented in Table 3-2.

**Table 3-1.** Reverse predicted concentration residuals of rhBMP-2

Run number	Nominal Concentration ( $\mu\text{g/ml}$ )									$R^2$
	0.500	1.25	1.50	5.00	10.00	25.00	50.00	75.00	100.00	
1	0.532	1.32	1.58	4.69	10.09	24.82	50.23	75.02	100.03	0.999
2	0.544	1.38	1.54	4.74	10.19	24.64	50.67	74.83	99.96	0.996
3	0.569	1.19	1.62	4.63	10.25	24.50	50.02	74.8	99.64	0.998
Mean	0.548	1.30	1.58	4.69	10.18	24.65	50.31	74.84	99.88	
S.D.	0.02	0.09	0.04	0.06	0.08	0.16	0.33	0.18	0.21	
%RSD	3.44	7.49	2.53	1.18	0.79	0.65	0.66	0.25	0.21	
%DFN	9.67	3.73	5.33	-6.27	1.77	-1.39	0.61	-0.22	0.12	

**Table 3-2.** Precision and accuracy data for rhBMP-2 QC's

Run Number	Nominal Concentration ( $\mu\text{g/ml}$ )			
	0.50	7.00	20.00	45.00
1	0.56	6.82	20.37	45.29
	0.54	6.71	20.05	44.29
	0.55	6.78	20.26	45.21
Mean	0.55	6.77	20.23	44.93
S.D.	0.01	0.06	0.16	0.56
%R.S.D.	1.82	0.82	0.80	1.24
%DFN	10.00	-3.29	1.13	-0.16
2	0.53	6.65	19.60	45.08
	0.57	6.68	19.63	44.93
	0.51	6.47	20.68	44.86
Mean	0.53	6.56	19.97	19.89
S.D.	0.03	0.12	0.62	0.11
%R.S.D.	5.36	1.77	3.08	0.25
%DFN	7.60	-5.73	-0.16	-0.10
3	0.55	6.83	19.65	45.67
	0.52	6.99	19.98	45.10
	0.47	6.74	20.04	45.01
Mean	0.51	6.85	19.89	45.26
S.D.	0.04	0.13	0.21	0.36
%R.S.D.	7.87	1.85	1.06	0.79
%DFN	2.67	-2.10	-0.55	0.58
<b>Global Calculation</b>				
Mean	0.53	6.74	20.03	45.05
S.D.	0.02	0.13	0.18	0.28
%R.S.D.	3.49	1.90	0.88	0.64
%DFN	6.67	-3.70	0.14	0.11

### 3.3.2.3 System Suitability and Recovery

System suitability helps to establish the appropriateness of the developed analytical method with respect to the chromatographic modules used on a routine basis. For the developed rhBMP-2 method, system suitability was evaluated at a concentration of 20 µg/mL with 6 replicate injections (n=6). Results of system suitability have been shown in Table 3-3. A global calculation was performed for all the injections and was reported in terms of mean and %RSD for all the parameters such as precision, retention time, efficiency and tailing factor. The %RSD on precision was found to be 1.45%; retention time 0.02%; column efficiency 0.23% and tailing factor 1.75%. The validated analytical method developed for rhBMP-2 in biorelevant media was also tested for recovery of rhBMP-2 drug in water: ACN (70:30) and Phosphate Buffered Saline (PBS). Known concentration samples (20 µg/mL) were spiked with 3 µg/mL of rhBMP-2 protein (n=3 replicates). a) Water: ACN (70:30) showed a recovery% of  $87.14 \pm 0.23$  b) PBS showed a recovery % of  $100.06 \pm 6.92$  and c) in modified HBSS a recovery % of  $102.07 \pm 4.11$  were observed.

**Table 3-3.** System suitability of 20µg/mL rhBMP-2 injection

Injection No.	Precision (20µg/mL)	Retention time (min)	Efficiency	Tailing factor
1	20.05	3.44	5309.69	1.80
2	20.26	3.44	5345.77	1.72
3	19.60	3.44	5323.68	1.75
4	19.63	3.44	5329.38	1.75
5	20.04	3.44	5333.79	1.72
6	20.25	3.44	5323.90	1.77
<b>Global Calculations</b>				
Mean	19.97	3.44	5327.30	1.75
% RSD	1.45	0.02	0.23	1.75

### **3.3.2.4 Stability of Drug Substance**

Stability was evaluated at low and medium concentration levels (i.e., 2 µg/mL and 15 µg/mL) and the data are shown in Table 3-4. Samples were stable when kept at 4°C (autosampler temperature), 22°C (room temperature) and 37°C (physiological body temperature). The recovery % of the samples was within the acceptable range of 90-110% for a period of 24hrs. Protein conformation (secondary and tertiary folding) stability has been known to be effected by freeze-thaw cycles, thereby leading to protein denaturation and aggregation (Bhatnagar et al., 2007; Pikal-Cleland et al., 2000). Hence, freeze-thaw stability of the samples was evaluated at the primary storage temperatures for the samples (-20°C and -80°C). The results for n=3 replicate samples are shown in Table 3-5. Although the recovery after one freeze-thaw cycle was within 90-110%, recovery after two freeze thaw cycles in both -20°C and -80°C indicated a reduction by 14.79% and 17.13%, respectively. To reduce the need for more than one freeze-thaw cycle for future use, the stock samples when required to be stored in solution form, were aliquoted into separate protein loBind tubes at low volumes for storage (0.100ml).

### **3.3.2.5 Stability Indicating Studies- Forced Degradation Analysis**

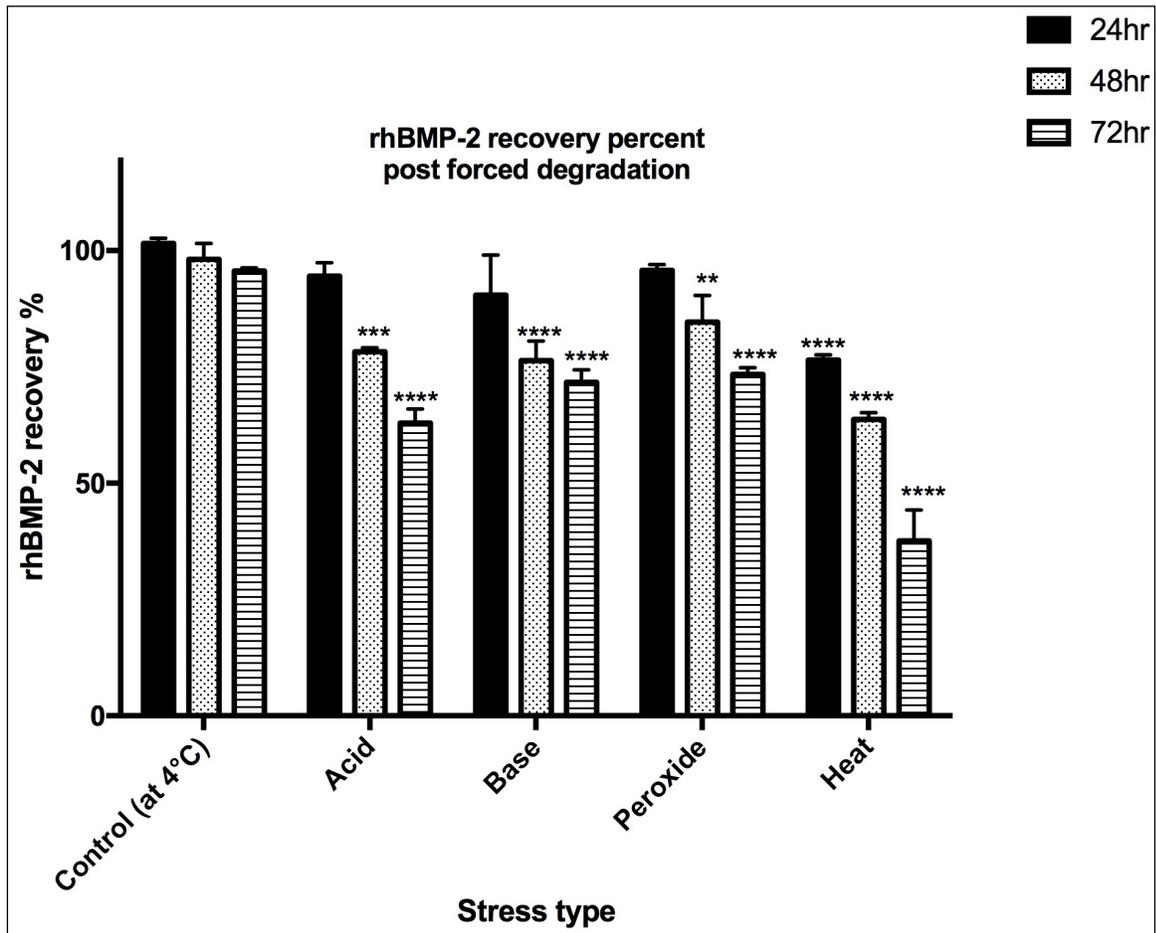
Forced degradation studies in the biorelevant release media helped in the characterization of rhBMP-2 drug remaining after 24hr, 48hr and 72hr under stress testing conditions. Results of the forced degradation studies have been represented in the bar graph in Figure 3-3. Acid forced degradation showed a recovery percentage of

**Table 3-4: Stability of rhBMP-2 in modified HBSS media (n=3 experiments)**

Nominal Concentration (µg/ml)	Autosampler temperature Stability (4°C) (Recovery%)		Room temperature Stability (22°C) (Recovery%)		Physiological temperature Stability (37°C) (Recovery%)	
	12hr	24hr	12hr	24hr	12hr	24hr
<b>2 µg/ml</b>						
Mean	93.71	98.43	91.06	90.12	91.00	92.51
SD	8.65	5.40	5.56	5.17	3.36	4.50
<b>15 µg/ml</b>						
Mean	99.97	99.96	100.24	99.13	102.32	95.82
SD	3.80	2.56	2.71	1.11	2.66	5.94

**Table 3-5: Freeze-thaw stability of rhBMP-2 at different temperatures (n=3 experiments)**

Temperature conditions	No. of freeze thaw cycles (Recovery% ± SD)	
	1 freeze thaw cycle	2 freeze thaw cycle
-20°C	100 ± 5.56	85.21 ± 4.79
-70°C	105.36 ± 2.87	82.87 ± 6.94



**Figure 3-3.** rhBMP-2 recovery % in stressed samples for acid, base, peroxide and temperature stress testing for 24hr, 48hr and 72hr time points.

All statistical comparisons between samples been made with the control at 4°C at their respective time points.  $p < .001$ (\*\*);  $p < .0001$ (\*\*\*);  $p < .00001$ (\*\*\*\*).

94.53 ± 2.85 %, 78.27 ± 0.85 % and 62.93 ± 2.99 % after 24hr, 48hr, and 72 hr, respectively. Degradation studies in base showed a recovery percentage of 90.40 ± 8.66 %, 76.37 ± 4.19 % and 71.66 ± 2.69 % respectively. Oxidative forced degradation yielded a recovery % of 95.77 ± 1.22 %, 84.66 ± 5.72% and 73.42 ± 1.41 % respectively. Temperature based forced degradation at 70°C showed a much lower level of recovery of rhBMP-2 protein. The recovery percentage of rhBMP-2 at 24hr, 48hr and 72hr were 76.47 ± 1.14%, 63.74 ± 1.42% and 37.62 ± 6.63% respectively. A one-way ANOVA with Dunnett's multiple comparisons test was performed at a significance level  $\alpha=0.01$ . The specific time points (24hr, 48hr, and 72hr) for each of the corresponding stress treated samples were compared with their control at 4°C at the same time point to observe if the means were significantly different. For the 24hr time point, the acid ( $p=0.1866$ ), base ( $p= 0.0266$ ) and the peroxide ( $p= 0.3163$ ) were not significantly different from the control at 4°C but the recovery percentage of rhBMP-2 for temperature was significantly less ( $p \leq 0.0001$ ) than the control. A one-way ANOVA with Dunnett's multiple-comparisons test at a significance level  $\alpha=0.01$  for 48hr however, showed a greater difference in means when compared to the 48hr control at 4°C. At the 48hr time point all the means were significantly different and decreased relative to the control: acid ( $p=0.0002$ ); base ( $p \leq 0.0001$ ); peroxide ( $p= .0035$ ) and temperature ( $p \leq 0.0001$ ). Similarly, for the 72hr time point, the data showed a further decrease in means as compared to the control sample at 4°C for 72hr. All the stress-tested samples had significantly different and lower means: acid ( $p \leq 0.0001$ ); base ( $p \leq 0.0001$ ); peroxide ( $p \leq 0.0001$ ) and temperature ( $p \leq 0.0001$ ). Temperature based stress testing also revealed the presence of three resolved degradation products (DP), which have been labeled as

DP1 (retention time= 1.8min, observed only in the 72hr stress tested sample at 70°C), DP2 (retention time= 3.8min) and DP3 (retention time= 5.1min). The representative chromatograms for the stress-tested samples have been shown in Figure 3-4A and B.

### **3.3.3 Qualitative mass spectral characterization of glycoforms of BMP-2 and changes observed in stressed samples during forced degradation**

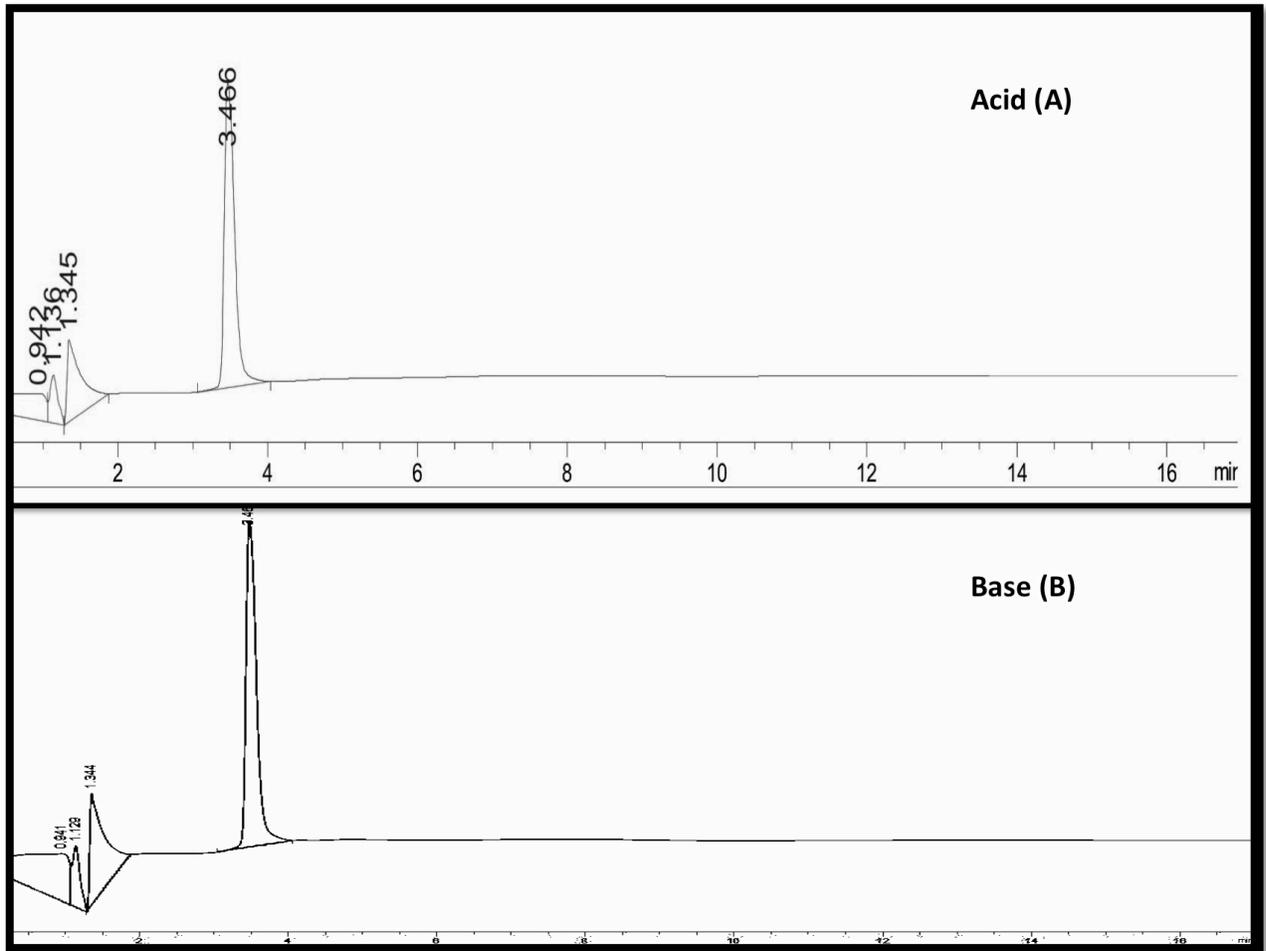
rhBMP-2 is expressed in Chinese Hamster Ovary (CHO) cell lines as a precursor propeptide. During post-translational processing, heterogeneity in the sequence of amino acids at its N-terminal end leads to a total of six different types of isoforms.

Therefore, the rhBMP-2 formulation consists of three distinct N-terminal isoforms:

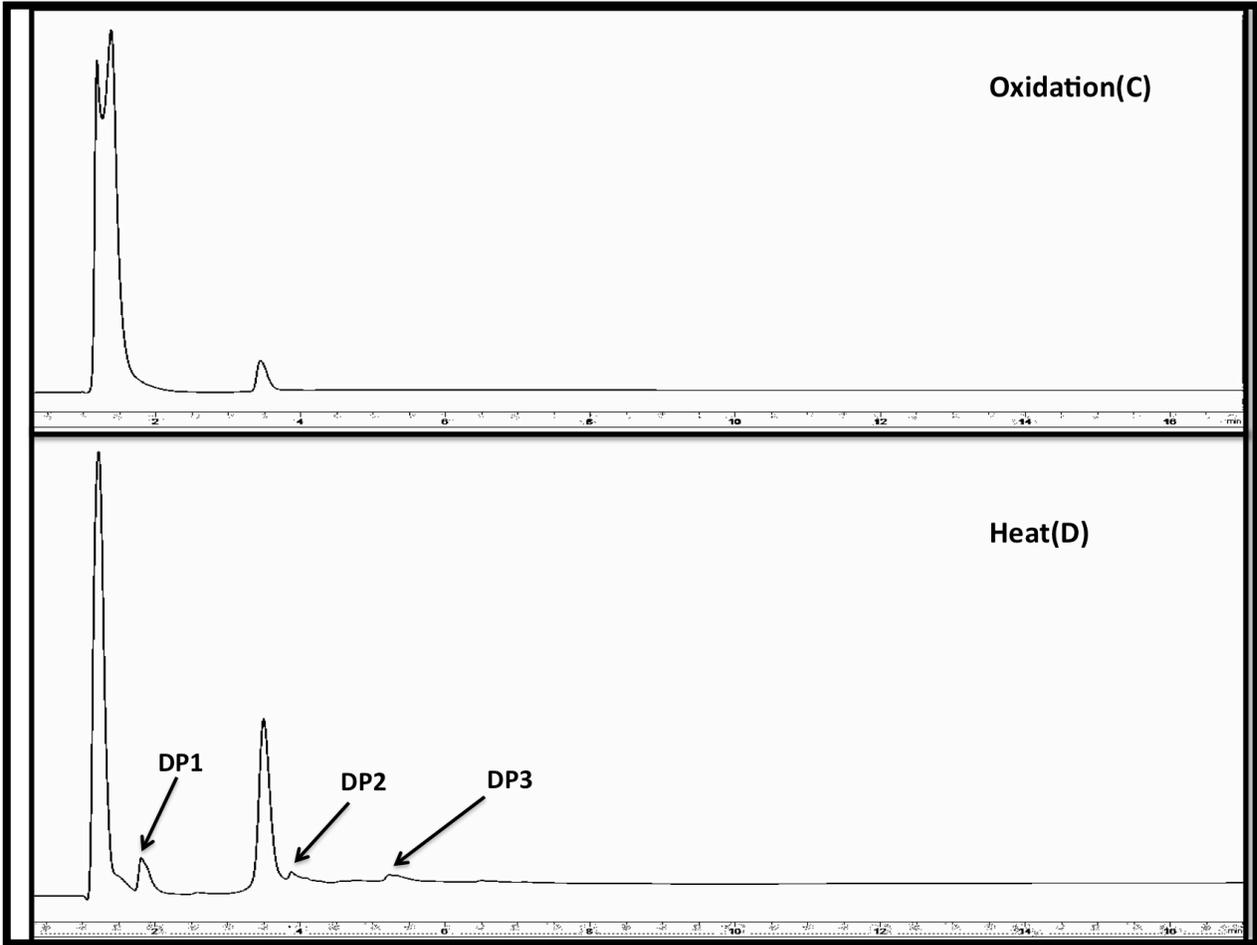
a) Q283/Q283 in which both monomers are processed at the glutamine 283 sites  
b) Q283/T266 one monomer processed at the glutamine site and one monomer processed at the threonine site and c) T266/T266 both monomers processed at threonine site (Israel et al., 1992). It has also been detected that the glutamine at the N-terminal end converts into pyroglutamate (Q') in solution form *in vitro* and *in vivo* and this leads to a loss of -17.03Da (Reimer et al., 2011). The glutamate to pyroglutamate conversion results in three additional isoforms: d) Q'283/Q'283 in which both glutamines are converted into pyroglutamate e) Q283/Q'283 in which one glutamine is converted into pyroglutamate f) Q'283/T266 with one pyroglutamate and one threonine (Porter et al., 2004). Individually the above isoforms also give rise to a diverse range of glycoforms depending upon the number of mannose residues (generally 5-9), attached to the N-linked glycosylation site. Table 3-6 is a list of some of the glycoforms with their

predicted and experimental molecular weights as observed in the rhBMP-2 non-stress treated control samples.

The effects of stress conditions on rhBMP-2 were explored with peak-trapped samples from an Agilent 1260 HPLC that were subsequently injected onto the LC-MS by direct infusion (at 15 $\mu$ L/min) into an AB Sciex 4000 Q trap. After deconvolution, a distribution is obtained covering a range of glycoforms from 29kDa- 32kDa. The deconvoluted mass reconstruction of the spectrum has been shown in Figure 3-5. The temperature based stress treated sample showed the major decrease in response area in chromatograms, presence of degradation peaks (DP) and also changes in the mass spectra.



**Figure 3-4A.** Representative chromatograms for rhBMP-2 stress studies (a) Acid (b) Base



**Figure 3-4B.** Representative chromatograms for rhBMP-2 stress studies (c) Peroxide (d) Temperature.

Degradant Peaks: DP1, DP2 and DP3 were observed only in temperature stress treated samples.

The major degradation pathways for a protein are deamidation, oxidation, hydrolysis and reduction (Patel et al.). These reactions frequently cause chemical conversions and degradation in the protein molecule. The chemical changes lead towards inability in maintaining the essential secondary and tertiary conformation of the protein structure. Distortion in folded structure of the protein molecule eventually results in fragmentation, aggregation, and precipitation of the biotherapeutic protein (Bhatnagar et al., 2007; Patel et al.). An in-depth survey and comparison of the deconvoluted mass spectra for the control and forced degraded samples revealed the most frequent and repetitive shifts in molecular weight was of +13Da; +16Da; +64Da. The predicted structural changes associated with these observed molecular shifts were as follows: *deamidation of asparagine*: deamidation of Asp leads to a gain of 0.982 Da due to the conversion of a  $-NH_2$  group to an  $-OH$  group (Yang and Zubarev, 2010).



**Table 3-6.** rhBMP-2 glycoform mass assignment

Type of differential Isoform	No. of Mannose residues	Predicted mass of glycoform (Avg. mass)	Experimental mass of glycoform (Avg. mass)	$\Delta m\%$
Q'283/Q'283	(Mannose) <sub>13</sub>	27945.433	27945.161	-0.0009
Q'283/Q283	(Mannose) <sub>13</sub>	27929.433	27928.131	-0.0046
Q283/Q283	(Mannose) <sub>13</sub>	27913.434	27913.603	-0.0006
Q'283/Q'283	(Mannose) <sub>18</sub>	28692.147	28692.602	0.0016
Q'283/Q283	(Mannose) <sub>18</sub>	28676.148	28675.572	-0.0020
Q283/T266	(Mannose) <sub>5</sub>	28696.591	28695.078	-0.0053
Q283/T266	(Mannose) <sub>10</sub>	29523.302	29524.047	0.0025
Q283/T266	(Mannose) <sub>12</sub>	29831.588	29831.852	0.0009
Q283/T266	(Mannose) <sub>12</sub>	29847.587	29847.536	-0.0002
Q283/T266	(Mannose) <sub>15</sub>	30286.016	30288.610	0.0086
Q'283/T266	(Mannose) <sub>15</sub>	30268.986	30271.580	0.0086
Q'283/T266	(Mannose) <sub>5</sub>	28631.562	28634.977	0.0112
Q283/T266	(Mannose) <sub>5</sub>	28615.563	28617.947	0.0083
T266/T266	(Mannose) <sub>8</sub>	31117.258	31116.446	-0.0026
T266/T266	(Mannose) <sub>9</sub>	31247.402	31251.383	0.0127
T266/T266	(Mannose) <sub>12</sub>	31701.830	31700.302	-0.0048
T266/T266	(Mannose) <sub>18</sub>	32690.683	32693.065	0.0073

**Table 3-7.** Mass shift in temperature stress treated samples of rhBMP-2

Predicted Degradation Reaction	Type of Glycoform	Non-stress treated Mass	Stress treated mass	Expected $\Delta m$ (Da)	Observed $\Delta m$ (Da)
1. Cyclization of Glu	Q283/T266	30205.988	30188.592	- 17.030	- 17.396
2. Deamidation of Asp	Q283/T266	30905.729	30919.477	+12.766	+12.360
3. Deamidation of Asp	Q283/T266	29847.587	29860.222	+12.766	+12.635
4. Oxidation of Met/Trp	Q283/T266	28891.850	28908.286	+16.000	+16.440
5. Oxidation of Met/Trp	Q'283/Q'283	28595.002	28659.512	+64.000	+64.500

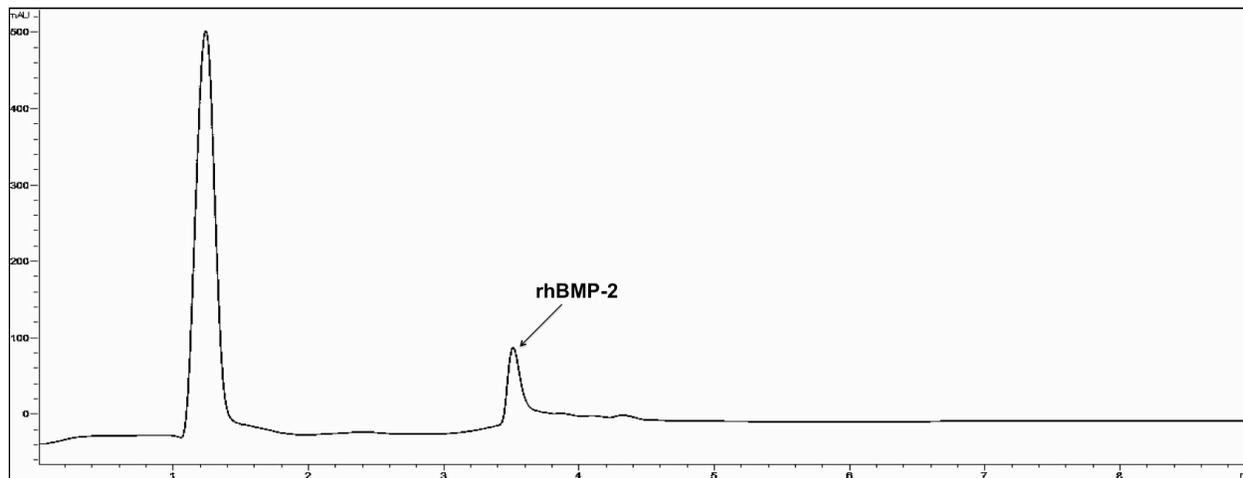
rhBMP-2 has 14 asparagine (Asp) residues in its sequence. Asp residues guarded by neighboring hydrophobic and branched chain amino acids exhibit lower rates of deamidation as compared to Asp residues with neutral, polar and hydrophilic amino acids such as glycine and serine (Tyler-Cross and Schirch, 1991). On comparison of the stressed samples to the non-stressed samples, the most frequent observation was a gain of +13 to +14Da. It was observed that the Q283/T266 glycoform with 10 and 15 mannose residues showed an increase in molecular weight of +12.36Da (from 30905.73 to 30919.48Da) and +12.64Da (from 29847.59 to 29860.22 Da), respectively. Therefore, this deamidation of 13 asparagine residues in rhBMP-2 is most likely due to the high stress temperature of 70°C for 3 days. Similar observations have also been reported in Porter et. al (Porter et al., 2004) when the protein was subjected to temperature stress at 37°C for 7 days. Further analysis by high resolution/accurate mass MS and NMR will be required to confirm the exact structural changes in the glycoforms for all the stress-treated samples.

*Oxidative degradation:* Another frequent pathway for degradation of proteins is by oxidative degradation. The amino acids, which are most susceptible to oxidation, are methionine, cysteine, tryptophan and tyrosine (Li et al., 1995). Oxidation generally entails the gain of oxygen and hence, increases in the molecular weight by +16Da; +32Da and +64Da depending upon the number of amino acid residues oxidized. rhBMP-2 dimer shows the presence of 2 tryptophan and two methionine residues on each monomer (hence a total of 4 tryptophan and 4 methionine on each dimer) (Ji et al., 2009). An analysis of the molecular weights of the different glycoforms predominantly

revealed shifts in molecular weights of +16 or +64 Da (that can be attributed to primarily methionine or tryptophan oxidation) (Patel et al.). Predicted oxidative molecular weight increase was mostly observed in Q283/T266 samples an increase in molecular weight of +16.44Da for a Mannose 7 glycoform (from 28891.850Da to 28908.286Da) and a Q'283/Q'283 glycoform of +64.50Da (from 28585.002 to 28659.512Da). The various mass shifts have been listed in Table 3-7. The qualitative characterization information generated from these stress studies will help in future in-depth structural conformation studies using high-resolution MS and NMR.

### **3.3.4 Bioanalytical method application to samples of 'biorelevant' *in vitro* release study**

The developed method was used to quantify release samples from a novel biorelevant model. Initial stability degradation studies addressed in section 3.2.4. and Table 3-4 reveals the stability of the formulation at 37°C in the biorelevant release media. The release was measured in modified Hanks Balanced Salts solution at a pH of  $4.5 \pm 0.1$  and a pH  $7.2 \pm 0.1$  at temperature conditions of 37°C. Approximately 400 samples have been analyzed using the above-validated method; Figure 3-6 is a real-time *in vitro* release study sample from the biorelevant *in vitro* model. Chromatographic interference from degradation products has not been observed in the samples thereby indicating the robustness of the method.



**Figure 3-6.** Chromatogram of 24hr. release sample from real time *in-vitro* study.

### 3.4 CONCLUSION

An HPLC stability indicating method was developed and validated for the first time for quantification of rhBMP-2 protein formulation in a biorelevant release media. The method will help facilitate analysis and quantification of rhBMP-2 protein released from a collagen scaffold based implant (INFUSE<sup>®</sup> Bone Graft) in a novel biorelevant *in vitro* model for release testing of implants. All six isoforms of rhBMP-2 and some specific glycoforms of each isoform were also detected in the release media using a triple quad/linear ion trap MS. Stressed forced degraded samples revealed shifts in molecular weights of the various glycoforms when compared to non-stress treated rhBMP-2. Future work will include use of tools such as high resolution/accurate mass (HR/AM) MS and NMR for extensive detection and characterization of specific structural and conformation changes that stress induces in the rhBMP-2 glycoprotein. This will be a step towards bridging the gap in higher order structural characterization changes induced by stress in complex biotherapeutic protein formulations and biologics.

## CHAPTER 4

### **4 A Novel *Biorelevant In Vitro* Drug Release Model to Characterize Release of Recombinant Human Bone Morphogenetic Protein-2 from an Absorbable Collagen Sponge Scaffold**

#### **4.1 INTRODUCTION**

##### **4.1.1 Biorelevant *In Vitro* Release Testing**

*In vitro* dissolution and release testing have been used as crucial tools for establishing bioequivalence between dosage forms and for biowaivers in immediate release solid oral dosage forms (Yu et al., 2002). Extended controlled release non-oral dosage forms also called as 'special/complex' dosage forms, such as carrier based protein implants, drug-eluting stents, liposomes, nano-particles are often positioned in unique physiological environment. Their primary functionality is to release the drug substance over an extended period of time in controlled and requisite quantities to meet the

targeted therapeutic range. After implantation drug biodistribution for 'complex' dosage forms occurs in surrounding tissues and compartments that are often difficult to access. This makes the sampling of the drug in all the tissue samples challenging. As a result exploration of the release mechanism and pharmacokinetic profile of the drug is considerably difficult in comparison to immediate release dosage forms such as tablets and suspensions. In such cases an *in vitro* release test can be of additional value in studying the release of the drug substance from the carrier scaffolds such as collagen, PLGA and liposomes. A dissolution or release test study design should ideally be a representative and surrogate test for *in vivo* release. It should be easy to perform; reproducible and robust yet circumvent the simulation of major physiological factors that might affect the dosage forms performance *in vivo*. Such dissolution or release tests can be said to be clinically biorelevant and can act as quality control tests. They can help in predicting batch-to-batch variability and minor formulation changes between batches during the manufacturing process.

Standard USP apparatuses that have been established and work well for immediate release dosage forms such as tablets and suspensions are often not relevant to controlled release parenterals. They fail to capture critical clinically biorelevant *in vivo* parameters such as low fluid volume surrounding the dosage form, vascularity and flow directionality, complications during the sampling procedure (e.g physical separation of the dosage form such as carrier hydroxyapatite particles, liposomes and nanoparticles from the release media). Amongst the USP apparatuses, USP-IV is the preferred apparatus in literature for release testing of complex release parenterals (Cardot and

Tomic, 2015; Sievens-Figueroa et al., 2012; Thomas, 2016). Various modifications have been applied in the past to standard USP-IV flow through cell for the release testing of microspheres, liposomes and drug eluting stents e.g dialysis adapter settings, design of vessel simulating flow through cell (Bhardwaj and Burgess, 2010; Seidlitz et al., 2011a; Zolnik et al., 2005). These adaptations have generally been made to facilitate ease of sampling and achieve representative cumulative release profiles. In many investigations such as Seidlitz and co-workers, 2011, the adaptations to the USP-IV were designed to better simulate physiological microenvironment and *in vivo* parameters that might affect drug release and biodistribution into complex tissue compartments (Seidlitz et al., 2011a).

The primary objective of this research was to develop a biorelevant release study design for a novel dosage implant INFUSE<sup>®</sup> Bone Graft (a collagen carrier based biodegradable scaffold with a protein rhBMP-2 as the drug substance). The first step was exploring conventionally available dissolution/release testing methods for implants such as 'sample and separate' and 'USP-IV flow through cell'. Due to high variability in results and incomparable release profiles obtained from sample and separate and USP-IV, our next step was to design and develop a novel biorelevant *in vitro* model for release testing of the implant graft. The model was designed to simulate a) low fluid volumes with b) membrane separated multi compartments of 3-8mL and c) flow directionality requisite for drug release in implants. It was primarily intended to facilitate the testing of carrier-based scaffolds (such as collagen and hydroxyapatite) in which the filler carrier material of the implant might occupy the central compartment mostly or

completely. It was also designed to simulate perfusion of the extended controlled release implants by parallel low flow rates. Lastly, it also simulated in its design if required physical separation of the dosage form from the media such as for release/dissolution testing of microspheres and liposomes. The model was expected to provide an evaluative *in vitro* relationship (IVR) between formulations of rhBMP-2 (the drug substance of the selected implant). The novel in house developed model was then evaluated by testing if it is discriminatory between two different formulations of rhBMP-2 using similarity (f2) and difference (f1) factors.

## **4.2 MATERIALS AND METHODS**

### **4.2.1 Materials**

INFUSE<sup>®</sup> Bone graft was generously donated by Medtronic (Minneapolis, Minnesota, USA). Osteograft/N-300 was purchased from Dentsply Sirona (Philadelphia, PA, USA). Modified Hanks Balanced Salts Solution (HBSS-without phenol red and sodium bicarbonate) and 4-(2-hydroxy ethyl) piperazine-1-ethanesulfonic acid (HEPES buffer, 1 mM) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade water was purchased from Fisher Scientific (Waltham, MA, USA). HPLC grade acetonitrile was purchased from VWR (Radnor, PA, USA) and trifluoro acetic acid was purchased from EMD Millipore (Billerica, MA, USA). Sample materials for creating the body of the model such as polycarbonate, polytetrafluoro ethylene (PTFE), teflon and poly ether ether ketone (PEEK) were provided by in-house engineer Mike Grieve at Virginia Commonwealth University, Richmond, VA, USA. The material for the construction of the

body of the model (polycarbonate) was obtained from Piedmont Plastics (Richmond, VA, USA). Three-way flow valves and polypropylene tubing connectors were purchased from Spectrum Laboratories (Rancho Dominguez, CA, USA). Protein low binding membrane materials: regenerated cellulose (47mm diameter; 0.45 µm pore size), poly tetra fluoro ethylene (47mm diameter; 0.45 µm pore size), polycarbonate (47mm diameter; 0.45 µm pore size), cellulose acetate (47mm diameter; 0.45 µm pore size) and poly ether sulfone (PES) (47mm diameter; 0.80 µm pore size) were obtained from GE Life Sciences (Pittsburgh, PA, USA). Protein low bind silicon pump tubings (Masterflex, platinized and formulation grade) were purchased from VWR (Radnor, PA, USA). Low flow peristaltic pumps (0.03 to 8.2 mL/min.) were purchased from VWR (Radnor, PA). Polysorbate 80 was purchased from Sigma-Aldrich (St. Louis, MO, USA).

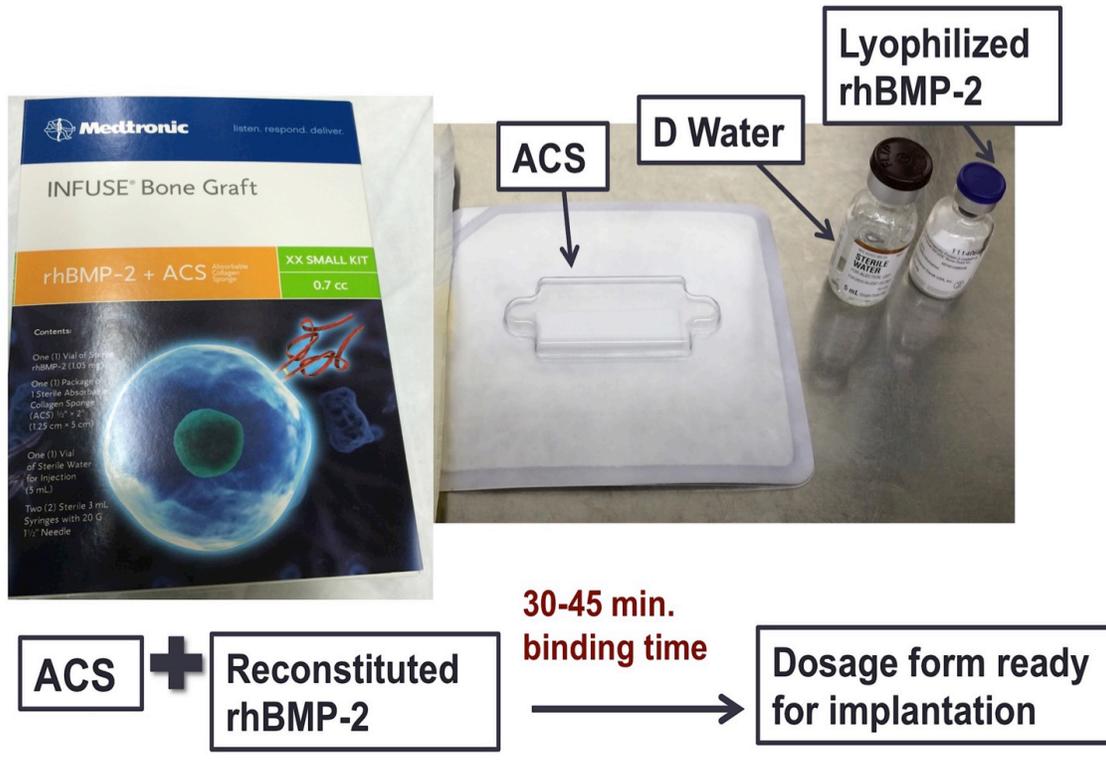
## **4.2.2 Description of the dosage forms tested**

### *INFUSE<sup>®</sup> Bone graft:*

INFUSE<sup>®</sup> Bone graft is a biodegradable locally acting implant that helps in osteoinduction and bone regeneration (FDA Rockville, 2007a; Urist, 1965). The dosage form has been used for oral maxillofacial surgery, healing of tibial shaft fractures and spinal fusion surgeries (McKay et al., 2007). It consists of two main components a collagen based scaffold for release over an extended period of time and bone morphogenetic protein-2 (BMP-2), the protein that helps in bone regeneration and healing (Carragee et al., 2011). BMP-2 is found endogenously in the body and is predominant during embryonic stages of growth and differentiation in humans

(Carragee et al., 2011). It is a cytokine belonging to the Transforming Growth Factor- $\beta$  family and due to its high rate of systemic clearance has a short residence time (FDA Rockville, 2007a; Urist, 1965). The recombinant human BMP-2 (rhBMP-2) in INFUSE<sup>®</sup> Bone graft is produced in Chinese Hamster Ovary (CHO) cell lines. The API formulation (rhBMP-2) is in the form of a lyophilized powder that has to be reconstituted with sterile water for injection (FDA Rockville, 2014). After reconstitution the protein solution is evenly distributed over the collagen scaffold and a period of 15-30 min is allowed for the binding of the protein with the scaffold before placing the implant in patients (FDA Rockville, 2014). The drug substance rhBMP-2 is generally provided as a total amount of 12mg of protein and after reconstitution is at a final concentration of 1.5mg/mL. The size of the carrier collagen sponges vary and are generally 1 X 2 in. or 3 X 4 in. depending upon the size of INFUSE<sup>®</sup> Bone graft kit chosen (FDA Rockville, 2014) . The dosage form with all its components have been photographed in Figure 4-1.

# Model Implant : INFUSE<sup>®</sup> Bone Graft



**Figure 4-1.** Dosage form INFUSE<sup>®</sup> Bone graft with its components.

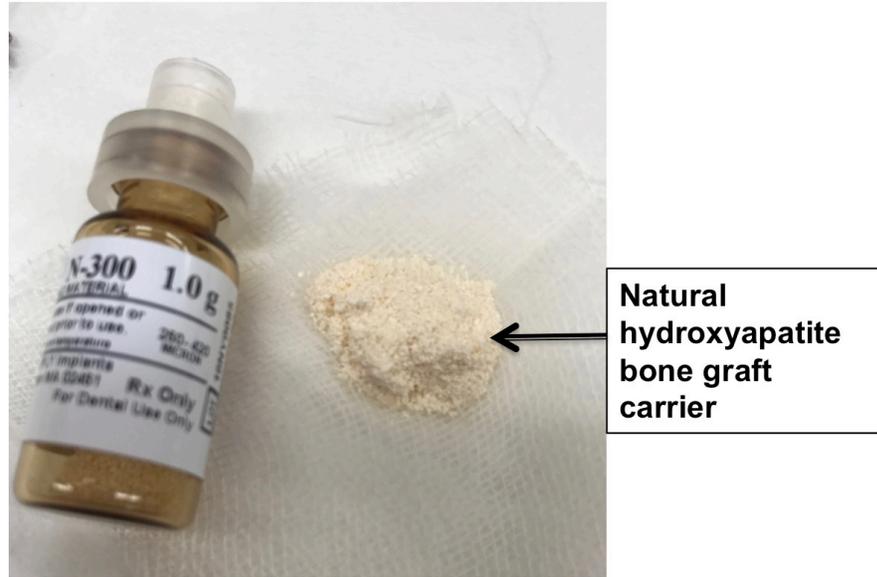
After reconstitution of lyophilized rhBMP-2 with sterile water and distribution over ACS a binding time of 30-45min was allowed for effective incorporation of rhBMP-2 in ACS.

The bovine collagen based scaffold in INFUSE<sup>®</sup> Bone graft called Absorbable Collagen Sponge (ACS) acts both as a carrier for BMP-2 and a supporting framework for invading osteoclast and osteoblast cells (Geiger et al., 2003). ACS thus helps in retaining the BMP-2 at the requisite local site of action over an extended period of time (Friess et al., 1999; Geiger et al., 2003). Due to its biodegradable nature eventually the scaffold is entirely replaced by new bone and there is no requirement to remove the implant with a post-operative surgery for the patient (Geiger et al., 2003).

#### *Osteograf-N/300:*

In this current study the release of rhBMP-2 from the ACS scaffold was also compared to its release from another natural hydroxylapatite-based scaffold called Osteograf-N/300. Similar to ACS, Osteograf-N/300 is also bovine derived and is a microporous anorganic hydroxylapatite (Hakimi, 2000). It has been used as a filling material/scaffold particularly in sinus floor elevation studies by clinicians (Froum et al., 1998). Osteograf-N/300 has been applied as a standalone dosage form for remodeling of bones or in several studies in conjunction with BMP-2 to help in bone remodeling (Hakimi, 2000). Therefore, the in-vitro release profiles of rhBMP-2 from two established scaffolds were compared to one another. This in turn was used for establishing an *In Vitro In Vivo* Relationship (IVIVR) in this study by comparison of rat in-vivo pharmacokinetic data (obtained from literature) with biorelevant *in vitro* release data (FDA Rockville, 2007a; Uludag et al., 1999a). Figure 4-2 is a photograph of Osteograf N-300 natural hydroxyapatite carrier material.

## Model Implant Product : Osteograf N-300



Natural hydroxyapatite bone graft carrier



Reconstituted rhBMP-2

30-45 min.  
binding time

Dosage form ready for implantation

**Figure 4-2.** Dosage form Osteograf N-300 with its components.

After reconstitution of lyophilized rhBMP-2 with sterile water a binding time of 30-45min between rhBMP-2 and natural hydroxyapatite bone graft carrier was allowed

## 4.2.3 Physiological environment affecting release of rhBMP-2 from ACS

### 4.2.3.1 Local effects of rhBMP-2

In order to design a clinically relevant *in vitro* release study design an in-depth understanding of the physiological microenvironment of the dosage form was critical. The local and systemic exposures of rhBMP-2 during bone regeneration process were therefore evaluated. Bone regeneration occurs in three stages. A) An inflammatory stage B) a repair stage and finally C) the remodeling stage (Kalfas, 2001)

*Stage 1 is the early inflammatory stage* that encompasses the first initial hours and can last up to a week. During this stage, the hematoma formation occurs and various inflammatory cells like macrophages, white blood cells are attracted to the fracture site. These cells release inflammatory molecules around the wound tissue, which are basically signaling molecules(Chen et al., 1997). The signaling molecules attract the mesenchymal cells, which establish the foundation for attracting the osteoprogenitor (bone regenerating) cells (Kalfas, 2001). The nutrient material to sustain these cells at the wound site is obtained by resorption of dead surrounding bone tissue by enzymes like collagenases (Ma et al., 2004). Table 4-1 and Table 4-2 is a list of the crucial *in vivo* and *in vitro* parameters affecting the release of rhBMP-2 from the collagen scaffold. The wound or fracture microenvironment is different from the normal physiological state. It is slightly acidic (pH of  $7.1 \pm 0.1$ ) as compared to physiological pH ( $7.4 \pm 0.2$ ); infused with inflammatory molecules and digesting enzymes(Hollinger and Wong, 1996).

In an implanted collagen scaffold like ACS during stage 1, the collagenases digesting the collagen will affect release of rhBMP-2, by causing a kind of matrix erosion. The degradation products of collagen digestion also end up in making the wound microenvironment more acidic and hence a decrease in physiological pH (Geiger et al., 2003). As a result the binding of rhBMP-2 to ACS is affected and a net increase in release of the protein from the scaffold during the stage 1/ early inflammatory stage has been speculated. What fraction of the *burst release phase* observed is a) due to binding effects of rhBMP-2 to ACS or b) is due to the changes in the wound microenvironment is not known but is considered as a complex interplay of the above factors. During this stage there is also increased temperature at the wound site: 1-2°C higher, which might affect the already released protein by causing aggregation. The higher temperature for a prolonged time of hours to a week has been speculated to affect the activity of the protein and hence decreases the local effect of rhBMP-2 (Geiger et al., 2003). There are also other factors like presence of serum and plasma proteins, which decrease the binding of rhBMP-2 to the ACS thereby increasing the release.

*Stage 2 is the repair stage and Stage 3 is the remodeling stage* of bone healing. It is during these stages that the vascularization process begins. During these two stages controlled or sustained release is required unlike the burst release phase. The osteoprogenitor cells, which have penetrated the matrix, cause differentiation of the cells thus slower controlled release into the local environment is best suited. The biorelevant a) *in vivo* and b) *in vitro* factors, which were considered significant in influencing drug release from the ACS scaffold, have been summarized in Table 4-1 and Table 4-2 respectively. Several of these factors have been explored in this research

investigation and incorporated into the in vitro release study design.

**Table 4-1.** Crucial *in vivo* biorelevant parameters affecting release of rhBMP-2.

- 
- 1 Enzymes and other large molecules e.g collagenases digesting the ACS scaffold.
  - 2 Hematoma formation on the ACS 3-6 days after implantation.
  - 3 Inflammatory response molecules.
  - 4 Physiological pH.
  - 5 Blood flow rate vascularization near the implant site.
  - 6 Flow direction.
  - 7 Masticatory pressure on the implant.
  - 8 Packing density of tissues and the scaffold at the surgery site.
-

**Table 4-2.** Crucial *in vitro* biorelevant parameters affecting release of rhBMP-2

---

- 1 Media pH-buffering system/capacity.
  - 2 Osmotic pressure.
  - 3 Media volume/Maintenance of sink conditions to maintain solubility.
  - 4 A) Flow rate. B) Flow direction.
  - 5 Stress application.
  - 6 Effect of packing with hydroxyapatite/glass beads on release.
  - 7 Effect of adding collagenase enzyme on release.
  - 8 Membranes-biomembrane vs. artificial membranes.
-

#### 4.2.3.2 Systemic exposures of rhBMP-2

During stage 1 of the wound healing phase the site has an enhanced blood supply due to inflammation. As a result, the rhBMP-2 molecule, which acts as a signaling molecule for attracting the mesenchymal stem cells and osteoprogenitor cell, is carried into the systemic circulation. To a certain degree burst release has been said to be desirable in the initial stages of bone healing so that the signaling molecules can reach the *systemic* circulation. Systemically the uptake of rhBMP-2 by organs like liver is rapid but the residence time is less (FDA, 2014). rhBMP-2 also has rapid catabolization and clearance. Within 24hrs approximately 92% of rhBMP-2 is recovered in urine. The peak maximal concentration was found to be less than 0.1% of the total dosage amount. Due to these factors, the amount of rhBMP-2 in systemic circulation should be minimal after a typical dosing.

However, depending on the implantation site, rhBMP-2 is known to cause numerous unwarranted *local* effects due to burst release such as: tumor formation, swelling (BMPs are a member of TGF- $\beta$  signaling family and hence inflammatory molecules), inability to breathe and morbidity when used in healing of cervical regions. These side effects can be due to the fact that rhBMP-2 is delivered at supra-physiological doses of 1.5mg/mL. Several factors were tested while determining the dose escalation when from moving from rats to canine to non-human primates. Factors like a) masticatory pressure in case of sinus augmentations, b) pressure applied when the surgeon packs the sponge into the trauma site were studied. An optimal concentration, which could withstand these effects, was determined to be 0.8 - 2mg/mL (Uludag et al., 1999a).

There is a scope for improvement in the release mechanism of rhBMP-2 from the scaffold to prevent some of the *localized* adverse events. The rapid systemic clearance, short residence time and low peak concentrations offer certain advantages against the entire body being exposed to supra-physiological doses of the molecule. The effect of rhBMP-2 in dosage forms such as INFUSE<sup>®</sup> Bone graft at supra-physiological doses are hence mostly limited to *local* bone growth and indications. Therefore, the adverse effects of rhBMP-2 are controlled to some degree (rapid systemic clearance) and it has been used as an ‘orthobiologic’ drug modality (local bone regeneration), since the net therapeutic benefits outweigh the risks.

Evaluation of the biorelevant parameters affecting the release mechanism and local release of rhBMP-2, was therefore, one of the core motivation of this research investigation. It led us to design and develop a multi-chambered biorelevant in vitro model/apparatus with design components allowing incorporation of clinically relevant parameters.

#### **4.2.4 Biorelevant *media* for in-vitro release studies and flow rate implications**

##### **4.2.4.1 *Characteristics of a dissolution or release media for implants***

*In vitro* release media for biorelevant testing is a representative of the physiological fluid conditions in the body. The medium should as closely resemble the fluid environment in the body surrounding the dosage form as possible. Various factors which are significant

when selecting a dissolution medium are the pH and the buffer capacity of the medium, the composition of inorganic ions responsible for maintaining this buffer capacity, the changes in the media components in response to temperature and its stability particularly at physiological body temperature 37°C, the osmolality of the media, the solubility and stability of the released drug or the dosage form in the media over the duration of the in-vitro release study. All these factors taken into consideration while selecting the media would enable a more predictive release/dissolution test with less variability.

Other significant factors associated with the media are the physiological flow rate of blood plasma or tissue fluids surrounding the dosage form. The flow rate of the fluids surrounding the dosage form ensures the diffusion and distribution of drug in the body. Hence, selection of a representative flow rate while designing a predictive dissolution/release test is also a primary contributing factor towards a predictive test. A preference is generally given towards a known composition media, which is practical and easy to recreate for everyday use in laboratories. Various media have been previously used in literature to characterize release of drugs from implants such as Dulbecco's phosphate buffered saline (PBS) media for characterizing release from PLGA scaffolds as in Kim and co-workers or release of rhBMP-2 from Poly (D-,L-lactide) disks in cell culture medium with 5%FBS as in Winn and co-workers (Kim et al., 2003; Winn et al., 1998). The core idea is to select a medium that facilitates solubility of the drug, is itself stable for the duration of study and also supports stability of the drug substance for a defined period of time.

#### **4.2.4.2 Selection of media for release study**

In this study we selected a previously characterized media modified Hanks Balanced Salts Solution to represent the blood plasma surrounding the biodegradable implant collagen scaffold (Iyer et al., 2007). Hanks Balanced Salts Solution is a common cell culture media that has been used in culturing osteoclasts and osteoblast cell lines to study bone regeneration and osteogenesis (Klokkevold et al., 1996). Modified Hanks Balanced Salts Solution (HBSS) was used as the *in vitro* media for the current release studies. The HBSS was modified by removal of phenol red to prevent the components interference in the analytical HPLC methodology. A second component sodium bicarbonate responsible for maintaining the buffer capacity of the media when CO<sub>2</sub> is purged for growing cells was also removed (since we did not require purging of CO<sub>2</sub> at intervals). Buffer capacity of the *in vitro* release media was in turn maintained with HEPES buffer. The *in vitro* media was prepared by addition of 9.8 g of modified HBSS to 975 mL of deionized water and 25mL of HEPES buffer. The modified HBSS has been previously characterized and used for *in vitro* release studies in Iyer et. al.,2007 for release characterization of a naltrexone implant (Iyer et al., 2007). Sodium azide at 0.01% was used as an antimicrobial agent.

Unique properties of the selected media, which make it a good candidate for being used in this study, are: the characterization of the medium for changes in pH and buffer capacity with temperature. The medium was observed to show only a 1.28 fold reduction in buffer capacity from 24°C to 56°C (Iyer et al., 2007). The net osmolality of freshly prepared media was 281.3 mOsm and varied between 282.3 -292.3 mOsm with changes in temperature from 38 °C – 45 °C over a period of 30 days. These values are

within the normal plasma osmolality range of 280-295 mOsm/kg (Iyer et al., 2007; Thompson et al., 1986).

#### **4.2.4.3 pH requirements of biorelevant media for the study and its significance in bone resorption, inflammation and bone remodelling**

The first step in bone regeneration is resorption and hence removal of the old dead and decaying bones by the osteoclast cells. This is brought about by extracellular acidification. The acidification causes dissolution of the organic and mineral constituents of the bone and helps in the activation of various lysosomal enzymes such as carbonic anhydrase (Baron et al., 1985). Bone regeneration with the help of 'orthobiologics' such as carrier scaffolds e.g. Absorbable Collagen Sponge (ACS) and PLGA often creates a localized acidic microenvironment. This acidic inflammatory osteogenic microenvironment was studied by Kohn et. al in 2001 to investigate its effects on tissue engineering of bone (Kohn et al., 2002). It was found that growth and differentiation of osteogenic cells such as osteoblasts is particularly sensitive to changes in pH. Bone resorption, regeneration and modeling are particularly dependent on the interstitial surrounding fluid pH and tissue pH. An acidic pH supports bone resorption and a basic pH supports regenerative activities. Since trauma sites are generally slightly acidic the media pH was also adjusted to a pH of  $7.1 \pm 0.1$  using 1N hydrochloric acid or 1N sodium hydroxide.

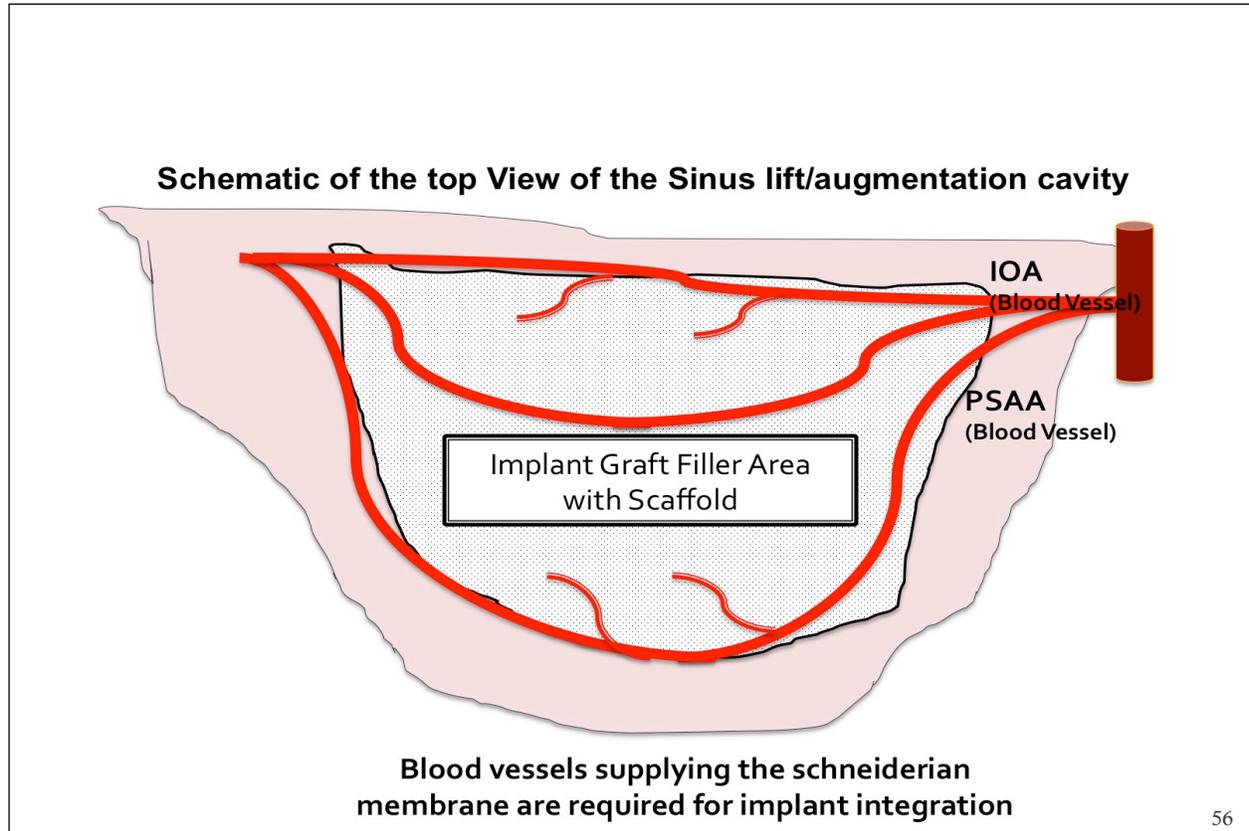
#### **4.2.4.4 *Flow rate selection and vascularization study of implanted graft material***

INFUSE<sup>®</sup> Bone graft application for bone healing and regeneration is an 'orthobiologic' approach as opposed to autografted bones. Continuous blood flow and supply to the implant graft area are significant for the survival of the graft. The continuous blood flow helps in carrying the neuropeptides and cytokines like bradykinins and rhBMP-2, which eventually help in the osteo-integration of the graft material (Dimitriou et al., 2011; Dimitriou et al., 2005). The placement of the graft in the sinus lift cavity induces acute and chronic inflammation in the area. Inflammation is characterized by enhanced blood flow due to vasodilation of the blood vessels surrounding the graft zone (Berggreen et al., 2007). The dilated blood vessels are also a result of release of neuropeptides, prostaglandins and bradykinins. Cytokines such as rhBMP-2 (which are chemo attractant molecules) activate a chemical signal to attract osteoclast cells and macrophages to digest the decaying surrounding bone tissues and replace with the help of new bone forming cells called osteoblasts (Dimitriou et al., 2005). During this entire process of bone growth acute inflammation is followed by chronic induced inflammation (Claes et al., 2012).

Chronic inflammation persists from weeks to months (Berggreen et al., 2007). The acute inflammation predominantly causes vasodilation and blood flow changes. Chronic inflammation however is dominated by cellular regeneration phases in addition to flow changes in the localized surrounding area (Berggreen et al., 2007). Normal resting blood flow rates in the pulpal area are between 0.17- 0.19mL/min called as pulpal blood flow (PBF)(Berggreen et al., 2007). During inflammation vasodilation can lead to a

200% increase in PBF and in turn vascular permeability for interchange of large molecules with the surrounding blood and interstitial fluid (Berggreen et al., 2007). The vasodilation is advantageous not only to deliver osteoinductive molecules to the site of regeneration but also to help in carrying away the heat generated (due to inflammation) and decaying by products of the regenerating bone (Dimitriou et al., 2011).

A detailed study in Solar et. al. showed that implanted grafts during sinus lifts and augmentations require blood supply from three major blood vessels: a) the vessels or blood flow to the schneiderian membrane (the lining of the maxillary sinus cavity); b) the posterior superior alveolar artery (PSAA) and the c) Infra Orbital Artery (IOA) (Solar et al., 1999). The PSAA also supplies the growing bone and the periosteal lining of the bone (Solar et al., 1999) and the schneiderian membrane. The measured net blood perfusion rate of the schneiderian membrane was shown to be between 0.09mL/min-0.99mL/min in a study in different animal models (Kumlien and Schiratzki, 1985). Hence, a flow rate of 0.8- 0.9 mL/min was selected for the biorelevant media in the designed model. This would be representative of the physiological flow associated with inflammation in the graft area at different stages of healing. A flow rate of 0.8- 0.9 mL/min would also ensure proper mixing of the drug substance rhBMP-2, with the low flow peristaltic pumps in the novel biorelevant in vitro model. A schematic representation of the blood vessels supplying the maxillary sinus cavity has been shown in Figure 4-3.



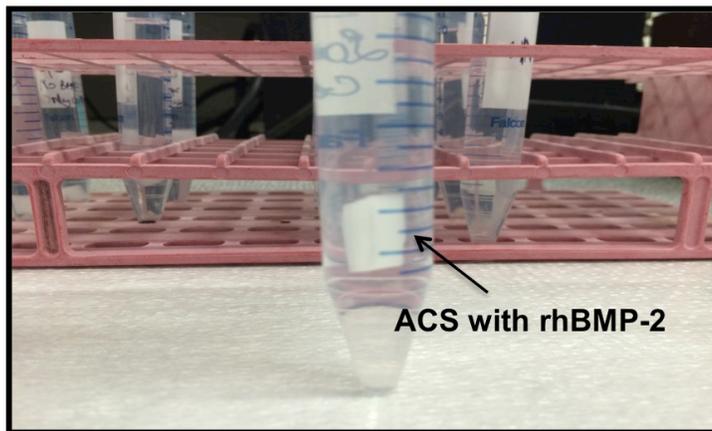
**Figure 4-3.** A schematic representation of the blood vessels supplying the maxillary sinus cavity during sinus lift and augmentation surgeries.

This picture has been modified from Solar et. al. (Solar et al., 1999)

## 4.2.5 Apparatuses for in-vitro release study

### 4.2.5.1 Sample and separate experimental set up

As mentioned previously in Chapter 1 of this document sample and separate studies help in establishing a baseline for in vitro release tests. Although due to lack of design and vessel hydrodynamics, they might not be able to simulate many biorelevant parameters (flow rates and directionality of physiological fluid, mixing) they can serve as an essential tool in observing the baseline performance of the dosage form. Many of the significant biorelevant parameters such as temperature, pH can be included in such experimental set ups. In our current study sample and separate experiments were performed in phosphate buffered saline (PBS). 200µg of rhBMP-2 was loaded onto 0.5 X 0.67 in of the absorbable collagen sponge scaffold at rhBMP-2 concentration of 1.5mg/mL. Each sample and separate tube had 5mL of media. Replicates of the tubes were prepared including blank controls (collagen sponge with no rhBMP-2). The entire media was replaced at 0.5hr, 1hr, 4hr and 6hr followed by once daily after the first day for up to 15 days. The tubes were incubated at 37°C. The pH of the medium was adjusted to  $7.1 \pm 0.1$ . The samples were analyzed using Quantikine ELISA kit (R & D Systems, USA). The linear dynamic range of the assay was from 62.5pg/mL-2000pg/mL. A sandwich ELISA was performed according to the kits manual and optical density measurements were recorded with BioTek ELISA microplate reader at 450nm. Background readings for the plate were measured and subtracted at 540nm. The amount of rhBMP-2 released in the *in vitro* study was plotted against time in days. A pictorial representation of the sample and separate set up has been shown in Figure 4-4. Table 4-3 summarizes the experimental conditions of the sample and separate set



Beads for temperature control at 37°C

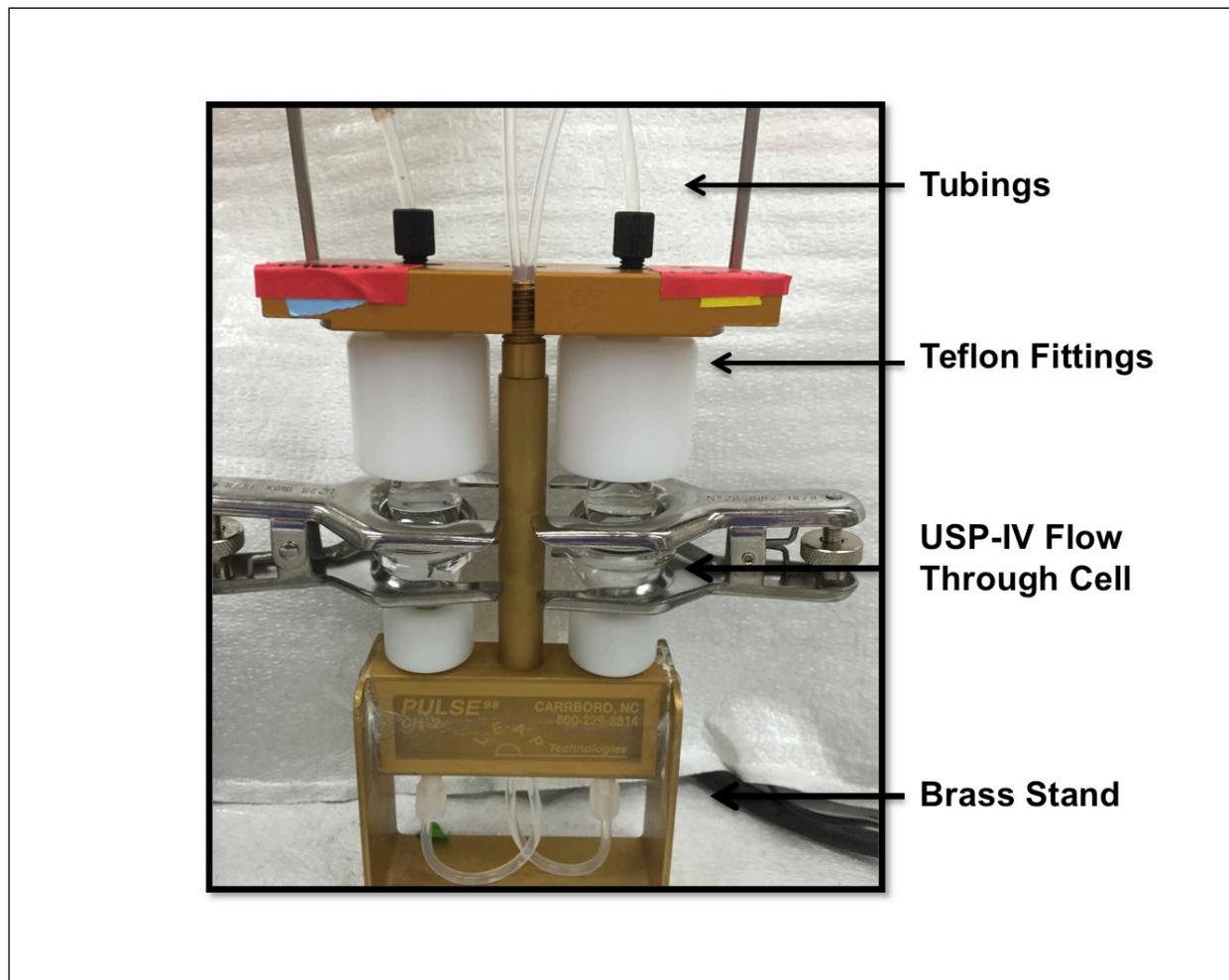
Incubator

**Figure 4-4.** A pictorial representation of the sample and separate set up at 37°C.

up and the biorelevant parameters incorporated while performing the test.

#### **4.2.5.2 USP-IV Flow through cell experimental set up**

In our current study of rhBMP-2 release from ACS scaffold (INFUSE<sup>®</sup> Bone graft) a modified flow through cell experimental set up was used after the initial sample and separate methodology. The modified USP IV flow through apparatus consisted of two conical glass cells of length 8.5cm each. Two Teflon fittings are present on the lower and upper end of size 4.5 and 1.5 cm respectively. The net volume of the two-half glass cell (clamped with o-rings and a stainless-steel clamp to render it leak proof) was 7.5 – 8 mL. An additional reservoir held 7.5ml of media and was used to complete the set up. Total media volume was 15mL. Two such USP-IV flow through cells were mounted on each brass stand to replicate the experiments (n=6 total experiments were performed). Glass beads served multiple functions: In addition to providing laminar flow within the apparatus, the beads also acted as a surrogate for simulating tissue packing and held the floating collagen sponge dosage form in position. Protein low bind cellulose acetate membranes were used to hold the glass beads and dosage form in the cell. Two kinds of media were tested using this experimental set up: traditionally used phosphate buffered saline (PBS) and biorelevant modified hank's balanced salts solution (HBSS) both monitored at pH  $7.1 \pm 0.1$  over the course of release study. A pictorial representation of the USP-IV flow through experimental set up has been shown in Figure 4-5. Table 4-4 summarizes the experimental conditions of the USP-IV flow through cell set up and the biorelevant parameters incorporated while performing the test.



**Figure 4-5.** A pictorial representation of the USP-IV flow through set up.

Two halves of the cells were clamped together. Each brass stand can mount two flow through cell. Three such settings comprising of six flow through cells were used.

**Table 4-3. Experimental conditions for sample and separate study design**

<b>Factors evaluated</b>	<b>Values</b>
1. Biorelevant factors	Temperature, pH
2. Temperature	37°
3. Media	PBS
4. Scaffold size	0.5 X 0.67 in.
5. Dosage amount	200ug
6. rhBMP-2 concentration	1.5mg/mL
7. pH	7.1 ± 0.1 (Biorelevant)
8. Volume (mL)	5
9. Sampling frequency	30min, 60min, 4hr, 6hr, once daily for up to 30days.
10. Sampling volume	5mL

**Table 4-4. Experimental conditions for USP-IV study design**

<b>Factors evaluated</b>	<b>Values</b>
1. Biorelevant factors	Temperature, pH, glass beads, membrane
2. Temperature	37°C
3. Media	PBS, HBSS
4. Scaffold size	0.5 X 0.67 in.; 1 X 2
5. Dosage amount	200 µg, 700 µg
6. rhBMP-2 concentration	1.5mg/mL
7. pH	7.1 ± 0.1
8. Volume (mL)	15 mL
9. Sampling frequency	30min, 60min, 4hr, 6hr, once daily for up to 30days.
10. Sampling volume	1 mL

<b>Dosage form component</b>	<b>Positive Control</b>	<b>Negative Control</b>
	(n=3)	(n=3)
<b>rhBMP-2</b>	+	-
<b>ACS</b>	+	+

### 4.2.5.3 Biorelevant *In Vitro* Drug Release (BIVDR) model

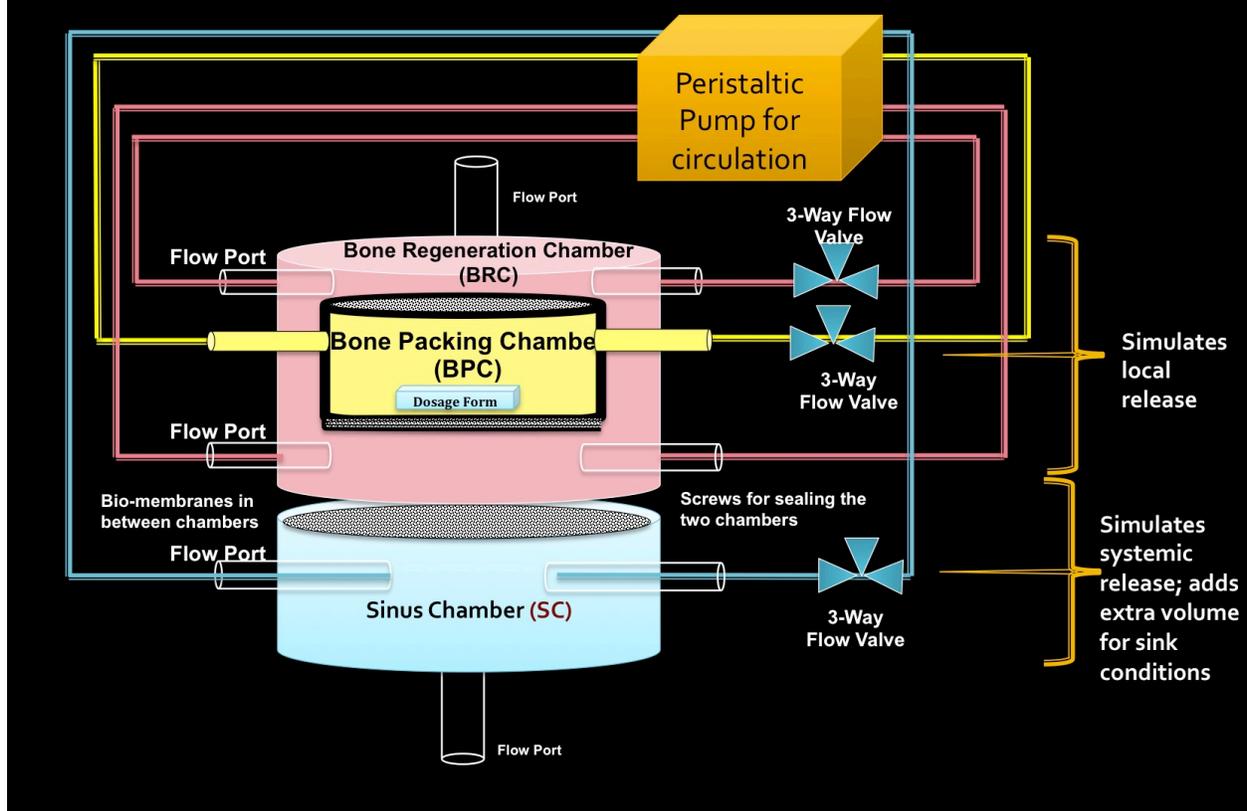
#### *Design of the BIVDR model:*

The in house designed and constructed biorelevant *in vitro* drug release (BIVDR) model has been represented in Figure 4-6 and Figure 4-7. The model design consists of three main compartments: an outer cylindrical bone regeneration chamber called BRC, a centrally located cylindrical core compartment housed inside the BRC called bone packing chamber or BPC and an optional sinus chamber called SC which can be used in case of extra volume requirements or the BRC can simply be capped off in case of smaller volume parenteral release testing requirements. The central donor compartment BPC opens into BRC on either side and is separated physically from BRC using membranes selected according to the requirements of the experiment. The membranes are screwed in position with the help of size 0 medical grade stainless steel screws, o-rings and a thin round donut shaped polycarbonate frame. The flow ports in each chamber are positioned horizontally and flow directionality is parallel in all the chambers. Additional flow ports have also been placed perpendicular to the compartment. They can be capped off or depending upon the needs of the experiment can be kept open (for perpendicular flow directionality, for an increased release rate for a dosage form or for turbulent flow).

Three- way sampling/flow valves were provided in all compartments (BRC, BPC and SC) for regular sample collection and regulating flow directionality. The BPC and SC have one pair of flow ports whereas the BRC has two sets of flow ports. Each pair of flow port in the BRC is positioned a few millimeters above and below the centrally

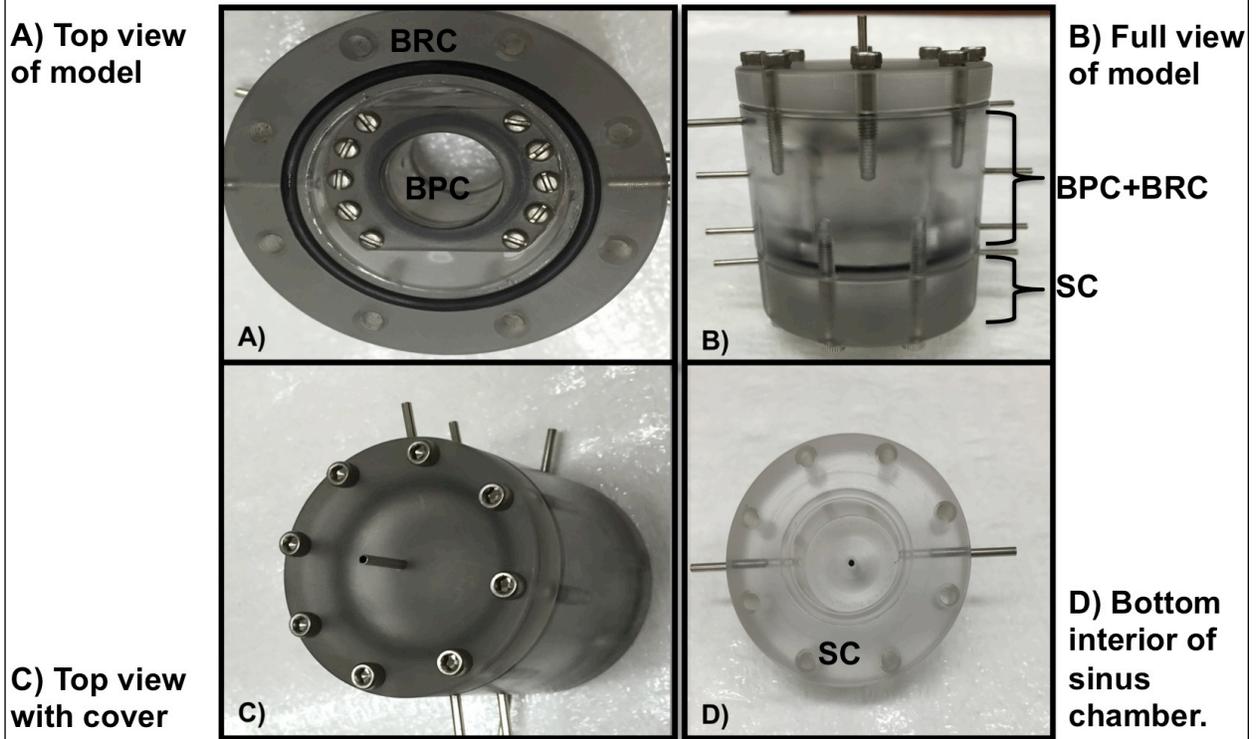
located core compartment BPC to allow for proper mixing of fluids and drug substance released from the donor compartment (BPC). The centrally located BPC along with the tubings in a closed loop mode has a net volume of 2.6 mL. The net volume of fluid in the BRC with the tubings is 8.9 mL. The additive volume of both the compartments (BPC+BRC) with the connecting tubings amounts to 11.5mL. If the sinus chamber is used it adds an additional 2.5mL to the model. The schematic representation of the complete BIVDR model and the experimental set up has been shown in Figure 4-6. The actual BIVDR model has been photographed in Figure 4-7.

## SCHEMATIC OF THE BIORELEVANT IN VITRO MODEL



**Figure 4-6.** A schematic representation of the biorelevant *in vitro* drug release (BIVDR) model experimental set up with its components: peristaltic pump, tubing connectors and three-way sampling valves for circulation in a closed loop configuration.

### Biorelevant In-Vitro Drug Release (BIVDR) Model



**Figure 4-7.** A pictorial representation of the fully constructed biorelevant *in vitro* drug release (BIVDR) model.

A) Top view of the model showing both the Bone Packing Chamber (BPC) and Bone Regeneration Chamber (BRC), B) Full lateral view of the model with all the three chambers: BPC, BRC and Sinus Chamber (SC). If SC is not in use the model can be capped off to use only the BPC and BRC for low volume release requirements. C) Top view with screwed in cover/cap of the model (note: the cap has an additional flow port for perpendicular flow if required) D) Top cross sectional view of the bottom SC.

#### **4.2.5.4 Biorelevant *In Vitro* Drug Release (BIVDR) model experimental set up**

*A) Arrangement and functionality of the chambers: Bone packing chamber (BPC) and Bone regeneration chamber (BRC)*

The central core compartment (BPC) acts as the donor compartment where the dosage form is placed. The BPC is used for simulating the tissue chamber where clinicians pack the implant carrier scaffold as shown in Figure 4-3. It can be a) completely packed with the carrier material e.g hydroxyapatite/ACS or b) partially packed with the remaining space filled up with media. During a sinus lift surgery the clinician normally creates a lateral window by incision, of approximately 13mm from the bony alveolar ridge of the sinus lift cavity (Rosano et al., 2011). This size of incision is for general sizes of implants of 11-13mm such as INFUSE<sup>®</sup> Bone graft, absorbable collagen carrier sponge. Therefore, a central compartment with a height of 11-13mm was selected; with a net volume of 2-3mL representing the central cavity during sinus lifts and sinus augmentation surgeries.

In experimental set ups where the bone packing chamber (BPC) is completely packed with filler or carrier graft material and no release media, the release media is found only in the surrounding bone regeneration chamber (BRC). Release samples then need to be collected only from the BRC and would represent the local release. If complete filling of the chamber is not required such as smaller size of carrier scaffolds, liposomes or microspheres the central BPC can have biorelevant media circulating through it. In such a case release samples collected from the BPC and additionally from BRC in totality

would represent the drug released at a sampling time point. In dosage forms such as ocular implants drug biodistribution occurs into multiple tissue chambers with different composition and osmolality of physiological fluids in each chamber (Bochot and Fattal, 2012). To represent physiological drug biodistribution in multiple tissue chambers two different compositions of media can be circulated through the compartments BPC and BRC. This would represent two different physiological fluid microenvironments. In our current research study since we were primarily concerned with the local release of rhBMP-2, hence, we used a single media in both the chambers BPC and BRC. Two peristaltic pumps circulate media within each compartment. Each of the pump is in a closed loop configuration i.e BRC circulates media in its own closed loop separate from the BPC. Each closed loop configuration has its own three-way flow valve, which is used during sampling at regular intervals. During sampling the peristaltic pumps are switched off for about 2 min to allow for the collection of the release samples. The volume lost during sampling is replaced into the BIVDR model using the same three-way flow valve with a graduated plunger syringe separately into each chamber.

The core compartment BPC was enclosed with membranes of low protein bind nature e.g. regenerated cellulose, cellulose acetate, polycarbonate membranes ensuring negligible adsorption and continuous permeation of rhBMP-2. The membranes had a pore size of 0.45  $\mu\text{m}$ . The net dimensions of the rhBMP-2 homodimer are 70  $\text{\AA}$   $\times$  35  $\text{\AA}$   $\times$  30  $\text{\AA}$  (Scheufler et al., 1999). In vivo pore diameter for pores found on the endothelial capillary vessels is 24  $\text{\AA}$  (Pappenheimer et al., 1951). Therefore, the porosity of the membranes used *in vitro* are in the order of  $10^6$  times the size of both: a) the rhBMP-2 molecule and b) in vivo pores found on the capillary walls. It can be safely stated that

the *in vitro* porosity of the membranes will not be limiting factor to the diffusion of the rhBMP-2 molecule. If required two variable flow rates such as mucosal flow rate and periosteal flow rates can be simulated in the BPC and BRC. However, in our present study these flow rates are almost similar between 0.8 - 0.9 mL/min (Berggreen et al., 2007; Solar et al., 1999). Therefore, the flow rates of all the chambers in our study were initially maintained at 0.8 - 0.9 mL/min as a starting point.

***B) Flow directionality selection between chambers:***

The flow directionality was controlled with the help of peristaltic pumps, three-way flow valves and connector fittings obtained from VDR, Cellmax and Spectrum laboratories respectively. These are special biocompatible flow valves to keep a tubing port open or closed. They are often used in cell culture experimental set ups to regulate the directionality of flow and help in easy sampling. Although all the chambers have inlet and outlet valves the valves were operated in combination, so as to simulate combinations of flow directionality. Flow directionality experiments would help us explore the affects of *in vitro* flow direction in the BIVDR model on drug release at a specific flow rate. A pictorial representation of the flow directionality choices have been shown in the Figure 4-8. The red line represents the flow direction of the media between the two compartments

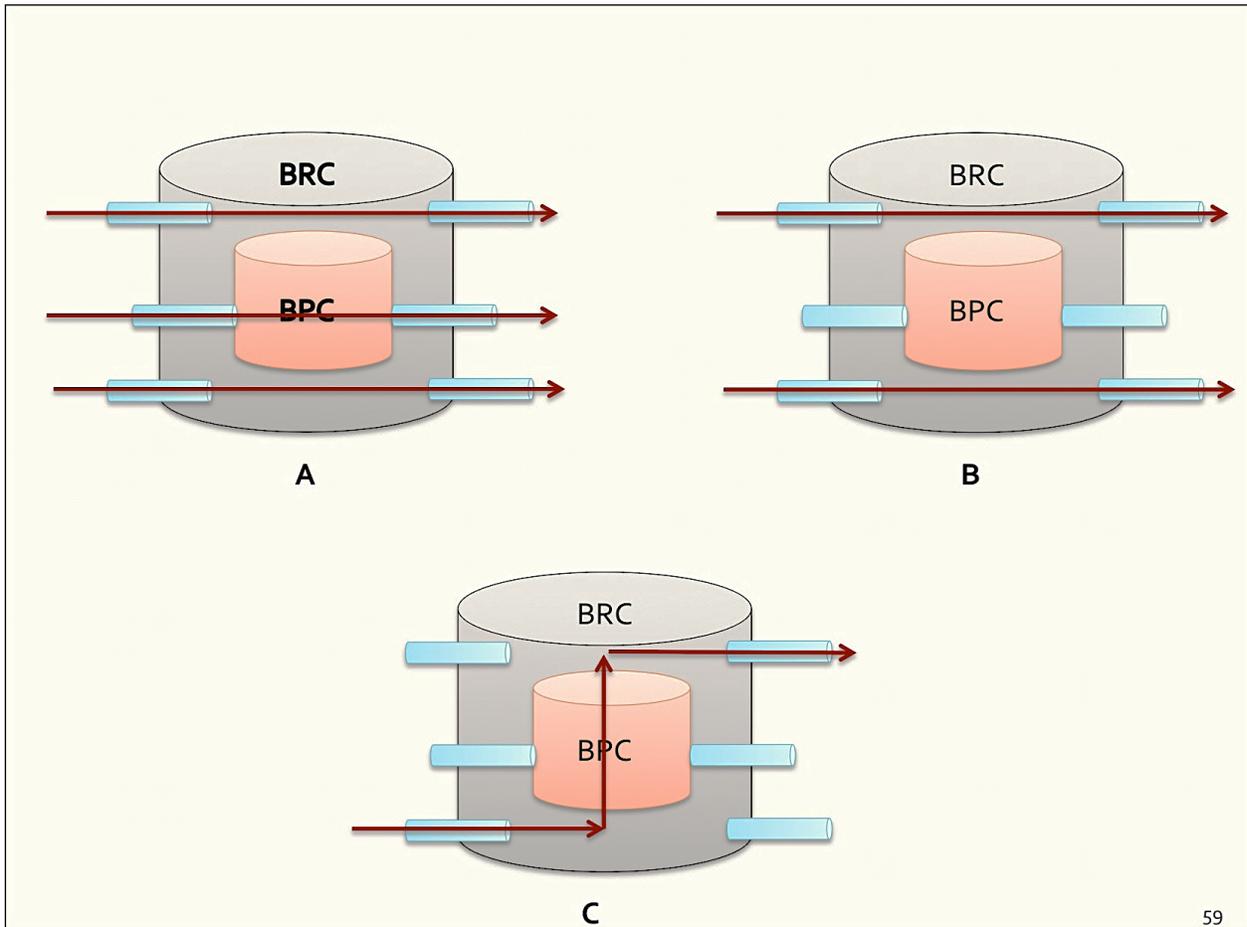
- i) Figure 4-8 (Module A): represents the situation in which the flow is maintained in parallel in both the chambers (BPC +BRC).
- ii) Figure 4-8 (Module B): represents a situation in which the flow is maintained only in BRC and BPC has no flow. This situation can be used when the entire

central BPC needs to be packed up with the carrier/dosage form e.g. natural/synthetic hydroxyapatite carriers for rhBMP-2 in sinus augmentation lift surgeries. Alternatively, it can also be used in a situation where we require some degree of segregation of the media and the carrier yet free exchange of drug is required e.g. microspheres, liposomes, nanoparticulate implants.

- iii) Figure 4-8 (Module C): represents a 'Z' patterned flow in which the flow in BPC is turned off and the flow in BRC, of the diagonal ends of the ports is maintained. The flow in BRC therefore indirectly drives the flow through BPC. Such flow directionality would help simulate a mixture of laminar and turbulent flow in the BIVDR model and can be used uniquely to offer flexibility to increase or decrease the release of drug substance from a dosage form in a single model.

A paired samples t test was conducted with pairwise alignment for each time point to compare the flow in (BPC + BRC) with a) flow only in BRC and b) Z patterned flow in BRC, at a significance level of  $\alpha = 0.05$ . Results were reported as a t test value with degrees of freedom (t(df)) and p values respectively. To summarize, the modular design of the novel biorelevant model was intended to better simulate *in vivo* physiological conditions like intra osseous vascularity, effect of tissue packing on the dosage form, change in pH and osmolarity in the surrounding environment and relate it to the release of the active ingredient from the dosage form. The combination of multiple flow directionality can also be used in the future if this model's application is extended further to other complex non-oral dosage forms such as liposomes, microspheres. Better incorporation of the *in vivo* conditions will enable us to better understand and predict the

bio-performance of the dosage form making the model more suited for clinical predictions of implants and novel dosage forms.



**Figure 4-8.** Flow directionality options between chambers in the BIVDR model.

BRC: Bone regeneration chamber; BPC: Bone packing chamber. The red solid line indicates the direction of flow of media.

#### **4.2.5.5 Experiments for selection of low protein-adsorption polymer material for construction of body of the *in vitro* model**

*In vitro* release experiments using USP-IV glass flow through cell showed considerable protein adsorption to the body of the model and has been discussed in-depth in the results section of this chapter. To prevent similar losses in drug substance due to protein adsorption, the polymer selection experiments for the body of the BIVDR model were performed. Polymer materials which are traditionally known in literature as having low protein adsorption properties such as a) poly ether ether ketone (PEEK) used in making of HPLC consumables, b) polycarbonate (PC) used in medical grade equipments and IV bags, and c) poly tetra fluoro ethylene (PTFE) were acquired from the vendor. They were neatly cut into small pieces of 1 X 1 X 1 cm<sup>3</sup> volume. Each of these pieces were immersed in 4mL of rhBMP-2 solution of concentration 10 µg/mL in modified HBSS and placed in nalgene tubes for incubation at 37°C. The control tube consisted of the protein rhBMP-2 solution but with no polymer block in it. 250 µL of sample was removed at 30 min., 24hr. and 72 hr. respectively. The samples were analyzed using an already validated HPLC-UV method as described in Chapter 3 of this thesis. The rhBMP-2 recovery percent was recorded and plotted against the time. ANOVA with Dunnett's multiple comparisons test was used for all purposes of statistical comparisons between different samples. The polymer material with minimal protein adsorption and maximal rhBMP-2 recovery percent that was also compatible in the desired working pH range of 6-8 was selected for the construction of the body of the model.

#### **4.2.5.6 Selection of low protein-adsorption membranes**

Low protein adsorption membranes were selected from vendors. Five such membranes which are traditionally known to be less interactive with proteins were: a) regenerated cellulose (RC) b) cellulose acetate (CA) c) poly ether ether sulfone (PES) d) etched polycarbonate (PC) and e) poly tetra fluoro ethylene (PTFE). Porosity of all the above membranes was 0.45 $\mu$ m. Table 4-5 is a summary showing the characteristics of the various membranes selected, their hydrophilic and hydrophobic nature to determine compatibility with the biorelevant media used, and their protein adsorption properties as known in literature or from information collected mostly from various vendor sites. The membranes were cut into pieces of 4cm<sup>2</sup> each, and incubated with rhBMP-2 solution at a concentration of 10  $\mu$ g/mL at 37°C. Total volume of solution in each tube was 3.5ml. 250  $\mu$ L of sample was withdrawn at 30 min. and 24 hr. to observe the adsorption of the rhBMP-2 to the membrane. 24 hr. was considered as the end point for the equilibration of the membranes with the rhBMP-2 solution. Control tube consisted of rhBMP-2 solution but without any membrane. The samples were normalized at each time point with respect to the control before calculating the recovery percent of rhBMP-2 in the respective solution. ANOVA with Dunnett's multiple comparisons was used for the purposes of statistical comparison. The top three membranes with minimal protein adsorption and maximal recovery at required pH of 6-8 were selected for incorporation into the model.

**Table 4-5.** Characteristics of different membrane types for incorporation into BIVDR model

<b>Type of Membrane</b>	<b>Nature of Membrane</b>	<b>Protein binding capacity and compatibility with solvents</b>
1. Regenerated cellulose (RC)	Hydrophilic membrane.	Compatible with HPLC solvents. Very low protein binding capacity, which makes it a good choice for protein recovery applications. Suitable for use with either aqueous solutions or organic solvents
2. Cellulose acetate (CA)	Hydrophilic membrane.	Limited solvent resistance. Very low protein binding capacity, which makes it a good choice for protein applications.
3. Polyethersulfone (PES)	Hydrophilic membrane.	Good solvent compatibility: aqueous and organic solvents. Low protein binding.
4. Polycarbonate (PC)	Hydrophilic membrane.	Extremely thin polycarbonate film, with a very narrow pore size distribution suitable for aqueous and some organic solvents
5. Poly tetra fluoro ethylene (PTFE)	Hydrophobic membrane	Very low protein adsorption. Compatibility with organic solvents and can be also used with aqueous solvents but only after considerable initial wetting with organic solvents.

#### **4.2.5.7 Protein adsorption studies on the biorelevant model experimental set up**

##### *Adsorption of rhBMP-2 to the model:*

Before incorporation of the membranes into the model it was essential to estimate the rhBMP-2 adsorption just to the BIVDR model experimental set up without the presence of the membranes. Analysis of the rhBMP-2 adsorption to the BIVDR model, with the introduction of membranes into the model would then elucidate if the adsorption effect was composite or a net additive effect was observed. For this the complete BIVDR model experimental set up with its components: the tubing and the pump assembly were arranged in a closed loop configuration. rhBMP-2 solution in media was circulated at a concentration of 10 µg/mL for a period of 24 hrs at a flow rate of 0.8 – 0.9 mL/min. Samples were collected at 30 min., 2, 5, 18, and 24 hr respectively, from the BRC chamber sampling port. The sampling time points were kept as close/similar as possible to the actual *in vivo* and planned *in vitro* sample collection time points. Control consisted of an aliquot of same rhBMP-2 solution incubated at 37°C in a nalgene tube. A paired samples t test was conducted to observed the recovery percentage difference between the control and BIVDR model at a significance level of  $\alpha=0.05$ .

##### *Adsorption of rhBMP-2 to the model with membranes included:*

The next step was to introduce the top two low protein adsorption membranes into the model and observe the recovery percentage of rhBMP-2. rhBMP-2 solution in media was placed in the BPC at a concentration of 15 µg/mL for a period of 24 hrs at a flow

rate of 0.8 – 0.9 mL/min. Samples were collected at 0hr, 0.25hr, 6hr and 24 hr from the BPC and BRC chamber sampling port. The net (BPC + BRC) percentage recovery of rhBMP-2 from the model was recorded and a membrane with better recovery, performance and lesser variability was selected for the *in vitro* release study.

#### **4.2.6 Sample Analysis**

*In vitro* release test samples were analyzed using a previously validated HPLC method described in Chapter 3 of this thesis. The dynamic range of the assay was from 0.5 µg/mL to 100 µg/mL. Minimal sample preparation was required and the assay helped in high throughput release sample analysis. Release samples collected were generally analyzed on the same day but the assay was also validated with regards to freeze-thaw stability, benchtop stability, autosampler stability at 4°C and forced degradation analysis for the presence and detection of degradation products in the sample.

#### **4.2.7 Evaluation of *In Vitro* Relationship (IVR)**

The dosage amount for rhBMP-2 was maintained at a constant concentration of 1.5mg/mL and was distributed uniformly onto the carrier scaffolds ACS and Osteograf N-300. The release studies were carried in the novel biorelevant model until approximately 80 % of dose was released from the carriers (Pillay and Fassihi, 1998; Shen and Burgess, 2015; Zolnik and Burgess, 2008). The cumulative *in vitro* release profiles from each of the formulations were compared to their existing *in vivo* rat data.

An *In Vitro* Relationship (IVR) was evaluated to observe if the cumulative release profiles are discriminatory amongst the carrier variants. It has been observed that a difference in the carrier scaffold influences the release profile and bone score of rhBMP-2 to a considerable extent (Sigurdsson et al., 1996). The release of rhBMP-2 from its scaffold depends upon the mechanical properties of the scaffold, the adherence level of the protein to the scaffold, which is the binding affinity of the protein to the scaffold, and the degradation rate of the scaffold in its environment (faster degrading scaffolds will release the protein sooner into the environment) which may or may not be a desirable property for the dosage form depending upon the application requirements.

#### **4.2.7.1 Time scaling and Levy Plots**

Time scaling and shifting are effective tools in developing a successful IVVC/IVVR/IVR for complex parenteral and extended release dosage forms. The primary purpose of time scaling is to rectify the rate between *in vivo* absorption/release and *in vitro* dissolution/release (Brockmeier, 1983). A time lag can be observed under two conditions while comparing the *in vitro* and *in vivo* data. A lag can be observed in the *in vivo* data due to delay in absorption (e.g. in case of immediate release dosage forms) or delay in release (e.g. many extended release parenteral dosage forms). This type of delay in *in vivo* data is generally observed as a negative intercept on the Y-axis. The other case of time lag is observed when a dosage form shows a delay with *in vitro* dissolution but there is *in vivo* absorption/release data during the period. This can occur due to an initial rapid burst release phase in the complex inflammatory *in vivo*

microenvironment due to plasma proteins, enzymes, collagenases (e.g. in special dosage forms such as liposomes, PLGA microspheres or carrier based scaffolds such as INFUSE<sup>®</sup> Bone Graft). This delay is generally observed as a positive intercept on the Y-axis. This lag in time, which, occurs, in either case is called as time lag or time shifting. Correction of time lag and rates of *in vivo* and *in vitro* release is commonly done by plotting Levy plots for normalization of data. A levy plot is created by plotting the *in vitro* dissolution/release time of certain specific percentages on the X-axis (e.g. time taken to release 10%, 20%, 30% of the drug substance) vs. the time taken *in vivo* for absorption/release of similar percentages of drug. A linear levy plot touching zero indicates similar rates of *in vitro* and *in vivo* dissolution/release. If the rates *in vitro* and *in vivo* are different, intercepts on the X and Y-axis might be observed as described above. Levy plots can be plotted more accurately by frequent sampling time points in *in vitro* dissolution/release data to allow for more accurate prediction of a relationship. Once a levy plot has been plotted and a time scaling factor is obtained this can then be applied to the normalization of data and *in vitro* and *in vivo* release data on the same time scale can be plotted against one another to study and establish a relationship (IVIVR/IVR).

#### **4.2.7.2 Similarity (f2) and difference (f1) factors as parameters to establish ‘discriminatory’ nature of a release profile**

Special dosage forms such, as extended controlled release parenterals e.g. nano particles, liposomes or carrier based implants often require establishing an *in vitro* relationship (IVR) instead of an IVIVC. For release / dissolution tests in addition to

establishing a release profile and an IVIVR, it is also crucial to show that the method is suitably *discriminatory* between formulations. Release rate profiles can generally be analyzed using model dependent approach, model independent approach (f1 and f2) or statistical analysis such as ANOVA (one way analysis of variance) and MANOVA (multivariate analysis of variance). The difference factor (f1) and similarity factor (f2) have often been used for establishing discriminatory nature of a dissolution/release test between its formulations (Moore and Flanner, 1996; Zolnik and Burgess, 2008). The similarity factor f2 and difference factor f1 provide quantitative measurements of the release profiles between the test and reference product. As the name suggests f1 measures the difference at each time point between the reference and the test. A value of zero for f1 suggests no difference in the release profiles and a value of 100 would suggest completely different releases profiles. According to FDA guidelines a value of  $f1 < 15$  is required for bioequivalence (Shah et al., 1997). Hence a value of  $f1 > 15$  suggests discriminatory profile. f2 (similarity factor) is measured between 0 and 100 and measures the similarity of the release profiles (Shah et al., 1998). It is a transformation achieved by logarithmic measures of the difference between the reference and test. A value of '0' for f2 would indicate completely dissimilar profiles and a value of '100' would indicate a completely similar release profile between the test and the reference formulations. These factors generally give an idea about the point-to-point percent predictive error in the release/dissolution between test and reference product or two different formulations. FDA guidelines require values of  $f2 > 50$  for similarity. Hence, values of  $f2 < 50$  indicate discriminatory release tests. Care has to be taken to not apply

f1 and f2 factors to the same formulation under varying release test conditions. The equations for f1 and f2 factors have been reported below (Pillay and Fassihi, 1998).

$$f1 = \left\{ \frac{\sum_{t=1}^n |R_t - T_t|}{\sum_{t=1}^n R_t} \right\} * 10$$

$$f2 = 50 * \log \left\{ \left[ 1 + (1/n) \sum_{t=1}^n (R_t - T_t)^2 \right]^{-0.5} \right\} * 100$$

In the above equations  $R_t$  is the cumulative percent release for the reference product or formulation, which in current case is INFUSE<sup>®</sup> Bone graft and  $T_t$ , is the test product, which in this research study is Osteograft N-300. The objective of our study was therefore, to design and develop a biorelevant model and show that it is discriminatory between formulations of INFUSE<sup>®</sup> Bone Graft and Osteograft N 300.

## 4.3 Results and Discussion

### 4.3.1 Sample and separate and USP flow through cell release profiles

For the sample and separate model the initial release of BMP-2 from ACS scaffold was observed to be  $45.21 \pm 2.53\%$  within a time of 5 days with a burst release profile of  $15.04 \pm 2.09\%$  over the first 24 hours. A total release of  $58.89 \pm 4.97\%$  over 30 days was finally observed. The Flow Through Cell also showed a similar burst profile as the sample and separate of  $15.23 \pm 2.99\%$  over the first 24 hours for PBS and biorelevant media studies but the difference being, this  $\sim 15\%$  burst release was achieved within

90min of starting of the experiment. Negligible release was observed in this model for the remaining 24hrs. The release almost reached an apparent asymptote over the next 24hrs. This can be due to two suspected reasons: 1) a uniform flow rate was not sufficient to maintain the controlled release rate in the USP-IV model after the initial burst release phase (possibly because of factors like packaging with glass beads of the dosage form- floating ACS scaffold) or 2) The released protein rhBMP-2 adheres to the glass USP-IV flow cell and to the glass beads. Upon further investigation it was found that similar problem has been reported in literature by investigators dealing with *in vitro* release testing of proteins (Xu et al., 2012). Therefore, the approach would be to study the release of the implant system in an eppendorf/protein low bind tubes and compare the release with that in a glass tube. The release can be studied over a period of 24hrs to 5 days for these adsorption studies. If the glass container gives substantially lower release profiles as compared to the eppendorf/protein low bind tube under the same minimal experimental conditions, it can be stated that the low release rate in the USP-IV cell is because of protein adsorption to USP-IV glass surface. From our stability analysis in Chapter 3 we already know that the stability of rhBMP-2 in modified HBSS media is >90% over 24 hrs.

In conclusion, the a) sample and separate and b) the USP-IV *in vitro* profiles were distinctly different from the *in vivo burst release* profile. In the *in vivo* rat system the loss of ~15% occurred only after the 2<sup>nd</sup> day. Comparison of the amount retained at the end of 5-day period also showed differences in the percentage yet to be released. The sample and separate model had approximately 55% retained; USP-IV for PBS and modified HBSS retained approximately 85%. Thus, it was observed that these *in vitro*

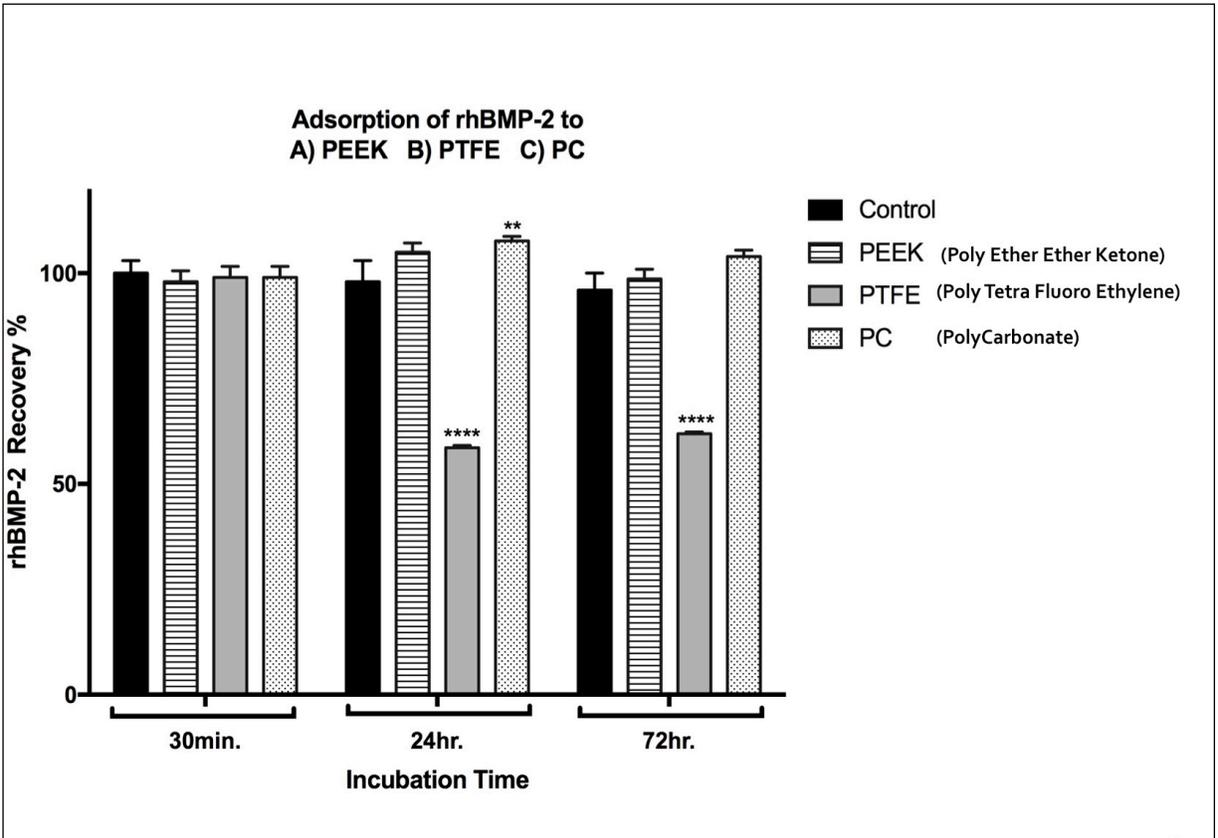
models did not compare to the *in vivo* profile. The sample and separate and USP-IV model percentage cumulative drug release profiles have been represented in Figure 4-21 later in this chapter (in conjunction with BIVDR model data).

## 4.3.2 BIVDR Model

### 4.3.2.1 Model body polymer selection

Adsorption of rhBMP-2 to three different polymer materials poly ether ether ketone (PEEK); Poly tetra fluoro ethylene (PTFE) and Polycarbonate was studied at 37°C.

Control set up constituted (no polymer cube incubated with rhBMP-2 solution). At 30min. no significant adsorption was observed to either of the polymer materials. ANOVA with Dunnett's multiple comparisons test was used for the purposes of statistical comparisons. At the end of 72hrs. the PEEK tube showed a recovery % of  $98.65 \pm 2.26$  %; PTFE  $61.89 \pm 0.47$  % and polycarbonate a net recovery % of  $103.95 \pm 1.52$  %. This revealed PTFE had the maximum adsorption to rhBMP-2 and hence the lowest recovery percentage. Therefore, based on the rhBMP-2 protein adsorption results polycarbonate (PC) polymer was selected as the material of choice for the construction of the body of the model. The results of the polymer for model body creation have been plotted as a bar graph in Figure 4-9. A one-way ANOVA with Dunnett's multiple comparisons test was performed at a significance level  $\alpha=0.05$ . The specific time points (30min., 24hr and 72 hr) for each of the corresponding incubated samples with the *polymer* were compared with their control (without polymer) at same day of incubation to observe if the means were significantly different. At the end of the



**Figure 4-9.** Selection of BIVDR body material: adsorption studies of rhBMP-2 to a) Polycarbonate b) PTFE c) PEEK.

All statistical comparison between samples have been made with ANOVA Dunnett's multiple comparisons test.  $p < .005(**)$ ;  $p < .00005(****)$ .

72hr. incubation, the mean recovery percentage for rhBMP-2 was not significantly different for PEEK ( $p=0.8454$ ) and PC ( $p=0.3146$ ); however, for PTFE samples the mean percentage recovery was significantly different ( $p=0.0001$ ) from the control sample.

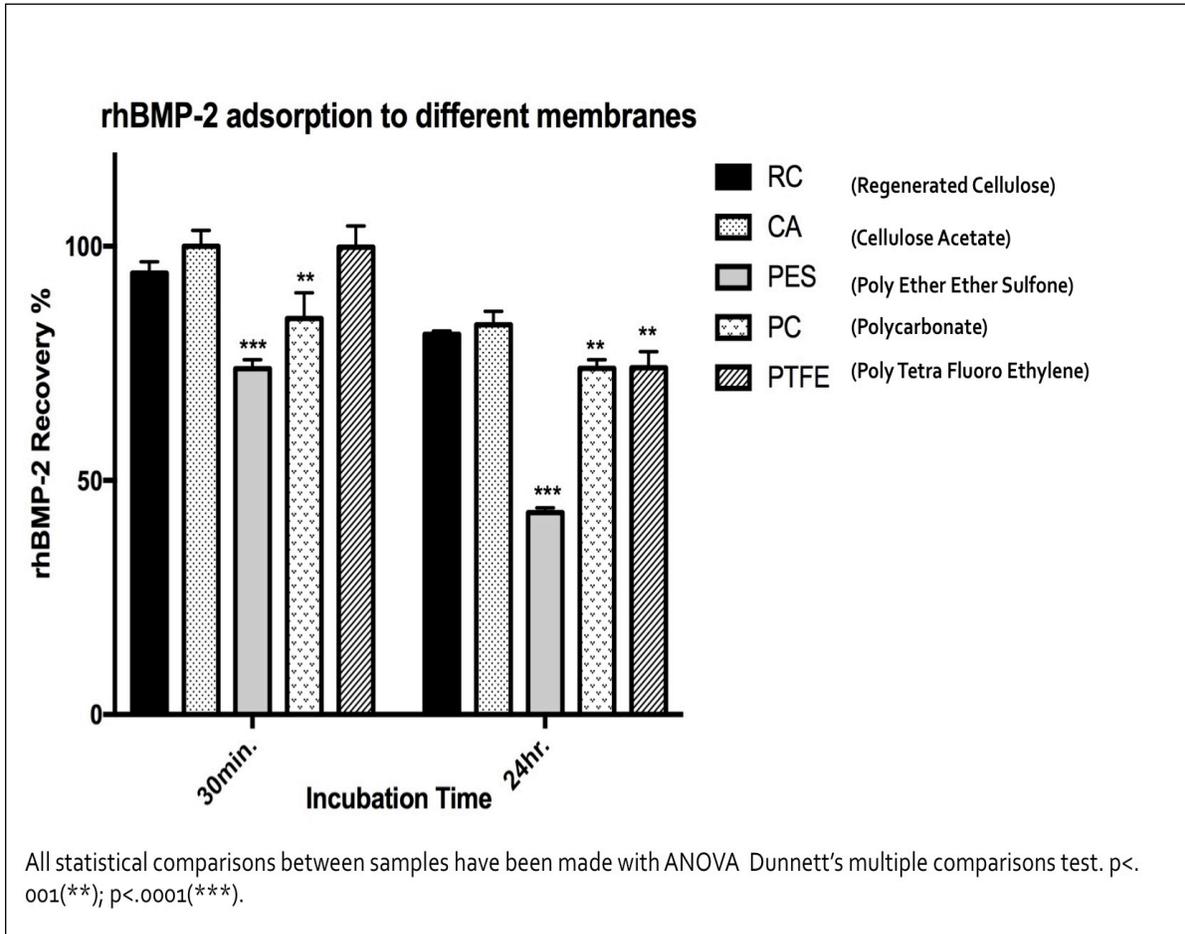
#### **4.3.2.2 Membrane selection**

Membrane selection experiments showed similar results as model body polymer experiments but with the exception that significant differences were observed in rhBMP-2 recovery after 30min. of incubation. Regenerated cellulose (RC) membrane had a recovery percent of  $81.22 \pm 0.68$ ; cellulose acetate (CA) of  $83.26 \pm 2.86$ ; poly ether ether sulfone (PES) of  $43.14 \pm 1.01$ ; polycarbonate (PC) of  $73.91 \pm 1.85$  and poly tetrafluoro ethylene (PTFE) of  $74.08 \pm 3.40$ . At the end of 24hrs RC showed a recovery percent of  $81.22 \pm 0.68$ ; CA a recovery percent of  $83.26 \pm 2.86$ ; PES a recovery percent of  $43.14 \pm 1.01$ ; PC a recovery percent of  $73.91 \pm 1.85$  and finally PTFE a recovery percent of  $74.08 \pm 3.40$ . Based on the above results RC, CA and PC/PTFE showed minimal adsorption to rhBMP-2 solution. However, PTFE is a hydrophobic membrane and since free exchange between the compartments of BIVDR model i.e. BRC (outer compartment) and BPC (inner compartment) is desired in the buffer media (HBSS), which has no organic components to it PTFE was eliminated as an option. PC membrane showed comparable results but was extremely thin and fragile, and difficult to handle. Since the BIVDR model required the membrane to be screwed in a round circumference using size 0 medical grade screws, some degree of robustness was required of the membranes to prevent tear, damage or perforation to the membrane

while setting up the *in vitro* model for testing. For this reason PC was also not selected as a choice. Regenerated cellulose (RC) and cellulose acetate (CA) membranes were selected for the experiments with the BIVDR model. The results of the membrane selection experiments have been plotted as a bar graph in Figure 4-10.

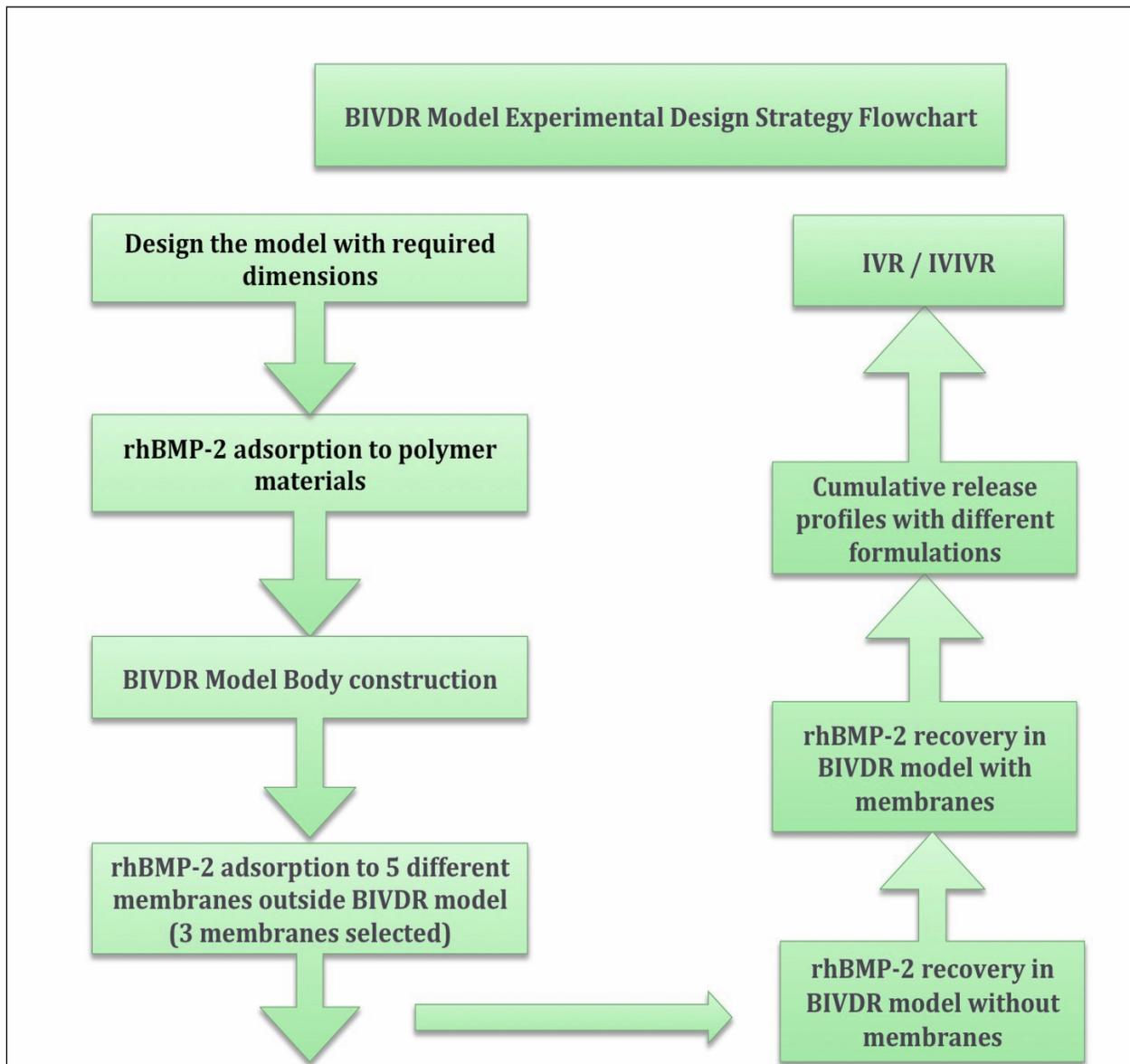
#### **4.3.2.3 Protein adsorption to the BIVDR model and model components and recovery**

Before beginning the *in vitro* release experiments in the BIVDR model it was important to assess the rhBMP-2 loss due to adsorption, to the various components of the experimental set up (BIVDR model, pump tubings and flow valves). This was carried out by a sequential experimental design plan. The schematic of the design plan has been shown in a flow chart in Figure 4-11. Since membrane was an external component added to the BIVDR model the rhBMP-2 recovery was first assessed in the model a) *without* the membrane followed by b) *with* the membranes, introduction of the two membranes: regenerated cellulose (RC) and cellulose acetate (CA). Samples were collected at regular intervals of 30 min., 2hr, 5hr, 18hr, and 24hr respectively.



**Figure 4-10.** Membrane selection for BIVDR model. Study of rhBMP-2 adsorption to five different membranes

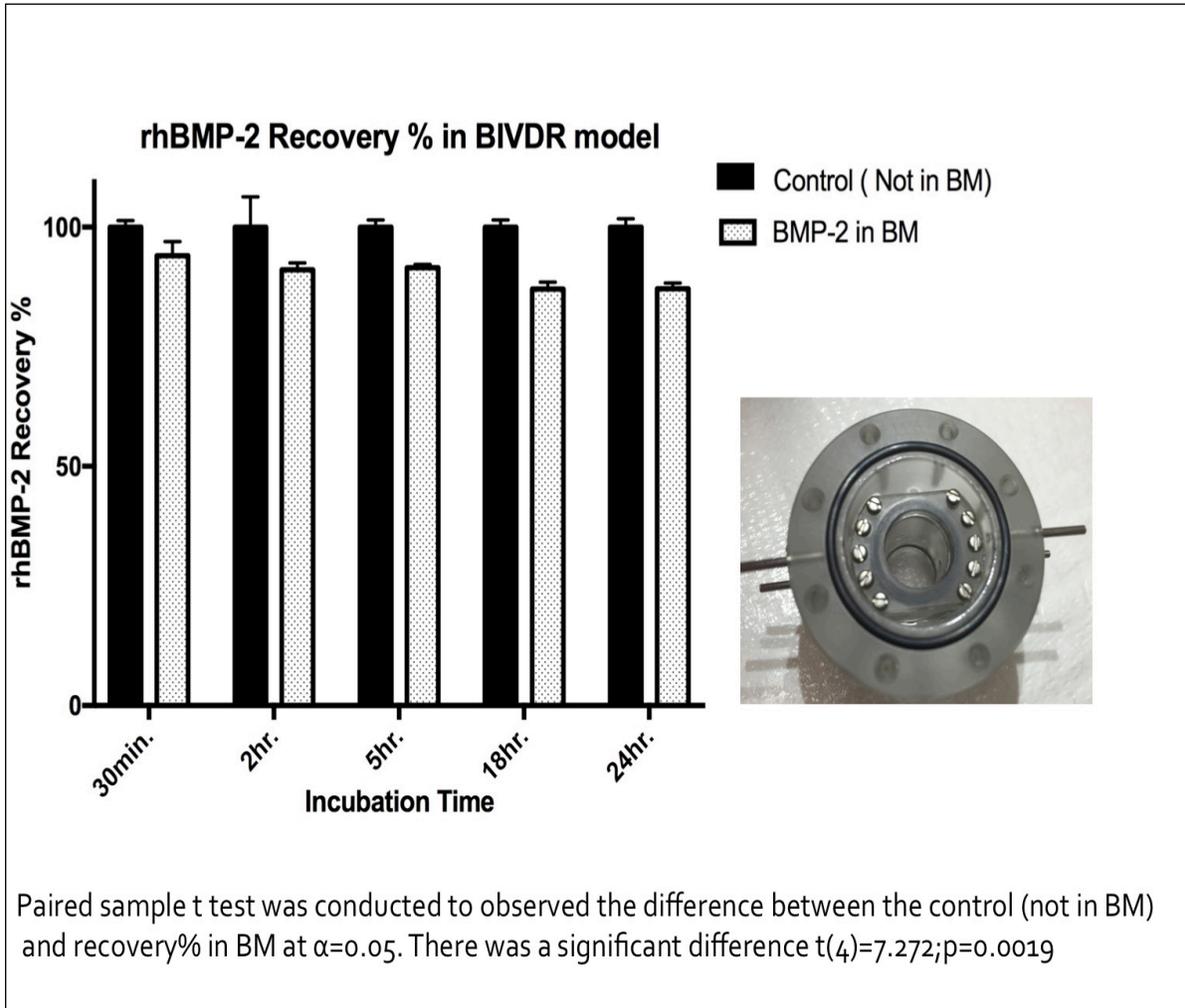
a) Regenerated cellulose b) Cellulose acetate c) Poly Ether Ether Sulfone d) Polycarbonate e) Poly tetra fluoro ethylene. All statistical comparison between samples have been made with ANOVA Dunnett's multiple comparisons test.  $p < .005$ (\*\*);  $p < .0005$ (\*\*\*).



**Figure 4-11.** Flowchart summarizing BIVDR model experimental study design.

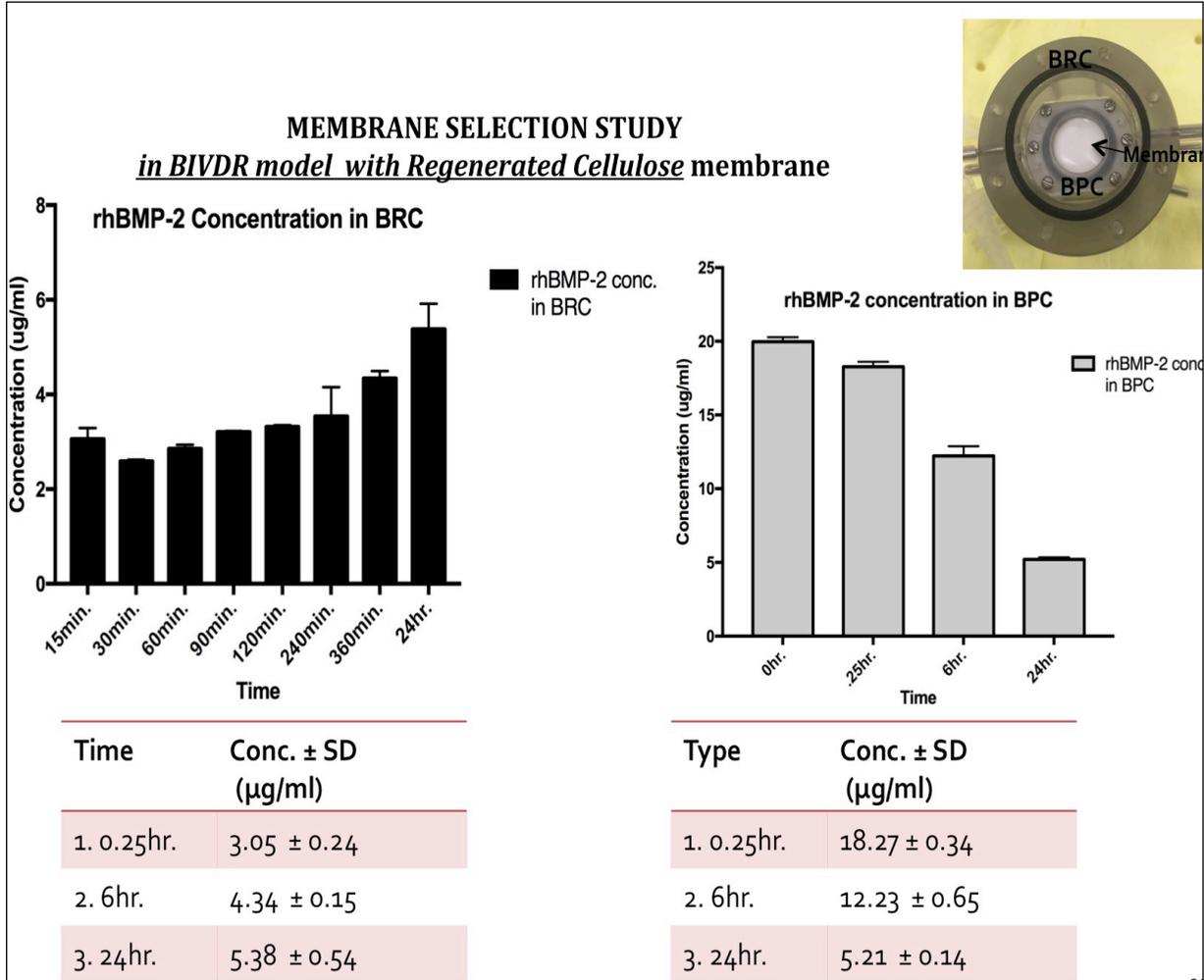
The rhBMP-2 recovery in the model with all the components of the experimental set up were recorded to be  $94.053 \pm 2.910$  after 30 min.,  $91.101 \pm 1.401$  after 2hr.,  $91.495 \pm 0.707$  after 5 hr.,  $87.092 \pm 1.414$  after 18hr., and  $87.116 \pm 1.181$  after 24 hr. respectively. A paired samples t test was conducted with respect to the controls incubated in an eppendorf tube incubated for the same duration of time to observe the difference between the control (rhBMP-2 solution not in BIVDR model set up) and the recovery percent in the BIVDR model at a significance level of  $\alpha=0.05$ . A significant difference was observed  $t(4)= 7.272$  with a p value of 0.0019. The bar graph for the percent recovery of rhBMP-2 vs. time has been shown in Figure 4-12.

The next step was to study rhBMP-2 recovery percent with the introduction of the membranes in the model set up. rhBMP-2 solution was placed in the core compartment (BPC) and modified HBSS media was circulated in outer compartment (BRC) at a concentration of 20  $\mu\text{g/mL}$ . Samples were collected at 0hr., 0.25hr., 6hr. and 24hr. respectively. The total recovery percent was calculated to be 94.58 at 0.25 hr., 90.79 at 6hr., and 84.31 at 24 hr. with RC membrane. The individual recovery percent in each chamber and total mean recovery percent (BPC+BRC) for the RC membrane has been shown in Figure 4-13 and Figure 4-14 respectively. The experiments were repeated with CA membrane to observe the recovery percent in the model. These were recorded to be 69.92 at 0.25 hr., 60.19 at 6 hr., and 86.23 at 24 hr. respectively. The individual recovery percent in each compartment of the BIVDR model and the total percent recovery (BPC+BRC) for CA membrane have been shown in Figure 4-15 and Figure 4-16 respectively.



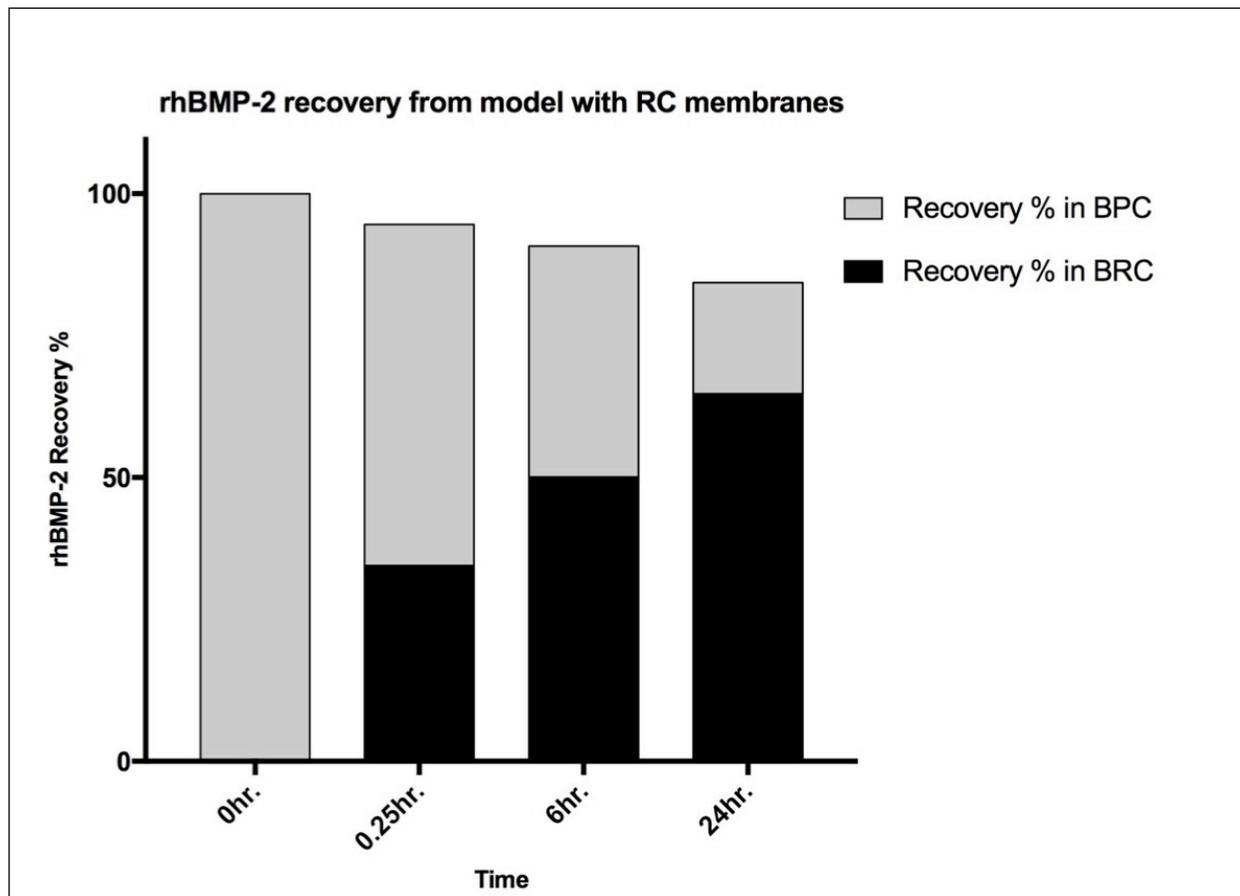
**Figure 4-12.** rhBMP-2 recovery percentage in BIVDR model experimental set up with pump tubings and flow valves.

Note: The membranes have not been introduced at this stage. The rhBMP-2 solution is (20  $\mu\text{g}/\text{mL}$ ) circulated in the model at a flow rate of 0.8 – 0.9 mL/min.

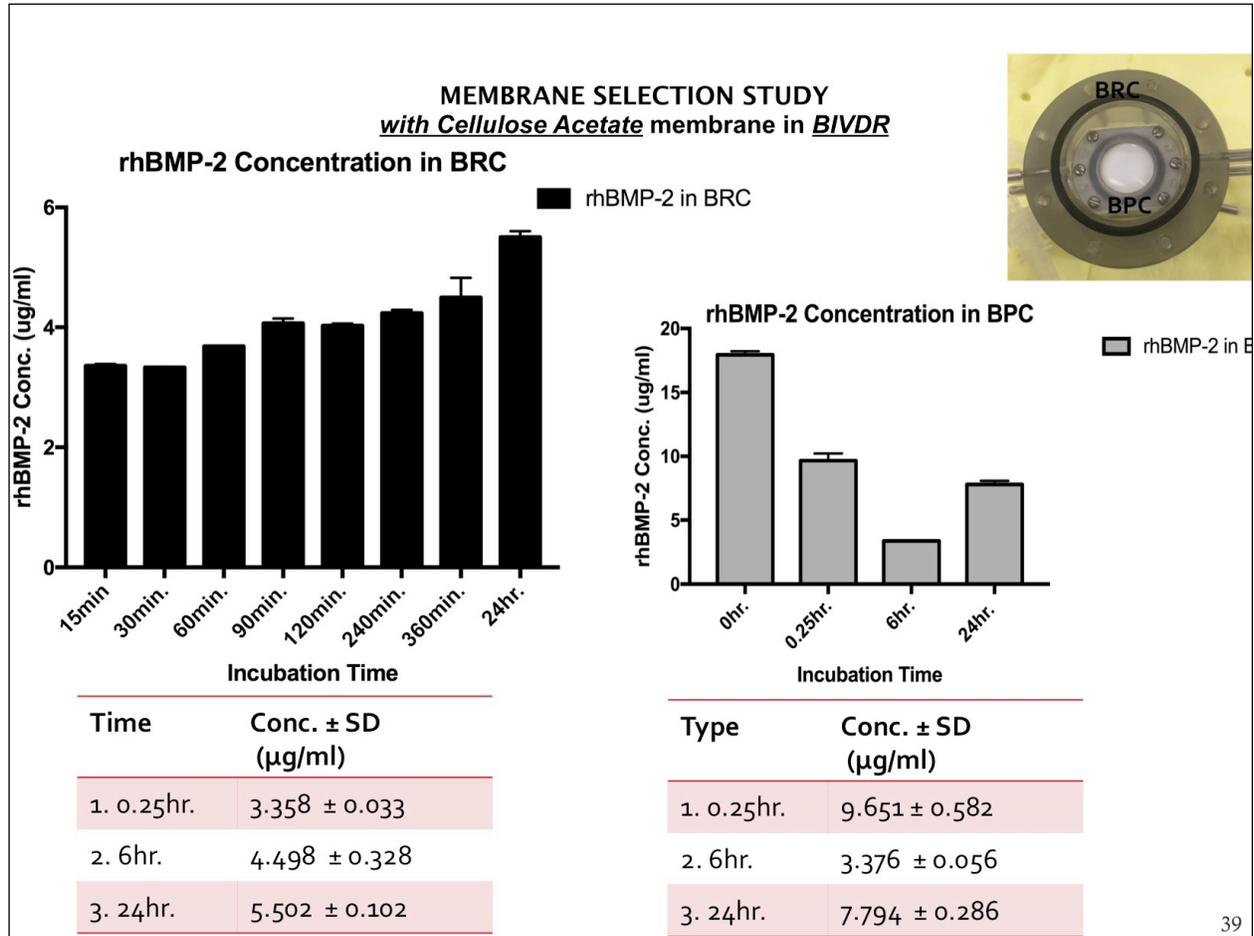


**Figure 4-13.** rhBMP-2 recovery percentage in each chamber of the model core Bone Packing Chamber (BPC) and surrounding Bone Regenerating Chamber (BRC) with the introduction of *regenerated cellulose* membrane into the experimental set up.

Note: the rhBMP-2 solution (20 µg/mL) was placed in the core compartment BPC.

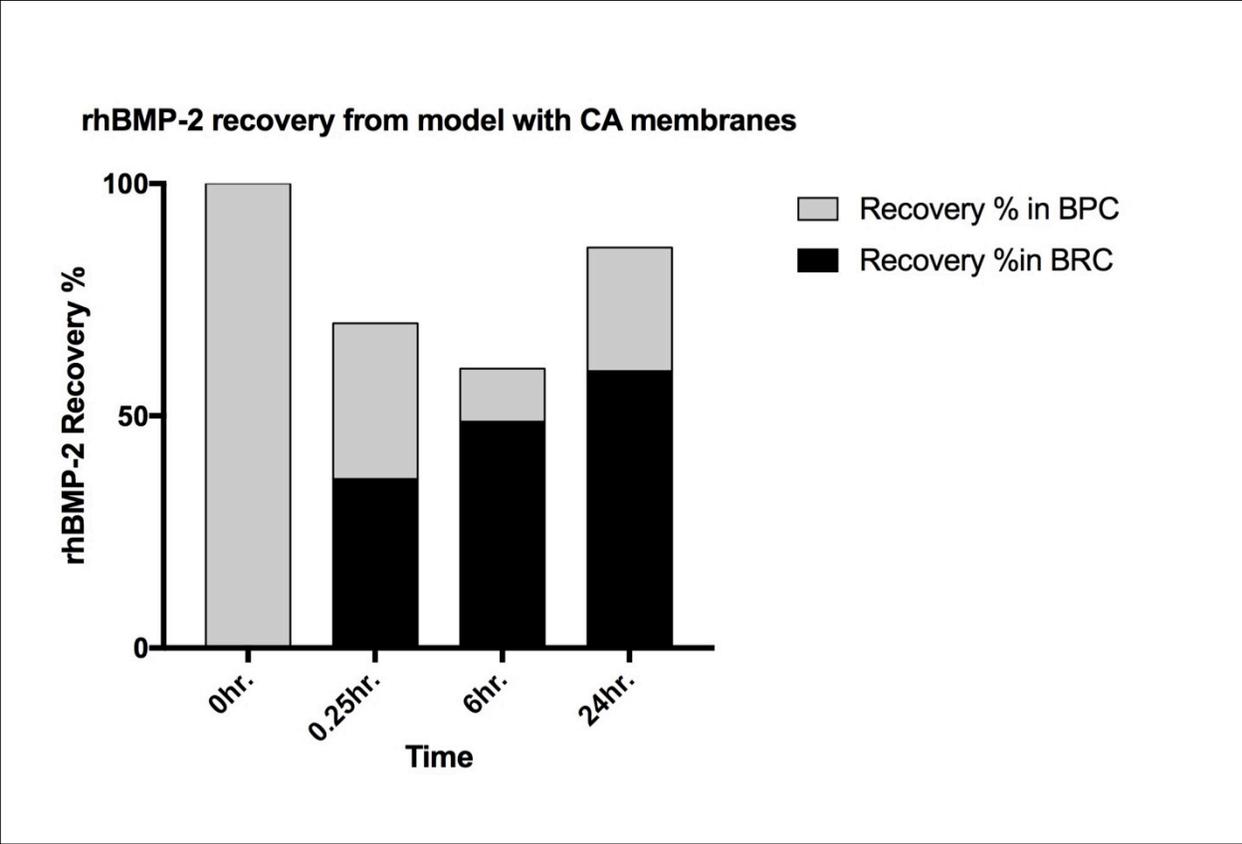


**Figure 4-14.** Net recovery (BPC+BRC) of rhBMP-2 in the BIVDR model experimental set up with the introduction of *regenerated cellulose* membrane.



**Figure 4-15.** rhBMP-2 recovery percentage in each chamber of the model core Bone Packing Chamber (BPC) and surrounding Bone Regenerating Chamber (BRC) with the introduction of *cellulose acetate* membrane into the experimental set up.

Note: the rhBMP-2 solution (10µg/mL) was placed in the core compartment BPC.



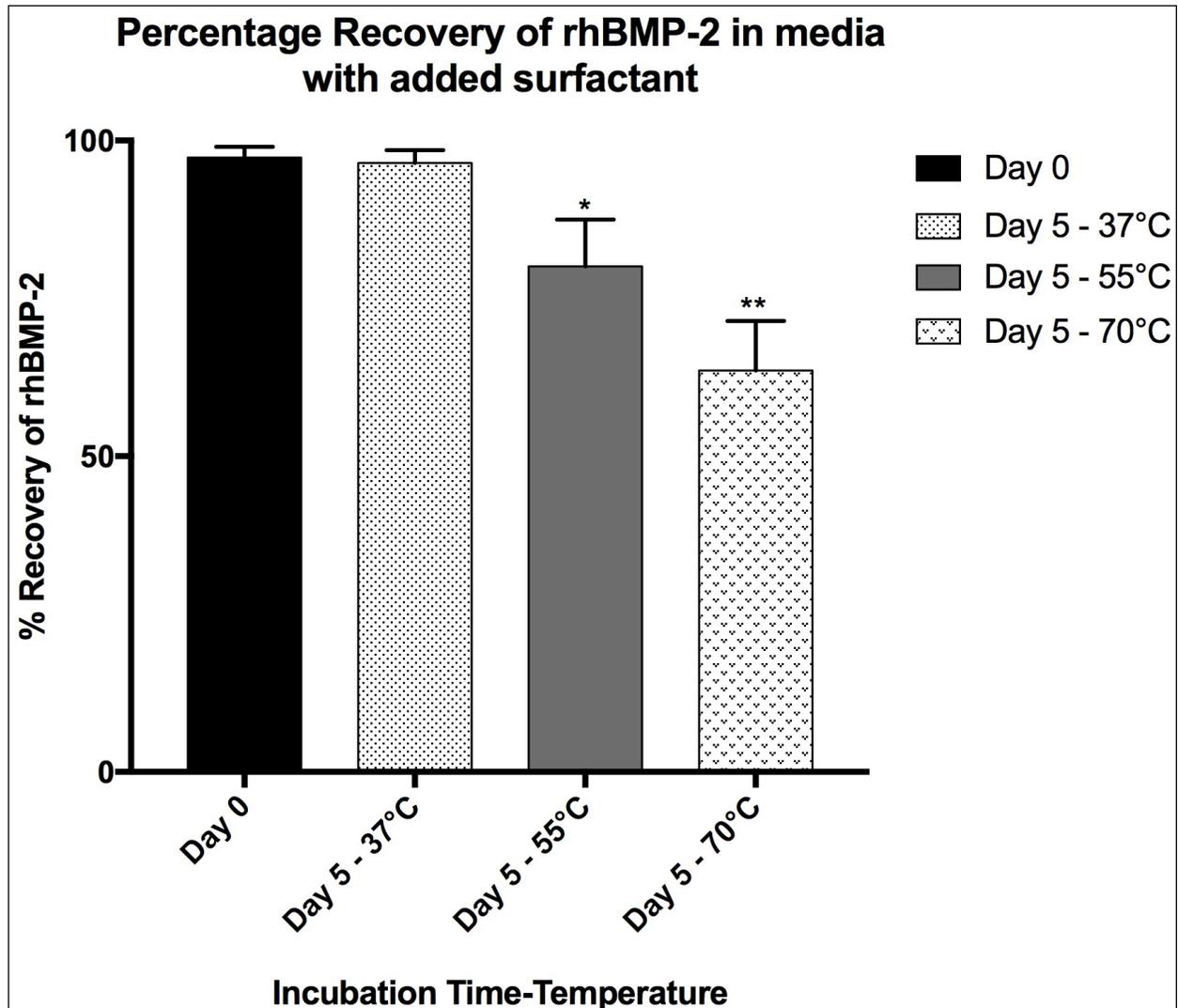
**Figure 4-16.** . Net recovery (BPC+BRC) of rhBMP-2 in the BIVDR model experimental set up with the introduction of *cellulose acetate* membrane.

In an attempt to increase the recovery percentage extremely low concentration of the surfactant polysorbate 80 (already present in the rhBMP-2 lyophilized formulation at 0.01%) was added to the media at 0.001% and the recovery was again tested with the RC and CA membranes. For the *analytical test* methodology (HPLC-UV) the recovery and stability of rhBMP-2 in modified HBSS with added surfactant at 0.001% was evaluated for three different concentrations (0.5, 5 and 15 µg/mL) outside the BIVDR model initially. Table 4-6 shows the recovery and stability evaluations for rhBMP-2 in modified HBSS media with added surfactant over two time points of 12 and 24 hrs. respectively. Percentage recovery of rhBMP-2 in the modified HBSS media with added surfactant was also examined at three different temperatures (37°C, 55°C, and 70°C) at the end of a 5-day period incubation in a Nalgene tube (outside the BIVDR model). Figure 4-17 shows the percentage recovery of rhBMP-2 in modified HBSS media for three different temperatures compared with rhBMP-2 controls at day 0. A one-way ANOVA with Dunnett's multiple comparisons test was performed at a significance level  $\alpha=0.05$ . The specific temperature settings (37°C, 55°C, and 70°C) for each of the corresponding 5-day incubated samples were compared with their control at day-0 to observe if the means were significantly different. For the 37°C sample, the mean recovery percentage for rhBMP-2 (in surfactant added media) in the 5-day sample was not significantly different ( $p=0.9971$ ). However, for the 55°C, and 70°C samples the mean percentage recovery of rhBMP-2 (in surfactant added media) when compared to day-0 sample were significantly different in both the cases: 55°C ( $p= 0.0365$ ) and 70°C ( $p= 0.0011$ ).

Once the stability evaluations for rhBMP-2 in the surfactant added media was performed outside the BIVDR model, the next step, was to introduce the surfactant added media to the BIVDR model (with the membranes) and observe the percentage recovery. In the BIVDR model experimental set up with the introduction of 0.001% of polysorbate 80 the percentage recovery with the RC membrane improved considerably. With the RC membrane the total recovery percent from both the BRC and BPC combined was observed to be 68.52 at 0.25hr., 93.20 at 6hr.,and 93.80 at 24 hr. respectively. The lower recovery percent 0.25 hr. can be attributed to time taken by the membrane to equilibrate with the rhBMP-2 solution. With the CA membranes the mean recovery percentage of rhBMP-2 from both the chambers combined was observed to be 91.42 at 0.25hr, 95.99 at 6 hr and 83.02 at 24hr. The final recovery percentage for the RC and CA membranes with added surfactant has been shown in Figure 4-18 and Figure 4-19 respectively. It was observed during these experiments CA membrane showed more variability in performance with regards to rhBMP-2 distribution between the two chambers and lower recovery percentage as compared to RC membranes. Therefore, *regenerated cellulose* membrane was selected as the membrane of choice in surfactant added modified HBSS media for *in vitro* release experiments, to achieve minimal variability and maximal recovery for the drug substance (rhBMP-2).

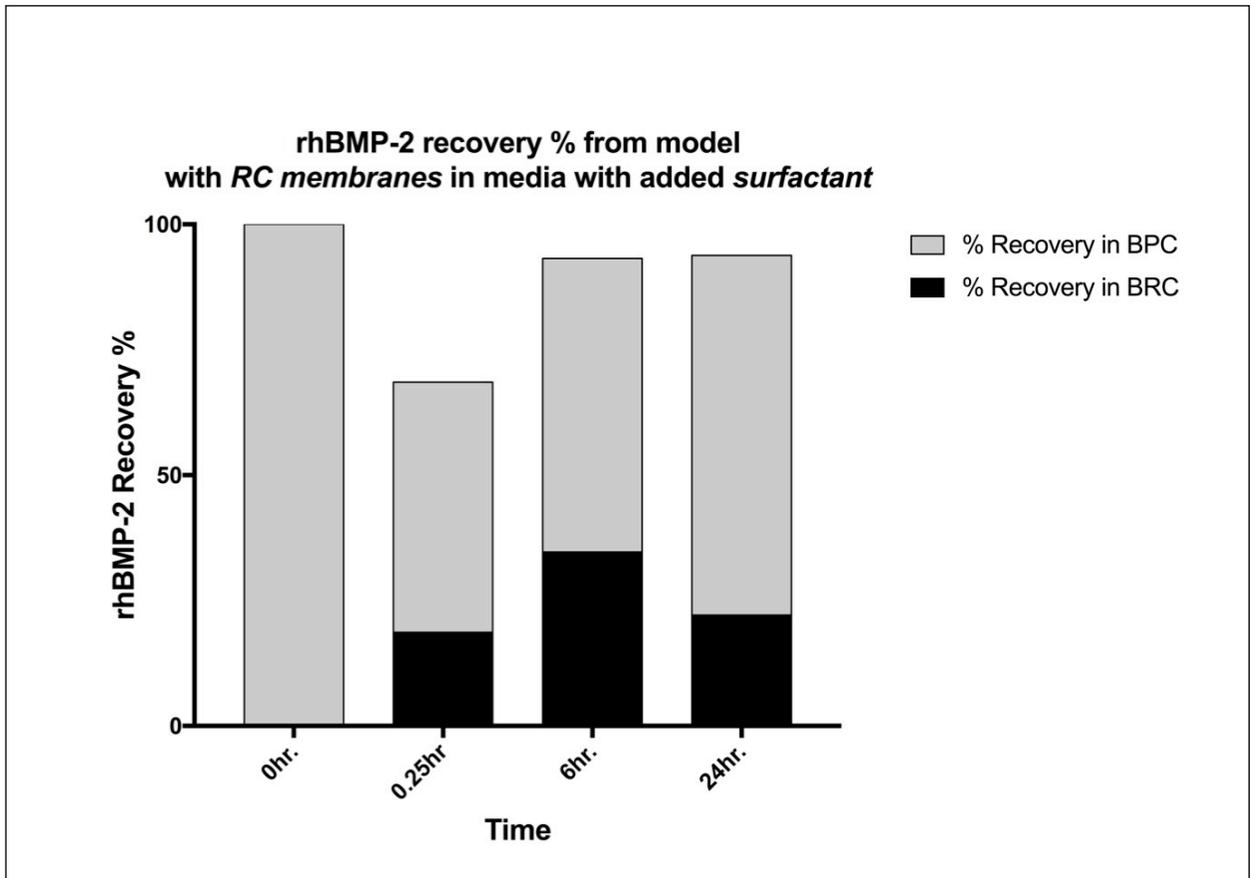
**Table 4-6.** Recovery and stability of rhBMP-2 in modified HBSS with added surfactant at 0.001%.

Nominal Concentration ( $\mu\text{g/ml}$ )	Modified HBSS media (Recovery%)		Modified HBSS with surfactant (Recovery%)	
	12hr	24hr	12hr	24hr
<b>0.5 <math>\mu\text{g/ml}</math></b>				
Mean	93.71	98.43	95.06	94.12
SD	8.65	5.40	5.56	5.17
<b>5 <math>\mu\text{g/ml}</math></b>				
Mean	99.97	96.96	100.24	99.13
SD	1.36	3.59	2.54	3.21
<b>15 <math>\mu\text{g/ml}</math></b>				
Mean	99.96	98.96	100.21	99.76
SD	3.80	2.56	2.71	1.11

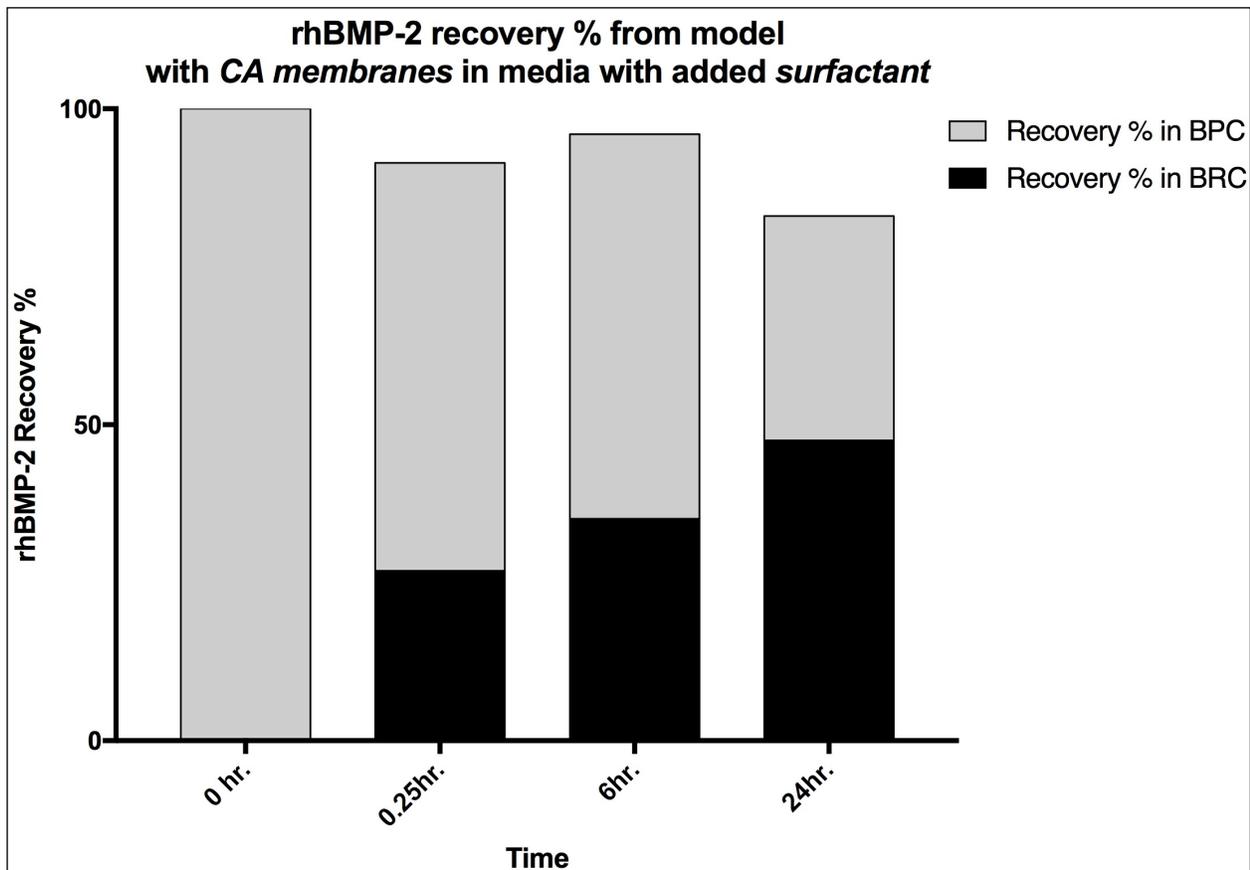


**Figure 4-17.** Recovery percentage of rhBMP-2 in media with the addition of surfactant.

All statistical comparisons between samples (with day 0 as control) have been made with ANOVA Dunnett's multiple comparisons test.  $p < .05$ (\*);  $p < .005$ (\*\*).



**Figure 4-18.** Net recovery (BPC+BRC) of rhBMP-2 in the BIVDR model experimental set up with the introduction of *regenerated cellulose* membrane with added surfactant of 0.001% polysorbate 80 in the media.



**Figure 4-19.** Net recovery (BPC+BRC) of rhBMP-2 in the BIVDR model experimental set up with the introduction of *cellulose acetate* membrane with added surfactant of 0.001% polysorbate 80 in the media.

### 4.3.3 BIVDR model System Performance

The BIVDR model experimental set up comprising of the model, peristaltic pumps, master flex platinized formulation tubings and three way flow valves worked seamlessly over a period of 15 days. Visual observation was performed each day for detecting presence of microorganisms. Sampling at each time point from the central chamber (BPC) was followed by replacement of 1mL of the media unless the timepoints were too close, e.g. during the first day (multiple time points). If more than two time points were collected over a period of 6 hrs.  $500 \pm 50 \mu\text{L}$  of the media was sampled from the central BPC. Sampling volume from the surrounding BRC was generally 1mL at each time point (unless more than 1 time point was collected during the day). In such cases (more than 1 time point during the day) the sampling amount was 300 – 400  $\mu\text{L}$ . Care was taken to replace back the fresh media volume as accurately as possible with the help of graduated piston syringes.

The flow rates of the peristaltic pumps were monitored every second day to avoid any variability. The flow rates were kept at 0.8 – 0.9 mL/min. About one-third of the media was replaced from the central BPC each day during sampling and one-eighth each day from the surrounding BRC. Complete media replacement was done at the end of a 5 day run. pH of the media was also monitored from these 5 day samples and the variability in pH was observed to be  $7.1 \pm 0.2$ . The small parts of the tubing wound around the wheel of each of the low flow (0.03 -8.2 mL/min) VWR peristaltic pump were the most fragile component of the experimental set up and required extra monitoring and visual inspection. These special pump overhead tubings were observed to be prone

to leakage. This was due to gradual development of perforations over the surface of the overhead tubing, due to peristaltic pressure over a period of 7 days. The tubings in these regions are required to be sensitive to pressure and too thick walled tubing would not be able regulate the peristaltic low flow rates. It was thus a tradeoff between thin walled tubings and tubing replacement every few days. Therefore, to avoid any media leakage accidents overnight due to perforations, wear and tear the tubings were replaced at the end of each 5th day (by stopping the flow with the help of three-way flow valves after sampling). This helped ascertain the system was steady in its performance and reduced chances of variability in data measurement.

#### **4.3.4 Flow directionality selection in the BIVDR model**

Figure 4-8 of this chapter in the methods section is a schematic representation of the three flow directionality options in the BIVDR model, which can be achieved as a function of alternating flow between the central BPC and surrounding BRC. In order to determine i) if the directionality of flow would affect the release of rhBMP-2 from ACS and ii) the most suitable flow direction (minimal variability) for the release of rhBMP-2 from ACS, an exploratory three day study at a flow rate of 0.8 -0.9 mL/min was conducted with the three flow directions:

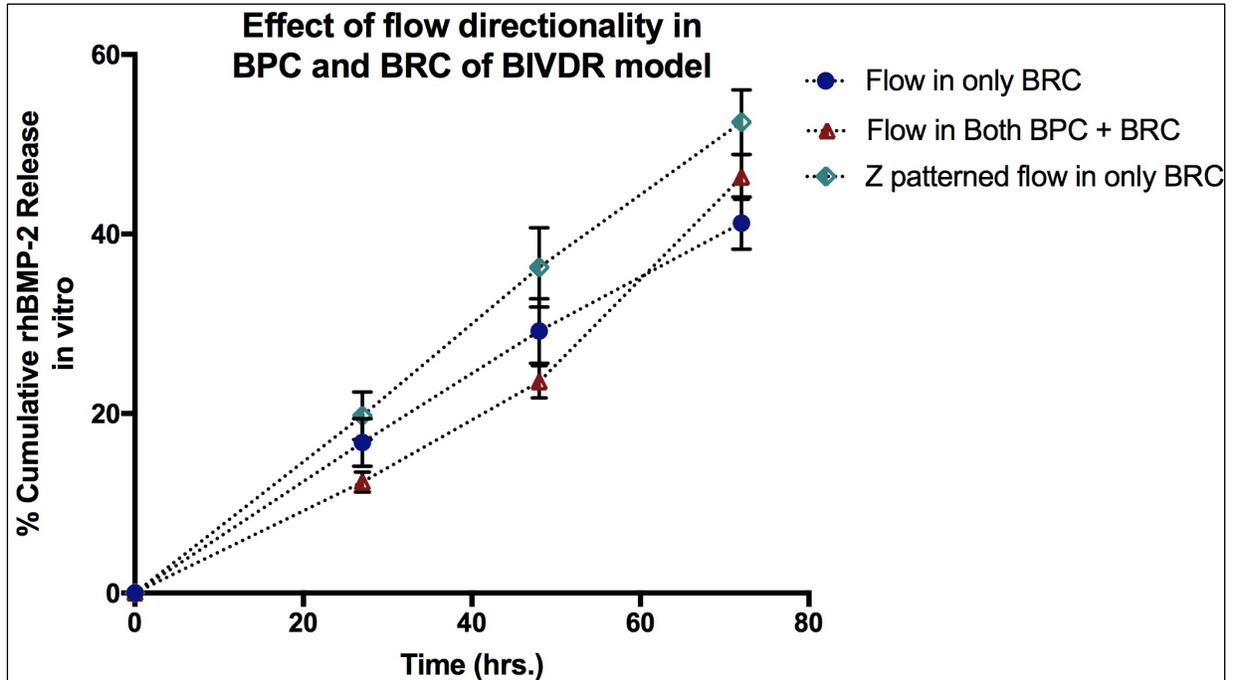
- A) Flow in both the chamber (BPC + BRC)
- B) Flow in only BRC (flow in BPC suspended)
- C) Z patterned flow in diagonal ports of BRC.

Figure 4-20 shows the results of flow directionality effects on the release of rhBMP-2 from ACS. Each point represents the mean and standard deviation of n=3 experimental

set ups. Table 4-7 shows the mean percentage cumulative drug released *in vitro*, standard deviation and %RSD of the measurements for each flow directionality study at 24, 48 and 72hrs. time points in the BIVDR model. A paired samples t test was conducted with pairwise alignment for each time point to compare the flow in (BPC + BRC) with a) flow only in BRC and b) Z patterned flow in BRC. Since situation A (the flow in both BPC + BRC) showed minimal variability (%RSD); it was selected as control for each time point. There was *no significant difference* in percentage cumulative release of rhBMP-2 for both the cases noted below:

- a) flow in (BPC + BRC) with flow only in BRC;  $t(3) = 0.5058$ ;  $p = 0.6748$  (at a significance level of  $\alpha = 0.05$ ).
- b) flow in (BPC + BRC) with Z patterned flow in BRC;  $t(3) = 2.505$ ;  $p = 0.0873$  (at a significance level of  $\alpha = 0.05$ ).

However, since scenario A (flow in both the chambers (BPC+BRC)) showed minimal variability (%RSD) at each time point we decided to use it as the flow directionality condition for the *in vitro* release experiments. This experiment also showed at a low flow rate of 0.8 – 0.9 mL/min flow directionality does not significantly affect the release of rhBMP-2 from ACS in the model. Higher flow rates need to be tested in future for other implants requiring greater blood flow circulation in physiological environment for release of drug substance.



**Figure 4-20.** Flow directionality study in the chambers BPC and BRC.

The flow rate was maintained at 0.8 – 0.9 mL/min. The flow directionality have been shown in detail in Figure 4-8. Each point represents mean and error bars represent SD (n=3 experiments).

A paired sample t test was conducted at each time point with the flow with BPC and BRC as control and a) Flow only in BRC and b) Z patterned flow at  $\alpha = 0.05$ . There was *no significant difference (ns)* in either case. a) Flow only in BRC:  $t(3) = 0.5058$ ;  $p = 0.6478$  b) Z patterned flow  $t(3) = 2.505$ ;  $p = 0.0873$

**Table 4-7.** Flow directionality study for BIVDR model at 0.8 – 0.9 mL/min.

<b>Flow directionality</b>	<b>Mean (% Cumulative drug released <i>in vitro</i>)</b>	<b>SD</b>	<b>%RSD</b>
<b>1. Flow in only BRC</b>			
24 hrs.	16.79	2.66	15.819
48hrs.	29.21	3.60	12.310
72hrs.	41.23	2.91	7.052
<b>2. Flow in both BPC and BRC</b>			
24 hrs.	12.38	1.11	8.966
48hrs.	23.54	1.79	7.608
72hrs.	46.36	2.50	5.389
<b>3. Z patterned flow (in only BRC flow ports)</b>			
24 hrs.	19.77	2.65	13.423
48hrs.	36.30	4.43	12.194
72hrs.	52.46	3.60	6.863

### **4.3.5 Model cumulative release profiles**

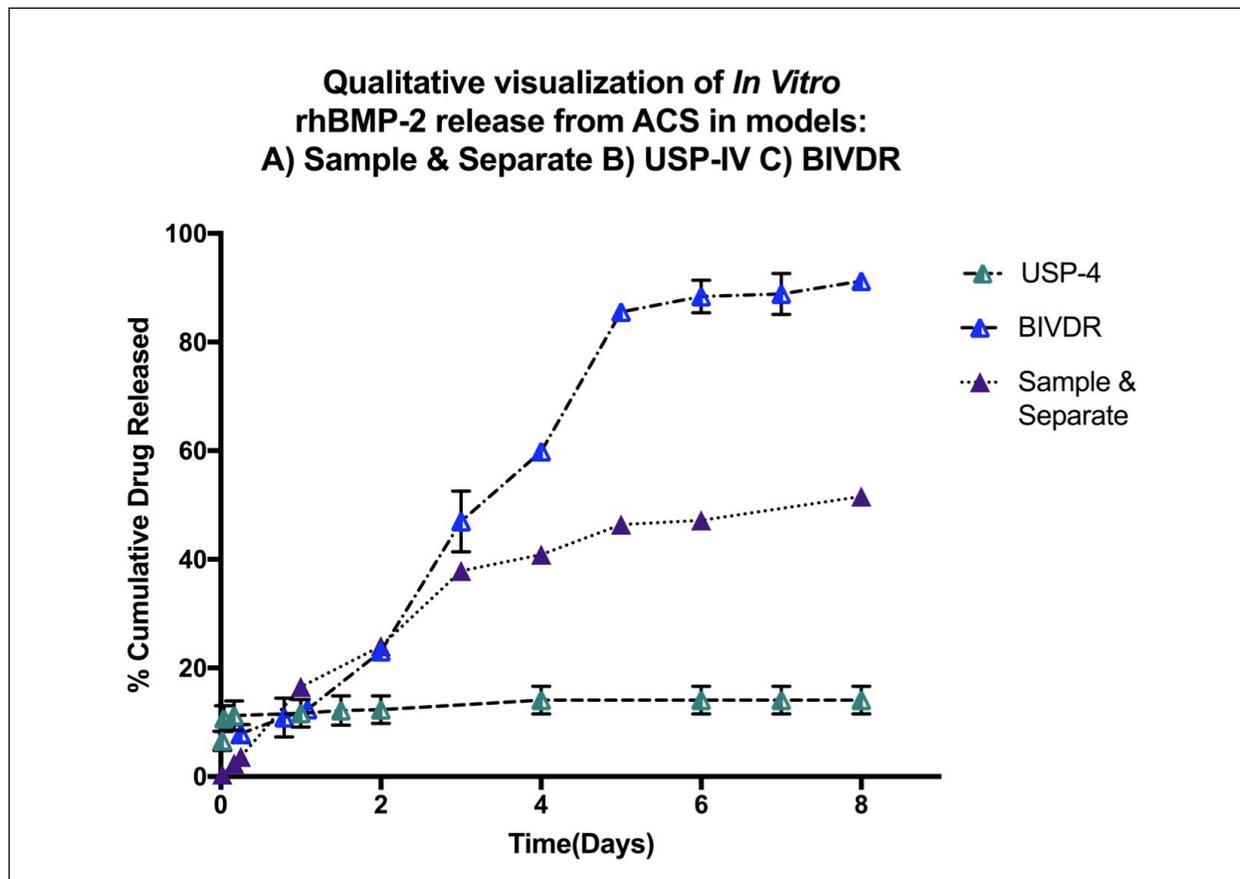
Drug release profiles can be plotted as either release rate profiles or percentage cumulative drug release profiles. Release rate profiles are indicative of quantitative pharmacokinetic parameters. These profiles are a function of time and their slopes might often help us in the determination of pharmacokinetic parameters. Release rate profiles especially in matrix assisted systems help in elucidation of the release behavior at each phase of the multi-phasic system. Release rates can be calculated per hour or per day depending upon the behavior of the dosage form.

Cumulative release profiles are particularly useful for matrix/scaffold assisted delivery systems. In such systems the release of the active moiety/drug might be either diffusion dependent or matrix erosion dependent or both. Such dosage forms often have complex multi-phasic release. Cumulative release profiles are particularly applicable for such polymer-based systems, during the initial phases of study where the intention might be simply to determine the presence of net amount of drug released into the surrounding (independent of mechanism of release). These profiles do not help us in the determination of any pharmacokinetic parameters such as  $t_{1/2}$ ; AUC or MRT unless they are converted and plotted. Neither does it help in the calculation of rate constants. Percent cumulative drug release profiles on their own, without deconvolution, are not elaborative of the kinetic order behavior of the drug. Such profiles are more for qualitative determination rather than quantitative, if not converted or deconvoluted. The information, which, they convey, is how much amount of drug is released over time. However, some amount of drug behavior such as burst release phases and sustained

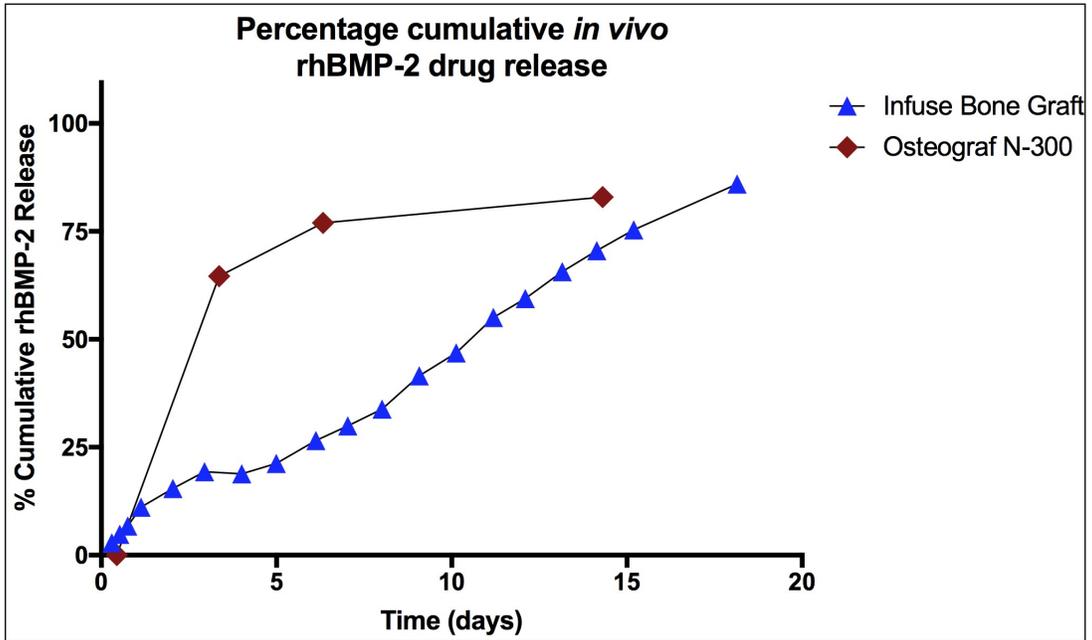
released phases can be visually inspected to get a fair idea about the behavior of drug, during early development phases. For many extended release complex dosage forms with local delivery and a rapid rate of systemic clearance it is difficult to compare an *in vitro* profile to *in vivo* profile with the help of pharmacokinetic parameters such as AUC,  $t_{1/2}$  and MRT. In such cases an alternative approach for a release or dissolution test would be to establish a simple, repeatable and predictable relationship of the *in vitro* data with the *in vivo* data using techniques such as levy plots, time scaling/shifting and making use of percent cumulative drug release profiles. The percentage cumulative drug released vs. time in days for comparison of the three models a) sample and separate b) USP-IV and c) BIVDR model has been shown in Figure 4-21A.

#### **4.3.6 *In vivo* rat pharmacokinetic data**

As mentioned in the methods section the rat pharmacokinetic data for the various formulations a) INFUSE<sup>®</sup> Bone graft b) Osteograf N-300 were digitized using Engauge digitizer and plotted using GraphPad Prism software. Figure 4-21B is a plot of the percent cumulative drug release *in vivo* against the time in days for qualitative visualization of the data. The profile has been adopted from the FDA submission of the Safety and Effectiveness data of INFUSE<sup>®</sup> Bone Graft approved application and Uludag et. al. 2005 (FDA Rockville, 2014; Medtronic, 2011; Uludag et al., 1999a). The profile has been acquired by implantation in rat models. At each time point two rats were sacrificed for obtaining the rhBMP-2 retained at the implant site. It had been shown that the site of implantation of rhBMP-2: orthotopic site, femoral onlay model, subcutaneous site did not



**Figure 4-21A.** *In vitro* percentage cumulative drug released over time (days) for three release study designs a) sample and separate b) USP-IV flow through cell c) BIVDR model.



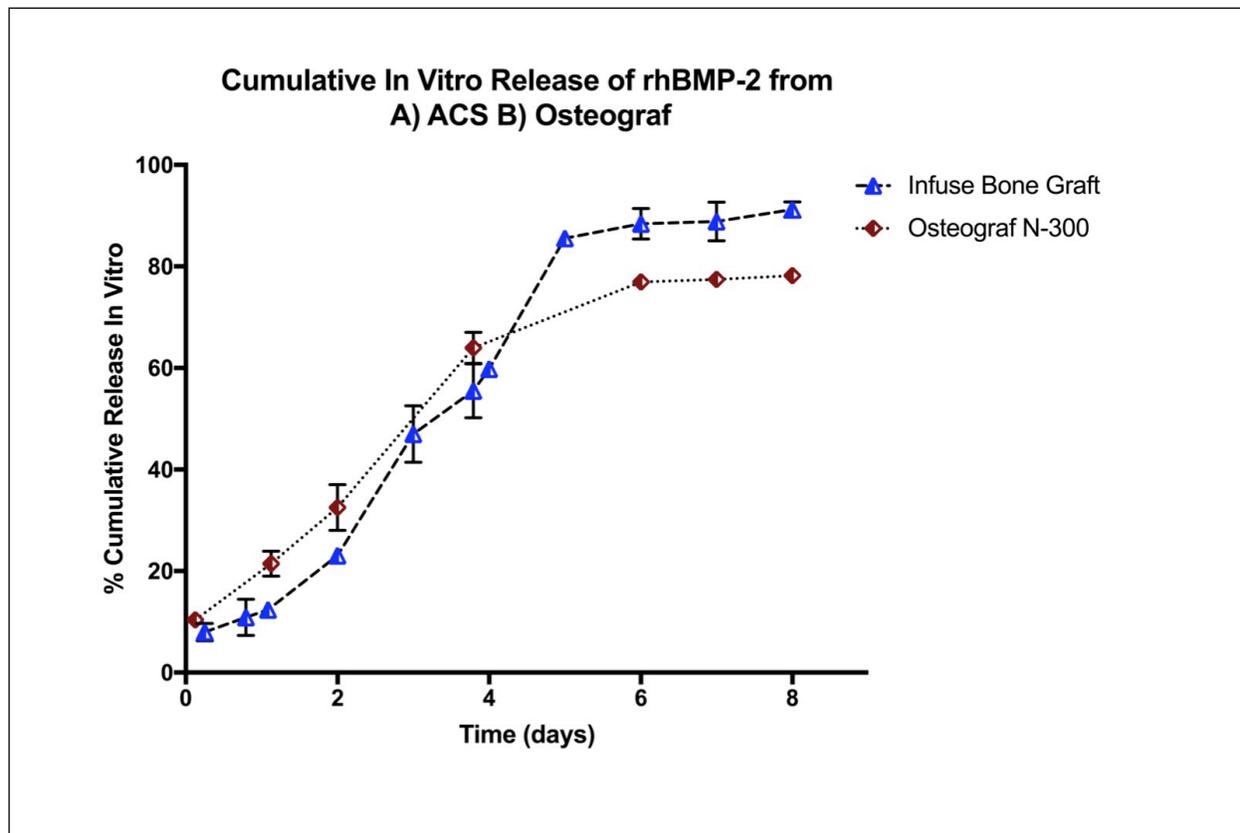
**Figure 4-21B.** *In vivo* percentage cumulative drug released over time (days) for A) INFUSE<sup>®</sup> Bone graft B) Osteograft N-300 in rat model.

(FDA Rockville, 2001, 2014; Uludag et al., 1999a)

cause significant differences (>20%) in the release profile of rhBMP-2, since most of these are deep seated osseous site and the pharmacokinetic parameters were "found to be in the same range" (Uludag et al., 2001). Analytical counts were calculated by gamma ( $\gamma$ )- counted scintigraphy as radiolabelled  $^{125}\text{I}$  was used for tagging the rhBMP-2 protein. The release of rhBMP-2 was observed to be diffusion controlled in the range of 0.8mg/ml - 2.0 mg/ml and was independent of concentration within this range.

#### **4.3.7 Evaluation of a relationship between *in vitro* and *in vivo* data**

The *in vivo* data was obtained from Uludag and co-workers which compares the rhBMP-2 release profiles between different carriers such as collagen sponge and hydroxyapatite (Uludag et al., 1999a). This data and the *in vivo* data obtained from the FDA submission document of INFUSE<sup>®</sup> Bone graft were digitized using Engauge digitizer (FDA Rockville, 2014). The digitization of the data was also verified using another software WebPlot digitizer. GraphPad Prism 7 and Microsoft excel was used for the purposes of all data analysis. The cumulative release percent of rhBMP-2 from ACS was plotted against time in days for all three models. The cumulative release percent of rhBMP-2 from two different scaffolds were compared a) ACS in INFUSE<sup>®</sup> Bone Graft and b) Osteograf N-300. It was observed that for the BIVDR model with ACS as the scaffold a cumulative percent release of  $88.40 \pm 3.02$  % occurred over a period of 6 days while for Osteograf N-300 a cumulative percent release of  $76.94 \pm 0.06$  % was observed. Figure 4-22 is a graphical representation of the percentage cumulative



rhBMP-2 released over time in days for the two formulations INFUSE<sup>®</sup> Bone graft and Osteograft N-300.

**Figure 4-22.** Percentage cumulative *in vitro* release of rhBMP-2 in BIVDR model from two different formulations of rhBMP-2 a) INFUSE<sup>®</sup> Bone graft and b) Osteograft N-300.

Each time point represents n=3 data.

#### 4.3.7.1 Time scaling and Levy Plot:

In order to compare the *in vivo* and *in vitro* data it was essential to normalize the data on the same time scale. This is called as time scaling. Time scaling is used as a tool when the *in vivo* and *in vitro* data exhibit different release rates. The data was normalized with the help of a Levy plot. A levy plot was created by plotting the *in vitro* dissolution/release time of certain specific percentages on the X-axis (e.g. time taken to release 10%, 20%, 30% of the drug substance) vs. the time taken *in vivo* for absorption/release of similar percentages of drug. Plotting the *in vitro* data against the *in vivo* data helped to normalize the *in vitro* data to the same time scale as *in-vivo* data. Time scaling and time shifting parameters were obtained. The Levy's plot has been shown in Figure 4-23. The X-axis of the Levy plot in our current study shows the time taken in days for *in vitro* cumulative release of rhBMP-2 in the BIVDR model. The Y-axis of the plot shows the time taken in days for release of similar percentages in the *in vivo* Sprague Dawley rat model. From the plot generated the time scaling factor was found to be  $3.78 \pm 0.238(\text{SE})$  and a time shifting factor of  $1.66 \pm 0.768(\text{SE})$  was calculated. The equation can be represented in a  $Y=mX +C$  linear regression format as:

$$\text{In vivo cumulative drug release time(days) = } \\ \mathbf{3.777 (In vitro cumulative drug release time) - 1.662}$$

With the help of the above equation the %cumulative *in vitro* release was plotted against the % cumulative *in vivo* release with the time on the same scale now for both groups of data. Figure 4-24A shows the evaluation of the *in vitro in vivo* relationship for the rhBMP-2 release from ACS.

$$\mathbf{\%Cumulative in vivo release = 1.005 (\% cumulative in vitro release) +2.212}$$

Slope was observed to be  $1.005 \pm 0.05192$  (SE). Y-intercept was  $2.21 \pm 3.138$ (SE)

The correlation coefficient was calculated to be 0.9816. The %prediction error for this model was calculated by the formula:

$$\%PE = [(Observed\ in\ vivo\ value - Predicted\ in\ vivo\ value) / Observed\ value] \times 100$$

(FDA Rockville, 1997). This was calculated to be 3.10% for INFUSE<sup>®</sup> Bone Graft using the above IVIVR. Figure 4-24B shows the percentage cumulative in vivo data for the observed and predicted rhBMP-2 release from ACS.

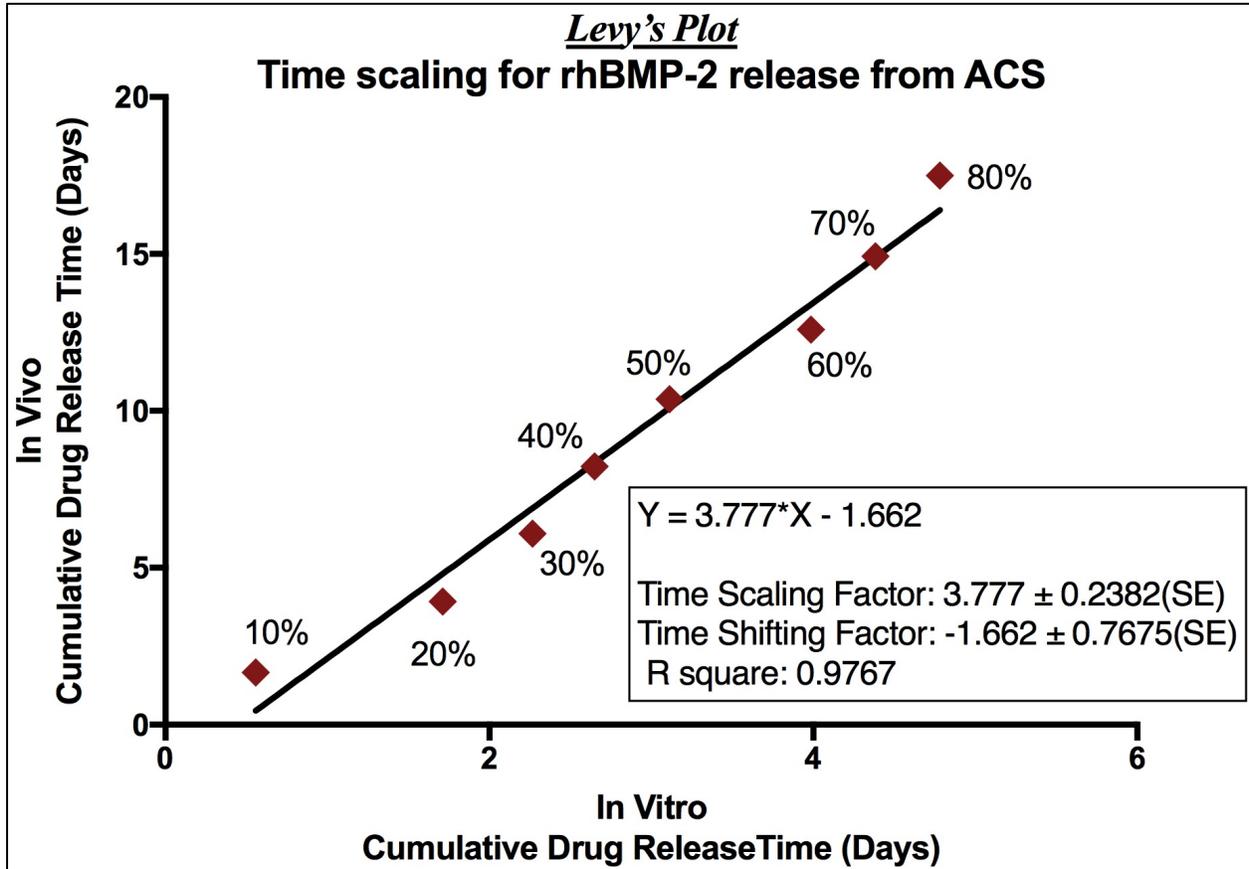
For the Osteograft N-300 a similar kind of time scaling/shifting was performed with the help of the Levy's plot (Figure 4-23) and a linear relationship between *in vitro* and *in vivo* data was obtained. Figure 4-25A is a representative plot of the % cumulative *in vitro* release against the % cumulative *in vivo* release. The parameters were evaluated according to the equation stated below:

$$\%Cumulative\ in\ vivo\ release = 1.036 (\% cumulative\ in\ vitro\ release) - 3.341$$

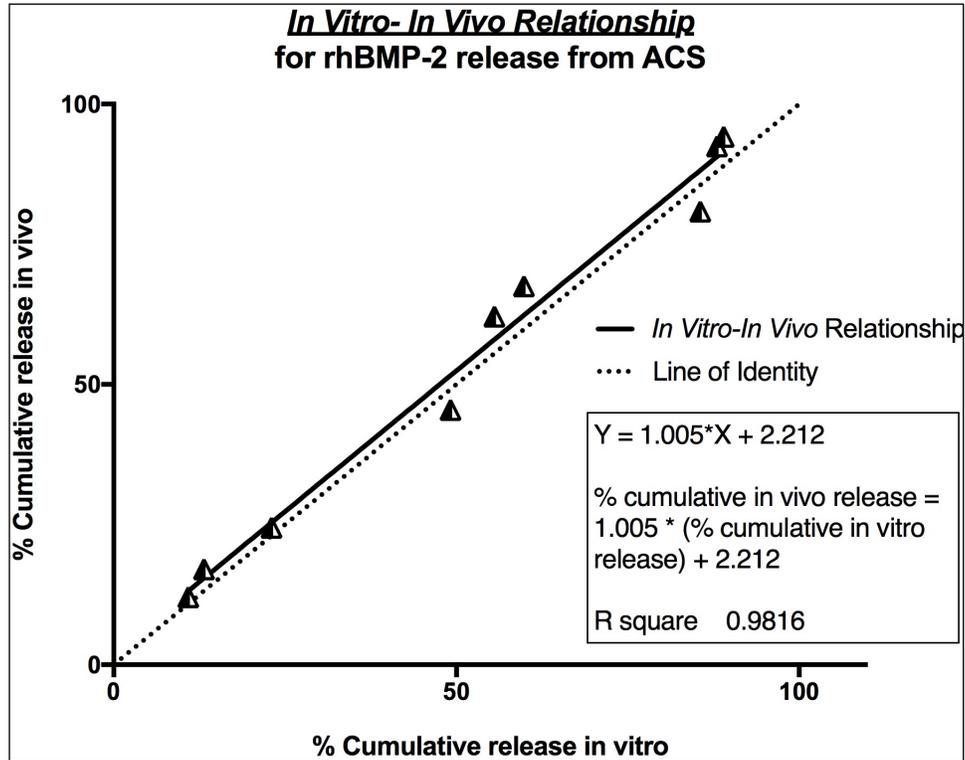
Slope was observed to be  $1.036 \pm 0.06941$ (SE). Y-intercept was calculated as  $-3.341 \pm 3.853$ (SE). The correlation coefficient was calculated to be 0.9824. The %prediction error for this model was calculated by the formula:

$$\%PE = [(Observed\ in\ vivo\ value - Predicted\ in\ vivo\ value) / Observed\ value] \times 100$$

This was calculated to be 1.11% for Osteograft N-300. Figure 4-25B shows the percentage cumulative in vivo data for the observed and predicted rhBMP-2 release from hydroxyapatite.

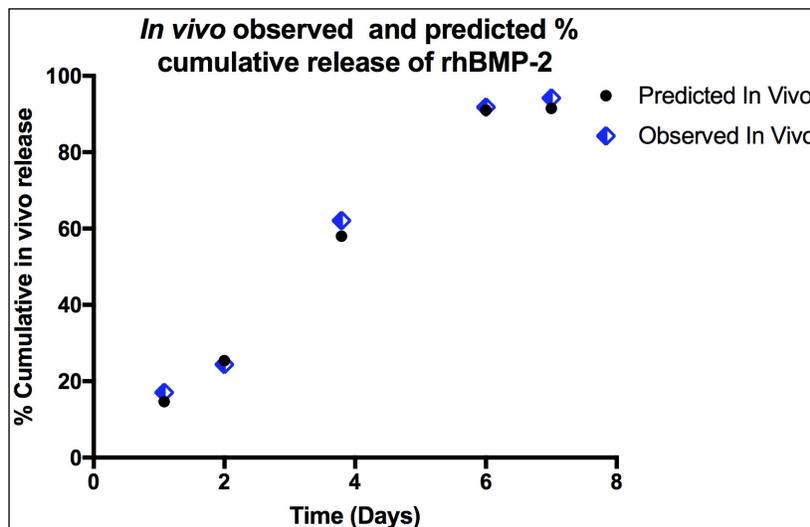


**Figure 4-23.** Levy's plot of cumulative *in vivo* drug release time (days) over cumulative *in vitro* drug release time (days) to normalize the *in vitro* and *in vivo* data to the same time scale.

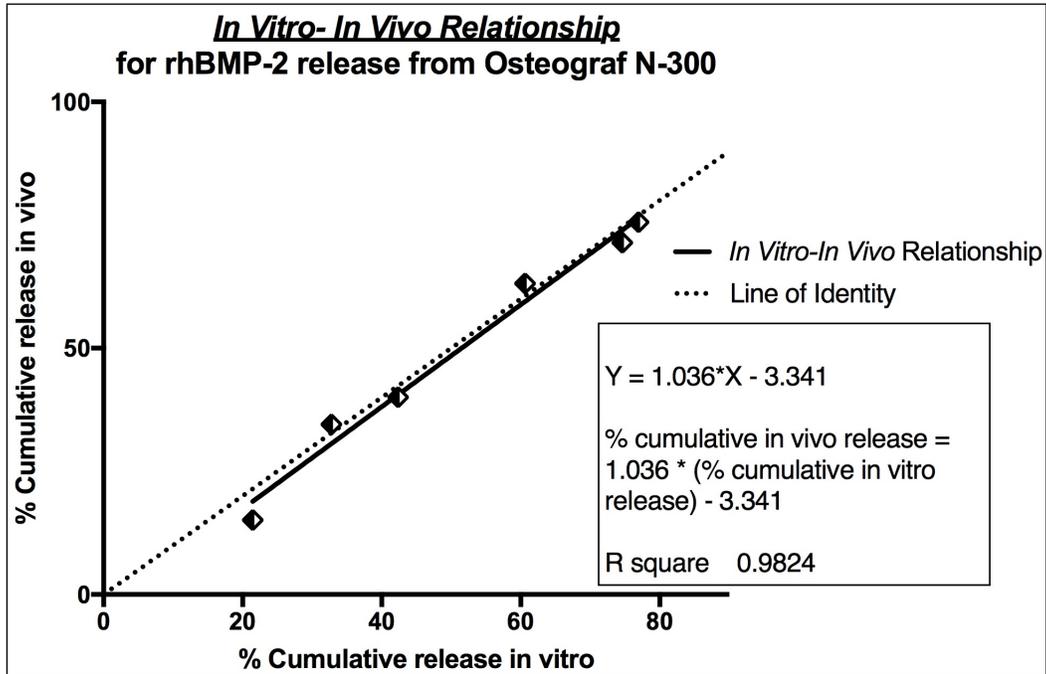


**Figure 4-24A.** Plots to show *In vitro-in vivo* relationship for rhBMP-2 release from ACS.

Slope was observed to be  $1.005 \pm 0.05192(\text{SE})$ . Y-intercept was  $2.212 \pm 3.138(\text{SE})$ . The correlation coefficient was calculated to be 0.9816.

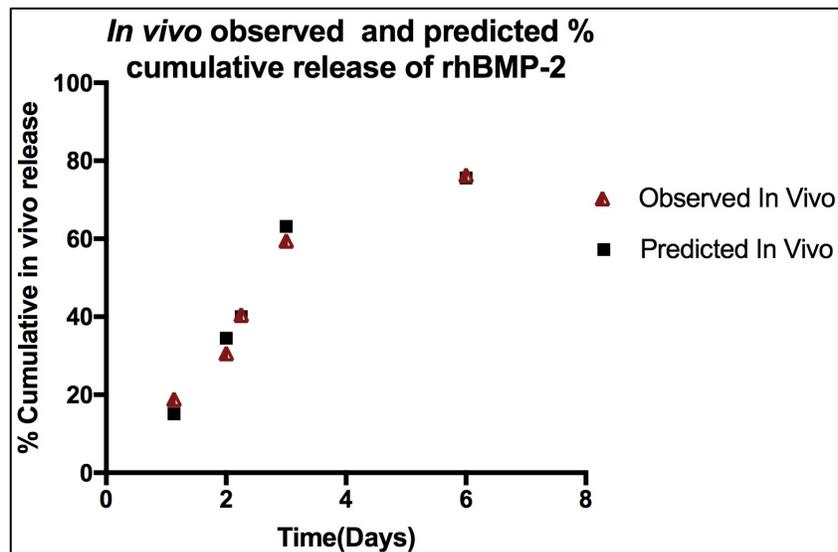


**Figure 4-24B.** Plots to show percentage cumulative in vivo observed and predicted in vivo for rhBMP-2 release from ACS.



**Figure 4-25A.** Plots to show *In vitro in vivo* relationship for rhBMP-2 release from Osteograft N-300.

Slope was observed to be  $1.036 \pm 0.06941(\text{SE})$ . Y-intercept was calculated as  $-3.341 \pm 3.853(\text{SE})$ . The correlation coefficient was calculated to be 0.9824.



**Figure 4-25B.** Plots to show percentage cumulative in vivo observed and predicted in vivo for rhBMP-2 release from Osteograft.

#### 4.3.7.2 Discriminatory nature of the model

The discriminatory nature of the model was studied by using a model independent approach. The model independent approach uses the calculation of a difference factor (f1) and similarity factor (f2). f1 and f2 values are used to compare the experimental *in vivo* and predicted *in vivo* data. They help in calculating a point-to-point comparison between the experimental and predicted curve and determine the relative error between them. For deciding if two formulations are equivalent or not FDA guidance on dissolution tests for immediate dosage forms suggests an f1- difference value of less than 15 and f2 – similarity factor values greater than 50. Though the equations given below have been defined by FDA for immediate oral dosage forms they have been applied in literature in relation to extended release dosage forms as in Burgess et. al for determination of dexamethasone release from two different molecular weight PLGA formulations (Shah et al., 1997; Zolnik and Burgess, 2008). The f1 and f2 equations for calculation with a model independent approach have been shown below:

$$f1 = \left\{ \frac{\sum_{t=1}^n |R_t - T_t|}{\sum_{t=1}^n R_t} \right\} * 100$$

$$f2 = 50 * \log \left\{ \left[ 1 + (1/n) \sum_{t=1}^n (R_t - T_t)^2 \right]^{-0.5} \right\} * 100$$

In the above equations  $R_t$  is the cumulative percent release for the reference product or formulation, which in current case is INFUSE<sup>®</sup> Bone graft and  $T_t$ , is the test product, which in this research study is Osteograf N-300.

Using the equations, a f1 (difference factor) value of 19.60 and a similarity factor f2 of 39.26 was calculated. For the model to be *discriminatory* between formulations an  $f1 > 15$  and  $f2 < 50$  is required. This suggested the model was discriminatory between the formulations. Table 4-8 shows the calculations for determination of f1 and f2 factor using the BIVDR model.

**Table 4-8.** f1 and f2 calculation for the reference product (INFUSE® Bone graft) and test product (Osteograf N-300).

Time (days)	Reference product (Rt) (INFUSE® )	Test product (Tt) (Osteograf N-300)	$ R_t - T_t $	$(R_t - T_t)^2$
0.25	7.96	12.36	4.40	19.37
1.08	12.38	21.45	9.85	97.08
2	23.05	32.51	9.47	89.67
3.79	55.53	63.97	13.80	190.35
6	88.40	76.94	8.66	74.99
7	88.86	77.45	11.41	130.20
8	91.21	78.20	14.40	207.36
$\sum_{t=1}^n  R_t - T_t $			71.99	
$\sum_{t=1}^n R_t$			367.38	
$\sum_{t=1}^n (R_t - T_t)^2$			809.14	
<b>Difference factor: <math>f1 = \left\{ \frac{\sum_{t=1}^n  R_t - T_t }{\sum_{t=1}^n R_t} \right\} * 100</math></b>			<b>19.60</b>	
<b>Similarity factor: <math>f2 = 50 * \log \left\{ [1 + (1/n) \sum_{t=1}^n (R_t - T_t)^2]^{-0.5} \right\} * 100</math></b>			<b>39.26</b>	

#### 4.3.8 Advantages and limitations of the study

The current research study had its own set of advantages and limitations. During the development of the *in vitro* release study design we faced some challenges while gathering *in vivo* data from literature particularly with respect to data for formulations of rhBMP-2 not involving INFUSE<sup>®</sup> Bone graft. A limitation of this study is the rat pharmacokinetic data obtained by digitization of Uludag et. al, 1999 involved radiolabelled quantitation methods for *in vivo* time points (Uludag et al., 1999a). <sup>125</sup>I and <sup>35</sup>S radiolabelled rhBMP-2 was used in the implants and  $\gamma$  and  $\beta$ - scintillation counters were used for calculating the radioactive counts for rhBMP-2 protein retained in the implant. At each time point two rats were sacrificed and the implants were extracted to calculate the retention of rhBMP-2 in the sponge (Uludag et al., 1999a) . While using the *in vivo* data we converted the *in vivo* percentage retention in ACS to *in vivo* percentage released. However, since destructive sampling was not a feasible option while developing an in house *in vitro* release study design we developed and validated a HPLC-UV method for the quantitation of rhBMP-2 in the release media. Although ELISA was used for quantitation during sample & separate and USP-IV, the release profiles were incomplete and non-representative of the in-vivo profiles for these methods and hence cross-validation was not necessary due to distinct differences in cumulative release in vitro profiles. Validation and stability analysis was evaluated in detail for the HPLC method in regards to precision, accuracy, selectivity, stability of rhBMP-2 in release media. This helped reduce the chances of our analytical methodology introducing any additional variability into the release study design and data. The *in vitro* release study design therefore helped us to avoid a destructive sampling mechanism at

each time point. Although this was time and cost efficient and one of the goals of developing the *in vitro* release study design was to avoid the path of destructive sampling we were mindful that during this study we are comparing the percentage cumulative drug release profiles obtained using two different analytical methods: radiolabelled counting for *in vivo* rat PK data and HPLC method for *in vitro* release data. In evaluation of the discriminatory profile of the BIVDR model between the reference (INFUSE® ) and test (Osteograft) product we use similarity factor (f2) as a parameter. While Polli and co-workers, in 1997 have evaluated the significance of f2 factor and its accuracy and applicability in dissolution study designs; the f2 factor gets affected by the “length of the dissolution profile” as mentioned in Pillay et. al, 1998 (Moore, 1996; Pillay and Fassihi, 1998; Polli et al., 1997). In the current research investigation we have attempted to reach a cumulative drug release percentage of > 80% as is suggested in the FIP/AAPS guidelines for novel dosage forms (Siewert et al., 2003). This helps us achieve a comprehensive release profile bracketing the different phases of the complex dosage form. Another limitation of the study is the *in vivo* sampling time points which are available for formulation carriers for rhBMP-2 other than INFUSE® Bone graft have only four time points. For developing a successful IVIVR and a Levy’s plot atleast 6 time points are required. Interpolation of two additional time point was required for the Osteograft N-300. This was performed between 0.44 day and 3.35 days: the first two time point for *in vivo* rat data; since the data was extremely sparse in this region and not evenly distributed. The sparse data points for the second formulation (Osteograft) therefore also posed a challenge in development of an evaluative *In Vitro* Relationship (IVR).

## 4.4 Conclusion

A step-by-step modular approach was adopted while designing the biorelevant *in vitro* drug release (BIVDR) model. Since our drug substance was a protein, which showed adsorption to conventional glass models such as USP-IV flow through cells, to make the model adaptable and deliver the best drug recovery percentage, each design component was tested for its adsorption to the protein component. The net recovery of the protein with the introduction of each specific component into the model was evaluated, and only then the design component was introduced into the BIVDR system. *In vivo* fluid inorganic ion concentration, osmolality, pH and temperature were simulated in the experimental set up. Flow directionality and flow ports were designed to simulate *in vivo* tissue vascularity. The flow ports were also designed to not subject the dosage form to be placed perpendicularly in the path of flow. Because of the presence of a centralized core compartment with membranes at both ends, the BIVDR model can also be applied for release or dissolution testing of complex dosage forms such as liposomes, nanoparticles and microspheres. There is physical separation of the dosage form from the other compartments with the help of porous filter membranes. The porous membranes allow for free exchange of media between compartments but limit the carrier scaffold of the dosage form within the core compartment. This helps in providing better sampling capabilities and no extra steps in physical separation of the dosage form and media is required at each sampling time point. We hope that the application of this model can be extended to dissolution and release testing of similar controlled release parenterals and protein based dosage forms.

## CHAPTER 5

### 5 SUMMARY AND GENERAL CONCLUSIONS

Drug formulation development has improved enormously over the past few decades. This has enabled targeted local drug delivery to complex physiological sites. Drug delivery systems have also seen an unparalleled growth. The active drug substance is no longer just a small molecule or chemical entity (200 - 2000Da); it can be a large molecule or protein (10 -150kDa). Biotechnology and genetic engineering has facilitated the development of recombinant 'orthobiologics'. 'Orthobiologics' are recombinant biologic products often having the same composition as a human endogenous protein but produced in other pre-clinical species or cell lines (such as Chinese Hamster Ovary cell lines) with the help of genetic engineering and recombinant DNA technology. The interplay of all these factors have also given rise to drug modalities that are able to deliver the active drug substance targeted at almost any location of the human body with a) minimal immunogenicity and b) controlled release over an extended period of time. Extended controlled release drug formulations often called as 'novel' dosage forms help in maintaining therapeutic levels of the drug substance and ensure patient compliance (Burgess et al., 2004; Burgess et al., 2002b).

*In vitro* dissolution and release tests are a part of the public specification tests of a dosage form. Their most significant use is in the SUPAC stages and for achieving biowaivers (clinical stages). However, a well-developed biorelevant release study design can also be used during the formulation development stages (pre-clinical stages). In many 'complex' dosage forms obtaining pharmacokinetic data from humans can be difficult even during clinical stages of the drug e.g. INFUSE<sup>®</sup> Bone graft, ocular implants. These implants generally carry drugs in their carriers targeted at local tissues and sites of healing and often have high rates of systemic clearance. Protein and peptide based drugs often show receptor mediated clearance -internalization / opsonization mechanisms of clearance (catabolic clearance) and do not follow the typical hepatic or renal clearance route (Chirmule et al., 2012). This also makes their elimination rates extremely variable. The biodistribution of these drugs at the local non-accessible tissue chambers enhances the dilemma of generating pharmacokinetic profiles in humans. 'Biorelevant' *in vitro* release tests can help with such 'novel' multi-chambered drug biodistribution study designs. These *in vitro* release tests add to the study design a combination of i) clinically relevant easy to reproduce media, ii) modular design of the release apparatus and iii) well researched biorelevant parameters represented in the study design with a robust analytical method.

The current research investigation focuses on the development of such an approach. The primary objective of this research was to design an *in vitro* release test that would be optimized for a 'complex' carrier based implant with a protein as the active pharmaceutical ingredient.

In Chapter 3 of the thesis a stability indicating HPLC-UV method was developed and validated for the quantitation of drug substance in this media. The media was comparable to plasma in terms of inorganic ion concentrations, osmolality, pH, buffering capacity with respect to temperature changes (Iyer et al., 2007b). The dynamic range of the method was from 0.50 -100 µg/mL with a %DFN of 9.67% at the LLOQ (0.50 µg/mL) to – 6.27%. The LOD for the method was 0.10 µg/mL. Stability of the drug was also determined in the media at 37°C, room temperature 22°C and at autosampler analysis temperature 4°C, at low and medium concentration level of 2 µg/mL and 15 µg/mL respectively. The stability percentages were found to be within the permissible range of 90-110%. In addition to the stability studies presence of interfering excipients and degradant peaks during the *in vitro* release test were investigated with the help of acidic, basic, oxidation and temperature based forced degradation analysis for 72hrs. The degradant peaks were trapped and injected into LC-MS/MS for qualitative characterization of the changes occurring to the protein molecule (oxidative degradation, deamidation of N terminal end and hydrolysis). The robustness of the method was also explored with inter and intra day accuracy and precision (%RSD within ± 10%), and system suitability for precision, retention time and tailing factor of multiple injections (n=6). The %RSD was found to be within ± 1.75%.

Chapter 4 of this research study focused on the primary objective of this research of developing a biorelevant *in vitro* release test for a 'complex' dosage form. This was performed by setting up a simple sample and separate with INFUSE® Bone graft. A cumulative release profile with a release percentage of 58.89 ± 4.97 % over 30 days was obtained for the sample and separate. An asymptote was observed in the

cumulative release profile after the first 15 days and drug release > 80% could not be achieved with this system. The next approach was to use the USP guideline suggested USP-IV flow through cell apparatus. For the USP-IV model the cumulative release profile showed an apparent asymptote after the first 15min of approximately 15% burst release and negligible release was observed for up to 5 days. USP-IV helped us investigate a step further the effects of:

- i) flow rates and flow directionality on the fragile, shear labile collagen sponge carrier based dosage form
- ii) nature of the cell container material (glass) on the active pharmaceutical ingredient (rhBMP-2 protein) recovery.

As a result, on visual inspection the placement of the dosage form (absorbable collagen sponge scaffold with the rhBMP-2 protein) also showed pieces of the scaffold being torn away and in the circulation even at reduced flow rates of 0.8 – 0.9 mL/min. Such a release study design also gives rise to chances of enhanced variability. Our observation was that, this occurred due to the fragile dosage form being placed perpendicularly in the path of flow. From our literature review, we know that the sinus augmentation cavity is innervated by blood vessels that have flow rates of 0.8 - 0.9 mL/min. The dosage form is not placed perpendicularly on the path of flow during the sinus augmentation surgery by the clinician. Therefore, in order to make the release study design more clinically relevant and less variable we incorporated all these factors to have an improved biorelevant *in vitro* release test.

A novel biorelevant *in vitro* drug release (BIVDR) model was designed for implant based dosage forms with novel construction and adaptability with the following components:

- i) Low protein adsorption material – Body of the model was constructed with the material (polycarbonate) having minimal protein (rhBMP-2) adsorption and maximal recovery thereby helping to reduce variability in cumulative release profiles as compared to USP-IV apparatus.
- ii) Multi-chambered model – the BPC and BRC helped to allow for physical segregation of the fragile dosage form from the media to help in sampling and media replacement at each time point. The chambers were separated by porous regenerated cellulose or cellulose acetate membranes (also optimized for minimal rhBMP-2 adsorption and maximal recovery with minimal variability). The membranes allowed free diffusion of the rhBMP-2 molecule between chambers while keeping the carrier scaffold in the centralized BPC.
- iii) Flow ports and flow directionality – Each chamber the BPC and BRC had flow ports, which, were placed parallel to the directionality of the dosage form. This simulated physiological blood vessel enervation and also reduced scaffold tearing off due to flow of fluid.
- iv) Low volumes – The chambers were designed to simulate implant physiological microenvironment of low fluid surrounding area. The central core compartment (BPC) had a volume of 2.99mL and the surrounding compartment (BRC) had a volume of 8mL. The core compartment's (BPC) height and width was designed to simulate the average physiological pocket of the maxillary sinus cavity during sinus augmentation or lift surgeries (net volume 2-3 mL).

Apart from these features, the other biorelevant parameters included in the study design were the pH and osmolarity of the media, and physiological body temperature 37°C.

With these components incorporated in the study design we obtained cumulative percent release profiles of > 80% release of rhBMP-2 from the ACS scaffold in the BIVDR model. Time scaling was performed to achieve normalization of the *in vitro* and *in vivo* data on the same time scale with a levy plot. The time scaling factor was 3.77 with a R<sup>2</sup> value of 0.97 for release percentages from 10% to 80% of rhBMP-2 from ACS.

With these factors the *In vitro in vivo* relationship was evaluated and found to be:

$$\%Cumulative\ in\ vivo\ release = 1.005 (\% cumulative\ in\ vitro\ release) + 2.212$$

with a correlation coefficient of 0.9816.

In order to observe if the model was discriminatory between formulations Osteograft N-300 a hydroxyapatite based carrier scaffold for rhBMP-2 was also evaluated. The IVIVR was calculated to be

$$\%Cumulative\ in\ vivo\ release = 1.036 (\% cumulative\ in\ vitro\ release) - 3.341$$

with a correlation coefficient of 0.9824. Similarity (f2) and difference (f1) factors were used for calculating the difference between the two formulations a) collagen carrier scaffold and b) hydroxyapatite-based scaffold of rhBMP-2 drug substance. A value of 39.26 and 19.60 was obtained for the similarity and difference factors respectively showing discriminatory release profiles for the BIVDR model and the release study design.

*Future application of the model:* The BIVDR model has been designed for the *in vitro* release testing of 'special' dosage forms such as liposomes, microspheres or any fragile carrier based implant. Generally implants are physiologically in surrounding low fluid

volumes. The net volume of the model is 11 mL with the central BPC and surrounding BRC. If required an additional chamber SC can be screwed in to add 2mL of fluid volume. This chamber can then be used for simulating a different tissue fluid and observing the distribution into this chamber. This kind of multi-chambered approach would also help for observing and studying biodistribution into tissue chambers (e.g. ocular chamber: vitreous humor) for complex dosage forms (such as for ocular implants) with controlled delivery using lipid nano-particles carrying drugs like dexamethasone (Souto et al., 2010; Xu et al., 2000). Another major advantage of the model is physical segregation of the dosage form in the BPC compartment with flow ports designed to enable media flow over and under the porous chamber. This would greatly facilitate sampling for dosage forms like microspheres, liposomes e.g. controlled delivery of proteins using PLGA, polyanhydride, chitosan microspheres (Cohen et al., 1991; Jameela et al., 1998; Tabata et al., 1993). The validated BIVDR model system has not used its full capability of multi-chambers (including the sinus chamber (SC) or utilization of different osmolality media in each chamber. Therefore, more data is required to be collected with diverse novel dosage forms to know how well the model performs. For our research investigation, we applied two implant based formulations of the rhBMP-2 drug substance and were able to develop a discriminatory and predictive relationship. We hope to use the model for other peptide and protein based parenteral formulations. The design of the model would help support the investigation of release profiles of 'complex' dosage forms.

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## VITA

Debina Biswas was born on 18<sup>th</sup> January 1983 in Kolkata, India. She earned her Master's in Science (M.Sc) degree in Microbiology from Bangalore University in 2007. She held the 2<sup>nd</sup> rank university wide (out of 4000 students) in M.Sc. Microbiology during her graduate studies in Bangalore University, India. Following her graduation she worked for 2 and a half years at Reliance Life Sciences, Navi Mumbai, India as an Executive for process development of recombinant biotherapeutic modalities. She gained her MS degree in Biological Sciences from State University of New York, Albany in 2013 prior to joining the Department of Pharmaceutics at Virginia Commonwealth University for her Ph.D in Pharmaceutical Sciences (with focus on biopharmaceutical analysis). She has been an invited speaker at Pfizer Inc. and worked as a Ph. D. Co-op at Sanofi Genzyme for the development of an LC-MS/MS analytical method for monoclonal antibodies in biological matrices. She also presented her developed method at scientific lightning talks at Sanofi, Waltham and received an excellence award for the same. She has presented several posters in various intramural (VCU, SUNY Albany) and extramural scientific conferences and presentations (AAPS 2016). In 2016, she won the AAPS Analysis and Pharmaceutical Quality (APQ) award for Graduate Research Excellence and the Peter and Sian Byron Graduate Student Travel Award. She is an elected AAPS Student Representative for 3 scientific committee's: In-Vitro Release and Dissolution Testing (IVRDT) group, the Analysis and Pharmaceutical Quality (APQ) group and the Non-Clinical Dosage Formulation (NCDF) group. She has organized and moderated two sessions in AAPS 2016. She has also served as the National Liaison for the Zeta Chapter of Graduate Women in Science (GWIS).