Evaluation of Manufacturing Robustness for a Biodegradable Subcutaneous Implant Containing Bictegravir for HIV Prevention

by

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According to World Health Organization (WHO), the global impact of human immunodeficiency virus (HIV) infection remains a significant concern, necessitating the development of effective prevention strategies. Oral pre-exposure prophylaxis (PrEP) is one method available for HIV reducing the risk of HIV transmission. However, several barriers such as patient adherence and persistence to product use have limited its widespread uptake. To address this challenge, a long-acting injectable PrEP product was recently approved by the FDA, as long-acting products are associated with improved adherence and compliance.

Toward this end, Research Triangle Institute (RTI) International is currently developing a long-acting HIV PrEP product in the form of a biodegradable implant made from polycaprolactone (PCL), which incorporates the antiretroviral drug Bictegravir (BIC). This thesis focuses on the characterization of the BIC-loaded PCL implants and the validation of manufacturing processes to support technology transfer activities.

Two high-performance liquid chromatography (HPLC) analytical procedures for both assay and dissolution study were qualified to be accurate and reliable. Formulation paste containing Bictegravir and castor oil was prepared and loaded into PCL tubes to fabricate the implants at Magee-Womens Research Institute (MWRI). Then, these implants were characterized and compared with those from RTI to validate the impact of different processing parameters.

The implants consistently achieved the target drug loading of 120 mg of Bictegravir per

implant, with uniform distribution within individual implants as well as between implants. Notably, significant differences in the in-vitro release profiles were observed between the two sets of implants after 2 weeks during the dissolution study, which indicated the importance of sealing integrity in ensuring reproducibility and quality of implants. Consequently, further investigations into testing the sealing integrity should be developed.

In conclusion, this thesis employed qualified HPLC methods for the characterization of BIC-loaded PCL implants. The cross-lab evaluation of implants underscored the significance of implant sealing integrity as a crucial processing aspect, emphasizing the need for ongoing research to investigate the testing and validation of sealing integrity.

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Preface

The work in this thesis was carried out in collaboration with Research Triangle Institute (RTI) International, Research Triangle Park, NC, USA and funded by National Institute of Health (NIH) National Institute of Allergy and Infectious Diseases (NIAID) (1R01AI154549-01). The purpose of this thesis is to characterize and validate a long-acting biodegradable subcutaneous implant containing Bictegravir, an antiretroviral drug, for HIV prevention. The scientific discoveries obtained from this thesis will be helpful for the scaling up and the manufacturing of this HIV pre-exposure prophylaxis (PrEP) product.

I would like to express my sincere gratitude to my advisor and mentor, Dr. Lisa C. Rohan, for providing me with the opportunity to join her laboratory and work on this project. Her scientific expertise and guidance helped in shaping me into a more proficient researcher. Furthermore, her unwavering support and encouragement have been instrumental in my growth.

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1.0 Introduction

1.1 Human immunodeficiency virus (HIV)/ AIDS

1.1.1 Epidemiology of HIV

Human immunodeficiency virus (HIV) is an infection which compromises the human immune systems, leading to acquired immunodeficiency syndrome (AIDS) if not treated. HIV can transmit through unprotected sex, sharing needles or syringes, pregnancy, childbirth, and breastfeeding. According to World Health Organization (WHO) in 2021, there were around 38.4 million people living with HIV, 1.5 million new HIV infections, and 650 thousand people dying from HIV-related causes[1]. The majority of current HIV infections are originating in Africa, but cases in Europe and America still rise. Compared to data from 2010, there are 30-40% new annual HIV infections across Europe and the Eastern Mediterranean regions.

1.1.2 Mechanism of HIV infection

There are several steps in the HIV viral life cycle[2]. First, HIV binds to receptors on the surface of a CD4 cell. The HIV envelope and CD4 cell membrane then fuse, which allows the virus to penetrate through host cell membrane. Inside CD4 cells, single-stranded HIV RNA is released to the cytoplasm and reversely transcribed to double stranded DNA with the assistance of reverse transcriptase released by HIV. The DNA will therefore be translocated to the nucleus and

integrated to the host DNA, and then transcribed to mRNA encoding viral proteins. Continuing with the translation of mRNA to proteins and post-translational cleavage by HIV protease, the HIV virus is finally maturated and budded. The replication and accumulation of HIV in the human body will lead to acquired immunodeficiency syndrome (AIDS), a chronic, potentially life-threatening disease[3, 4].

1.1.3 Treatments for HIV infection

There is still no cure for HIV. However, it is preventable and controllable by antiretroviral therapy (ART), involving a combination of HIV treatment regimens[5, 6]. The goal of therapeutic implementation of ART is HIV viral suppression, which is defined as less than 200 copies of HIV per milliliter of blood. Generally achieving viral levels of less than 200 copies requires 3 to 6 months of continuous antiviral drug therapy. If viral suppression is achieved, HIV transmission can be prevented between sex partners, perinatal transmission, and HIV transmission through sharing needles or syringes.

1.2 Antiretroviral (ARV) drugs

1.2.1 Classification of ARV

More than 30 HIV medications have been approved by the U.S. Food and Drug Administration (FDA)[6]. This group encompasses different mechanistic classes of antiretroviral drugs[7]. Examples of drug classes include nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), integrase inhibitors (IIs), and protease inhibitors (PIs). NRTIs compete with natural deoxynucleotides to incorporate into a growing viral DNA chain, which causes a chain termination in DNA synthesis. NNRTIs bind to reverse transcriptase that are critical to DNA synthesis for direct inhibition. IIs block the action of integrase to avoid the insertion of viral genome to host DNA. PIs bind to HIV protease to stop the proteolytic cleavage of protein precursors essential to the production of viral particles. These classes all interfere with the enzymes necessary for HIV to replicate, leading to a lower viral load. Some classes of antiretroviral drugs disable HIV infection of CD4+ cells. Examples of this include fusion inhibitors, which interrupt HIV virions to bind, fuse, and enter human cells; CCR5 antagonists prohibit CCR5 receptors on T-cell to hinder viral attachment; post-attachment inhibitors bind to CD4 receptors on host CD4 cells to block viral entry[8].

With excellent efficacy, better tolerability, and most importantly, fewer drug-drug interactions (DDIs) than NNRTI and PI-based regimens, integrase strand transfer inhibitors (INSTIs) are now recommended by the WHO as a first-line treatment for people with HIV[9]. Integration is a crucial factor in the HIV replication cycle. It entails the formation of a preintegration viral DNA complex, 3’ processing, and strand transfer[10]. The mechanism of

INSTI is to prohibit HIV integrase from incorporating proviral DNA to human host cells, which block the HIV-catalyzed strand transfer step and thus inhibit integration[11]. Up to now, multiple INSTIs have been investigated, including raltegravir (RAL), elvitegravir (EVG), dolutegravir (DTG), bictegravir (BIC), and cabotegravir (CAB)[12].

1.2.2 Bictegravir (BIC)

Bictegravir (BIC; GS-9883) is a second-generation integrase strand transfer inhibitors (INSTIs) developed by Gilead in 2016 (**Figure 1**), which inhibits the replication and propagation of HIV-1 virus into the human genome. According to the biopharmaceutics classification system (BCS), BIC is classified as a BCS class II compound, showing low solubility and high permeability. It is lipophilic with weak acidic nature, and a partition coefficient cLogP of 2.7 (determined by ALOGPS 2.1 software)[13, 14]. BIC exhibits high potency against HIV even at low concentrations, as demonstrated by a 50% effective concentration (EC_{50}) at a range of 0.02 – 6.6 nM[15]. BIC is primarily eliminated through UGT1A1 glucuronidation and CYP3A4 oxidation, which can lead to drug-drug interactions when combined with potent dual CYP3A4/UGT1A1 inhibitors and CYP3A4 inducers[16]. Additionally, its increased plasma protein binding ability contributes to lower clearance[17]. However, clinical studies have shown that bictegravir is more efficient and has much broader antiviral profiles than other integrase strand transfer inhibitors (INTSTIs) including raltegravir and elvitegravir. Previous research also demonstrated the ability of BIC to inhibit a broad range of INSTI-resistant integrase mutants to a greater extent than dolutegravir and cabotegravir[18].

The first BIC product approved by the FDA was a co-formulated product- Biktarvy®, containing BIC 50 mg, tenofovir alafenamide (TAF) 25 mg, and emtricitabine (FTC) 200 mg in a single daily tablet regimen (STR) for HIV treatment[19, 20]. A phase III clinical study demonstrated approximately 92% efficacy, making it an advantageous treatment option. This product was found to be highly resistant to mutations in the HIV virus and due to BIC's high potency, sufficient drug loading can be achieved in a small tablet size. Additionally, its primary metabolism mediated by UGT 1A1 avoids many drug-drug interactions[21, 22]. This further proves BIC's potential as an effective and optimal HIV prevention option.

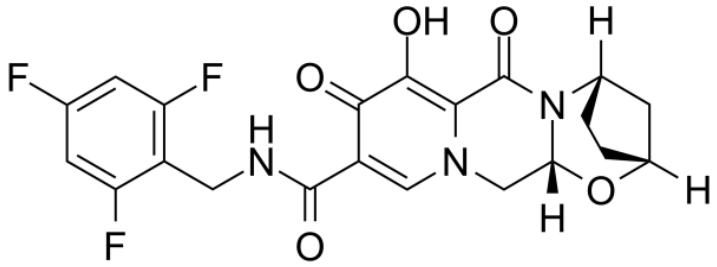


Figure 1 | Bictegravir (BIC; GS-9883), IUPAC name: (1S,11R,13R)-5-hydroxy-3,6-dioxo-N-[(2,4,6-trifluorophenyl)methyl]-12-oxa-2,9-diazatetracyclo[11.2.1.02,11.04,9]hexadeca-4,7-diene-7-carboxamide

1.3 HIV prevention

As previously discussed, successful antiretroviral therapy achieves sufficient drug plasma concentration levels for suppression of HIV viral load to levels which can also prevent HIV transmission to uninfected individuals. Despite the efforts of 'treatment as prevention' using ARV

in addition to safe sex education and postexposure prophylaxis (PEP), the incidence of HIV is still high in defined risk groups including men who have sex with men (MSM), heterosexually active adults (HET), and persons who inject drugs (PWID)[23, 24]. Hence, the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) both recommend the use of pre-exposure prophylaxis (PrEP) to be a part of HIV prevention in 2016[9, 25]. Application of several antiretroviral drugs to uninfected individuals as PrEP has been implemented.

1.3.1 Existing oral PrEP

Currently, there are many different modalities of PrEP available. According to the HIV guidelines from World Health Organization (WHO), Truvada® (emtricitabine/ tenofovir disoproxil fumarate; FTC/TDF) pericoital or daily regimen, was highly recommended for people at substantial risk. Beside Truvada®, Descovy® (emtricitabine/ tenofovir alafenamide; FTC/TAF) is also used as oral PrEP for daily use[26]. Although the oral route is a convenient method for drug administration, continuous adherence to a daily tablet can be challenging.

One important factor which has been shown to impact PrEP efficacy is medication adherence. Compliance to medication regimens can be enhanced through modalities which require less frequent product administration[26-28]. As we can see, patient adherence is critical for PrEP efficacy, continues to be crucial as long as the subjects are at high risk of HIV infection[29]. However, it was reported that adherence and persistence are challenges[30]. Therefore, PrEP options which provide long-term protection are essential.

1.3.2 Other PrEP and the unmet needs

To improve patient compliance of HIV PrEP, different options and delivery systems which achieved extended duration of protection are being developed. Examples include Apretude®, an injectable product containing cabotegravir, which has been approved by the FDA and the dapivirine vaginal ring which has been approved for use in a number of African countries[31-35]. Currently, Apretude® (CAB 200 mg/mL) is the only extended-release injectable suspension available on the market as HIV PrEP. It is available in a single-dose vial containing 600 mg/3 mL. The utilization of Apretude® as a long-acting PrEP option is administered every two months but requires a clinical visit for dosing which may be challenging in regions with limited access to healthcare. Although this product achieves less frequent dosing there is still need to develop improved and longer duration options for PrEP.

1.4 Long-acting subcutaneous implant for HIV prevention

To improve patient adherence of patients to HIV prevention and to avoid the first pass metabolism of oral delivery, other delivery systems are being developed as the alternatives options. Since sustained release delivery systems can maximize drug efficacy, minimize side effects, provide zero-order drug release kinetics, and enhance patient compliance by decreased frequency of drug administration, the application of long-acting devices would be a satisfactory option for HIV prevention[39].

1.4.1 Long-acting subcutaneous implants

Prior studies demonstrated that a non-visible implant PrEP option was preferred among HIV PrEP formulations, while oral PrEPs were least favored [40]. Implants have been applied for various uses, such as ocular or orthopedic implants, tissue regenerative implantable compositions, intrathecal implant pumps, and subdermal implants[41-45]. Some indications for the employment of implants include hormonal contraception, prostate cancer, and coronary artery disease [46].

Implantable devices can achieve therapeutic effects with lower concentrations of drug, leading to fewer adverse effects. In addition, subcutaneous implants tend to have increased bioavailability compared to oral formulations due to the prevention of hepatic first pass effects and chemical degradation in the stomach and intestine[47]. Moreover, there is a chance for early removal of implants with a minor surgical procedure if urgent termination of treatment is needed [48]. Other benefits of parenteral implants include the use in clinical conditions where oral medications cannot be applied, such as gastrointestinal surgery, malabsorption, or swallowing dysfunction[49].

1.4.1.1 Categories of long-acting subcutaneous implants Matrix-style and reservoir-style implants

The two main categories for subcutaneous implants are matrix (monolithic)-style and reservoir-style implants, shown in **Figure 2**. Matrix-style implants are made from a polymer matrix

in which the active pharmaceutical ingredient (API) is homogeneously dispersed[50]. The drug release is governed by Fickian diffusion, which is impacted by concentration gradient, diffusion distance, and the degree of swelling [51]. For example, Probuphine® is a matrix-style subcutaneous implant that contains buprenorphine distributed through poly(ethylene-vinyl acetate) (EVA) rods, is one of a matrix-type subcutaneous implant for maintenance treatment of opioid addiction[52, 53]. Conversely, reservoir-style implants contain a compact API core encapsulated by a permeable membrane[54]. The release rate of reservoir-type systems is driven by membrane thickness and permeability of the API through the membrane. Nexplanon®, which contains etonogestrel in a rod of EVA-based polymer, is a popular reservoir-type implant for contraceptives on the market [54].

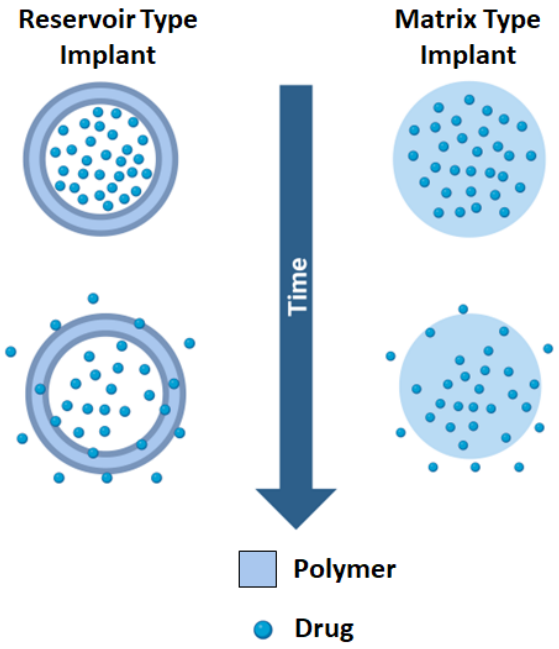


Figure 2 | Illustration of reservoir and matrix type implants.

To further differentiate the type of implants, polymer composition can impact the biodegradability of the formulation. Non-biodegradable implants are made of materials such as

silicones, polyvinyl alcohol (PVA), and poly(ethylene vinyl acetate) (EVA)[55-57]. The general mechanism of drug release from implants is mediated by diffusion through the polymeric matrix, water-mediated transport process, and polymer hydrolysis and erosion. Depending on the style and the materials of implants, water mediated transport properties, polymer hydrolysis and rate of erosion differs[58]. Although non-biodegradable materials demonstrate long-term bio-compatibility, they need to be excised after the drug load is depleted to prevent adverse events.

Biodegradable implants differ in this fashion because they can be broken down, and excreted or absorbed by the human body. In addition, as the polymeric membrane eroded in the body, the rate of drug release changes. These implants are prepared by biodegradable polymers including poly(caprolactone) (PCL), poly(lactic acid) (PLA), and poly(lactic-co-glycolic acid) (PLGA)[50]. This is advantageous because the rate of drug release can be modified by adjusting the degradation kinetics of polymers.

1.4.2 Long-acting biodegradable implants developed by RTI

Dedicated to the achievement of an AIDS-free generation, RTI International has been working on the development of a long-acting implantable system for HIV prevention. Their effects include development of a subcutaneous biodegradable implant for HIV pre-exposure prophylaxis (PrEP) for long-term delivery of tenofovir alafenamdie (TAF). This system exhibited zero-order release kinetics for around 120 days *in vitro*[60]. Also, a long-acting biodegradable implant demonstrating sustained release of 4’-Ethynyl-2-fluoro-2’-deoxyadenosine (EFdA) for 6 months and etonogestrel (ENG) for 12 months *in vivo*, was prepared as an HIV multipurpose prevention

technology (MPT) for HIV prevention and contraception[61]. To develop these long-acting, biodegradable, and implantable devices, RTI International manufactured reservoir-style implants with a biodegradable polymer, PCL, and loaded the implants with different antiretroviral drugs or contraceptives.

1.4.3 Poly(ε-caprolactone) (PCL)

PCL is a synthetic, biodegradable, linear polyester consisting of repeating hexanoate units [62]. This semi-crystalline polymer has a glass transition temperature of -60°C and a melting point between 59°C and 64°C based on the crystallinity[63]. PCL has been widely applied in tissue engineering and drug delivery applications [64, 65]. Due to its viscoelastic and rheological properties, PCL has been used in the fabrication of scaffolds for bone, cartilage, blood vessel, and tendon, with the purpose of tissue repair or replacement [66]. Regarding drug delivery, PCL had been used for implantable biomaterials and injectable implants for controlled release delivery systems. Capronor™ is an example of a PCL-based 1-year contraceptive implantable capsule, which is eliminated from the human body after 2 to 3 years[67]. ther examples of PCL applications include ciprofloxacin-loaded PCL implantable matrices for sustained release, praziquantel-loaded PCL cylindrical implants with long-sustained release rate, TAF within subcutaneous PCL implants as sustained release delivery systems[68-70].

The degradation of PCL is dependent on the molecular weight, degree of crystallinity, and condition for degradation. Two phases are included during the degradation of PCL: First, the amorphous phase is degraded, which increases the degree of crystallinity but retains the molecular

weight[71]. PCL degradation then follows an autocatalytic process, which is the hydrolysis of ester bond. This leads to loss of mass[72]. At high temperatures, PCL is degraded by end chain scission, while at low temperatures, it is degraded by random chain scission [73]. The properties of PCL are superior for drug delivery systems because of its specific structure, which is composed of five methylenes and one ester group (**Figure 3.**). The characteristics include strong hydrophobicity, slow hydrolysis, resulting in comparably slower degradation than other polymers, and good biocompatibility[69]. CL is highly permeable to small molecules and does not generate acidic environment that might adversely impact drugs. Therefore, PCL has been applied to various drug delivery devices, including micro-/ nano-particles, films, hydrogels, and implants[64, 74-76].

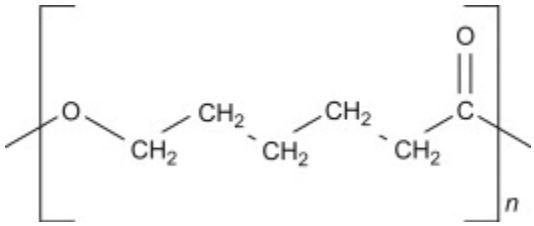


Figure 3 | Structure of Poly(ε-caprolactone) (PCL).

1.4.4 Long-acting PCL implants

As previously mentioned, PCL is biocompatible, biodegradable in physiological conditions, and has good mechanical integrity for shaping and manufacturing[14]. Therefore, PCL is suitable for design of subcutaneous implants that do not need to be removed in the end of the therapy.

Since the mechanism of drug release from implants includes diffusion through the polymeric matrix, water-mediated transport processes, polymer hydrolysis, and erosion[58], the release kinetics of reservoir-based architectural PCL implants is governed by polymer properties.

These include composition, molecular weight, and membrane thickness. Physicochemical properties of API such as solubility, drug particle size, and the molecular weight are also important to consider for optimizing drug release [38, 69, 77].

According to the laws of Fickian diffusion, drug release rate was found to be inversely proportional to the wall thickness and diameter of tubes. Increased wall thickness and diameter of tubes demonstrated slower drug release because of longer diffusion path for drug diffusion from PCL implants[38]. Meanwhile, the release rate showed proportional correlation with surface area, indicating the membrane-controlled release. More drug load also showed faster drug release. Moreover, smaller crystalline size and higher mass % crystallinity within PCL would slow down the diffusion of API through PCL.

1.5 Project hypothesis and objective

Long-acting subcutaneous implants offer advantages over injections for HIV prevention due to increased patient adherence over long periods of time, possibility for early removal, and biocompatibility. PCL is a biocompatible and biodegradable material in physiological conditions with good mechanical integrity for shaping and manufacturing, making it suitable for use in design of long-term released subcutaneous implants that do not require removal[14]. It is optimal for subcutaneous devices to be small in size. This limits the overall capacity for drug loading and thus highly potent drugs are more compatible with this technology. Given the high potency of BIC it is an excellent drug candidate for incorporation into an implantable device. By combining BIC and

PCL, a small, discrete, biodegradable subcutaneous implant that provides effective protection against HIV can be developed.

According to biopharmaceutics classification system (BCS), BIC is classified as a BCS class II compound, showing low solubility and high permeability. In this work, BIC was mixed with castor oil (CO), which will act as an emulsifying and solubilizing agent, and helps improve the solubility of BIC[78]. Drug release from reservoir-based architectural PCL implants follows a membrane-controlled release mechanism, where drug dissolution and diffusion through the PCL membrane predominantly govern release kinetics[79]. Since the bulk erosion of PCL is slow, at the early stages the rate of drug delivery is decoupled from biodegradation. By encapsulating BIC in a PCL implant, a consistent drug release profile can be achieved, which overcomes solubility challenges.

This project builds upon previous studies conducted by RTI International on biodegradable implants [38]. The overarching goal of the larger project is to develop a long-acting biodegradable PrEP implant which incorporates the antiretroviral drug bictegravir (BIC). The implant is intended to have controlled drug release, providing a sustained release of BIC over a period of 6 months to 1 year. This implant was designed by RTI International as a subcutaneous reservoir-style delivery system for HIV prevention which demonstrates zero-order release kinetics.

To facilitate future manufacturing activities to support preclinical and clinical studies, MWRI is being utilized as the secondary site for product manufacturing. This thesis focuses on the characterization of the BIC-loaded PCL implants and the validation of manufacturing processes to support technology transfer activities. The following specific objectives were proposed to accomplish the established goal:

Objective 1: To qualify the analytical methods for Bictegravir using high-performance liquid chromatography (HPLC) The methods will be used to characterize BIC-loaded PCL implants, determining BIC content in the device, and to quantify BIC in dissolution studies to assess in-vitro drug release profiles of the implants.

Objective 2: To replicate the manufacturing process developed by RTI and conduct a cross-lab comparison of long-acting BIC-loaded PCL subcutaneous implants prepared at both labs. Parameters, including drug content, content uniformity, and in-vitro drug release, will be characterized to assess the transferability of methods and identify potential process parameters that could impact product quality.

2.0 Qualification of High Performance Liquid Chromatography (HPLC) methods

2.1 Introduction

2.1.1 High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is an essential analytical chemistry technique to assess drug products, including separation, identification, and quantification of compounds in a chemical mixture. The pharmaceutical application of HPLC contains potency/ purity/ performance assays, pharmacokinetics/ bioanalytical testing, purification, high-throughput screening, and quality control testing[80]. By injecting the sample into a column filled with porous materials, known as the stationary phase, the mobile phase is pumped through the column at high pressure. The separation of the sample is based on the differences in the rates of migration through the column, which arises because of different partition of the sample between the stationary and mobile phases[81]. Sample compounds that have higher affinity to the stationary phase would move at a slower pace, while those with less affinity would move faster[82]. The high performance refers to high speed or high resolution separations, achieved by utilizing small particles in the column packing. The smaller the particle size is, the higher pressure it is needed to push the eluent through the column at a specific flow rate. Therefore, HPLC is sometimes mentioned as high pressure liquid chromatography.

There are several classifications of chromatography based on the mode of separation. Normal phase chromatography (NP-HPLC) utilizes polar (hydrophilic) stationary phase and non-

polar (hydrophobic) mobile phase, reverse phase chromatography (RP-HPLC) achieves separation using non-polar stationary phase and polar mobile phase, size exclusion chromatography (SEC) uses size differences between analytes, and ion-exchange chromatography (IC) relies on charge of the analyte and stationary phase[83, 84]. Of the different forms of HPLC, reversed phase HPLC (RP-HPLC) is most commonly used one. In RP-HPLC, the mobile phase is more polar than the stationary phase. A lipid-like or non-polar stationary phase is covalently bonded to the solid support, for example, C4, C8, C18 and phenyl columns are usually used. Also, a mobile phase comprising of buffered aqueous and water-miscible organic modifier such as methanol or acetonitrile are employed, which are safe and readily available[85].

2.1.2 Qualification of analytical procedures

There are several types of analytical procedures, the most common types include identification tests, quantitative tests for determination of impurities, limit tests for the control of impurities, and quantitative tests of the active moiety in samples of drug substance or drug product. To demonstrate the suitability of an analytical procedure for its intended purpose, method validation or qualification is critical. Validation and qualification were mentioned in reference to International Council for Harmonization (ICH) 7 Good Manufacturing Practice Guidance for Active Pharmaceutical Ingredients Guidance for Industry September 2016 and FDA 2011 Guidance for Industry – Process Validation: General Principles and Practices[86, 87]. Validation was defined as a documented program, providing a high degree of assurance that a specific process, method, or system will consistently produce a result meeting predetermined acceptance criteria.

On the other hand, qualification states the action of proving and documenting that equipment is properly installed, works correctly, and actually leads to the expected results, which is part of validation. While validation is more complex and time-consuming, qualification focuses on ensuring that equipment and processes would function properly before being applied to the determination of drug product.

According to Analytical Procedures and Methods Validation for Drugs and Biologics (Guidance for Industry), issued by Center for Drug Evaluation and Research and Center for Biologics Evaluation and Research, validation characteristics such as accuracy, precision, specificity, limits of detection (LOD) and limits of quantitation (LOQ), linearity, and range would be assessed during method development to evaluate the robustness of an analytical process[88, 89]. Specificity is the extent to which a method can determine a particular analyte in a mixture without interferences from other components which may be expected to present. Accuracy of an analytical method demonstrated the closeness of agreement between the mean of a set of measurements and the true value of analyte concentration. Precision indicated the degree of scatter between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed condition, which can be described as repeatability, intermediate precision, and reproducibility. Limit of detection (LOD) is the lowest concentration level which can be determined significantly different from a blank at a specified level of confidence but not necessarily quantitated as an exact value, while limit of quantitation (LOQ) is the lowest amount of analyte in a sample above which quantitative results can be determined with the specified degree of accuracy and precision[90, 91]. Linearity is the ability to elicit test results directly proportional to the concentration of analyte within a given range.

2.1.3 HPLC methods for bictegravir (BIC) determination in this study

Since the preparation of the bictegravir (BIC) formulation and drug product was still under exploration with a limited scope, qualification rather than validation of the analytical methods for BIC determination was considered to be more appropriate for this work. In this study, two RP-HPLC methods were developed for different purposes and were both qualified. Bictegravir is classified as BCS classs II, showing low solubility and high permeability, with log P value 2.7. For the characterization of BIC formulation and final drug product, one HPLC method is an assay procedure expected to be a quantitative measurement of BIC. The other HPLC method is for the dissolution study, investigating the drug release profile of BIC from a drug product. To qualify the assay procedure and the method for dissolution study, the specificity, accuracy, precision, LOD and LOQ, linearity, and range were conducted[92]. Besides, pre-formulation studies including stock solution stability and filtration validation were also confirmed.

2.2 Materials

BIC API (Catalog# N16998) with 99.72% purity was manufactured by AstaTech Inc, shipped by RTI International and stored in a plastic container sealed with Parafilm at 4°C refrigeration when not in use. Castor oil (Catalog# SR40890) was acquired from Croda International (USA). Hypersil GOLD™ HPLC Column, Column Format=Analytical, I.D. x L=4.6 x 150 mm, Phase=C18, Pore Size=175 Å, Packing Material=Silica, Particle Size=5 µm was

obtained from Thermo Scientific (Pittsburgh, PA, USA). OPTIMA™ LC-MS grade Acetonitrile and HPLC grade Methanol were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). HPLC grade (≥99.0%) Trifluoroacetic acid was provided by Sigma-Aldrich (Sigma-Aldrich, USA). Phosphate-Buffered Saline (PBS) (10x) pH 7.4 was purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). HPLC grade (≥99.%) dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (Sigma-Aldrich, USA). Milli-Q water was obtained from the Milli-Q ® system (Millipore Sigma Advantage 10), installed in the laboratory.

2.3 Methods

As mentioned above, two HPLC methods, with the same mobile phase gradient program but different matrices, were conducted for BIC determination. One method was for assay procedure for BIC quantitation in drug product, and the other was for dissolution study. In the HPLC system, 0.01% (v/v) trifluoroacetic acid and 100% acetonitrile were employed because of their volatility (easily removed from collected fractions) and their little UV absorption at low wavelength, which means they would not be detected in the range of the analyte being detected. In terms of assay procedure, the calibration curve had BIC at the concentration range of 1 µg/mL to 100 µg/mL, while for dissolution study, the calibration curve was within the concentration of 0.417 µg/mL to 50 µg/mL.

2.3.1 Preparation of mobile phase

The mobile phase of the HPLC method for BIC analyis was comprised of 0.01% (v/v) trifluoroacetic acid in water and 100% Acetonitrile. 0.01% trifluoroacetic acid was prepared by dissolving 200 µL of >99% trifluoroacetic acid in 2 L of Milli-Q water.

2.3.2 Preparation of standard solution

2.3.2.1 Assay procedure for BIC quantitation in drug product

To prepare the primary stock solution with 500 µg/mL BIC in 100% Methanol, 10 mg of BIC was accurately weighed and transferred to a 20 mL volumetric flask. 100% Methanol was then added to reach the calibration line on the 20 mL volumetric flask. The solution was vortexed and sonicated for 20 minutes to make sure BIC was completely dissolved in 100% methanol. A secondary working stock solution with 200 µg/mL BIC was prepared from the primary stock, with 720 µL of 500 µg/mL BIC in 100% methanol primary stock being diluted by 1080 µL of 50% methanol in water. The weights of both primary stock solution and 50% Methanol diluent were recorded to calculate the concentration of secondary stock by the weights and densities of 100% Methanol (0.791 g/mL) and 50% methanol in water (0.916 g/mL). The range of calibration curve was from 1 µg/mL to 100 µg/mL. The calibrators were prepared from the Bictegravir 200 µg/mL secondary stock solution, and serially diluted with 50% methanol, as shown in **Table 1**. By plotting the theoretical concentrations of BIC versus the area under the curve (AUC, µV*sec), the amount of BIC in samples could be analyzed by the calculation based on the calibration curve.

Table 1 | Calibrators of the calibration curve for analysis of BIC.

Standard solution	BIC concentration (µg/mL)	Concentration of BIC stock solution (µg/mL)	BIC stock solution (µL)	Diluent: 50% Methanol (µL)	Total volume (µL)
8	100	200	600	600	1200
7	75	100	750	250	1000
6	50	75	667	333	1000
5	25	50	500	500	1000
4	10	25	400	600	1000
3	5	10	500	500	1000
2	2	5	400	600	1000
1	1	2	500	500	1000

2.3.2.2 Method for BIC determination in dissolution study

The stock solution with 50 µg/mL BIC in 1x PBS was comprised of 10 mg of BIC in 200 mL of 1x PBS. To prepare the stock solution, 10 mg of BIC was accurately weighed and transferred to a 200 mL volumetric flask. 1x PBS was prepared by 10 times dilution from 10x PBS, and then added to reach the calibration line on the 200 mL volumetric flask. To dissolve BIC thoroughly in 1x PBS, the solution in the volumetric flask was stirred with magnetic stir overnight, covered by aluminum foil to prevent it from light. A secondary stock solution with 12.5 µg/mL BIC in 1x PBS was prepared by directly diluting 150 µL of the primary stock with 1650 µL of 1x PBS. The range of the calibration curve was between 0.417 µg/mL and 50 µg/mL, with calibrators directly being diluted from the both 50 µg/mL primary stock solution and 12.5 µg/mL secondary stock solution, as presented in **Table 2**. The plot with theoretical concentrations of BIC in 1x PBS versus the area

under the curve (AUC, µV*sec) allowed the measurement of BIC amount in dissolution study by calculation according to the calibration curve.

Table 2 | Calibrators of the calibration curve for dissolution study.

Standard solution	BIC concentration (µg/mL)	Concentration of BIC stock solution (µg/mL)	BIC stock solution (µL)	Diluent: 1x PBS (µL)	Total volume (µL)
1	0.417	12.5	60	1740	1800
2	0.694	12.5	100	1700	1800
3	1.042	12.5	150	1650	1800
4	2.083	50	75	1725	1800
5	4.167	50	150	1650	1800
6	6.944	50	250	1550	1800
7	12.5	50	450	1350	1800
8	25	50	900	900	1800
9	50	50	1800	0	1800

2.3.3 HPLC system and chromatographic conditions for BIC determination

The chromatographic conditions of the RP-HPLC methods were presented in **Table 3**. A high performance liquid chromatography (HPLC) system (Waters Alliance e2695) equipped with an autosampler, a quaternary pump controller, and a photo diode array (PDA) detector was utilized to quantify BIC. The gradient method used a reverse phase chromatography with a Thermo Fisher Hypersil Gold column (4.6 x 150 mm) at a wavelength of 320 nm. The retention time of BIC is 15 minutes, within a total of 40-minute run time. The mobile phase consisted of A) 0.01%

Trifluoroacetic acid and B) 100% acetonitrile, with a flow rate at 0.8 mL/min. The gradient program of mobile phase was 0 min-5% MPB, 25 min-90% MPB, 25.1 min-100% MPB, 35 min-100% MPB, 35.1 min-5% MPB (**Table 4**), and the injection volume was 10 µL. Empower software was applied to capture the results generated by the HPLC system. The temperature of the autosampler was set at 4°C, and the column temperature was maintained at 25°C. An extended needle wash with 80% methanol was utilized to decrease BIC carry-over between injections.

Table 3 | Chromatographic conditions for RP-HPLC analysis of BIC

HPLC system	Water Alliance 2695
Column	Thermo Fisher Hypersil Gold, 4.6 x 150 mm
Mobile phase	A: 0.01% Trifluoroacetic acid in water B: 100% Acetonitrile
UV Wavelength	320 nm
Injection volume	10 µL
Flow rate	0.8 mL/min
Retention time	15 min
Run time	40 min
Autosampler temperature	4°C
Column temperature	25°C
Extended needle wash	80% Methanol
Column storage	70% Methanol

Table 4 | Gradient program used for analysis of BIC.

Time (in min)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0	0.8	95	5
25	0.8	10	90
25.1	0.8	0	100
35	0.8	0	100

35.1	0.8	95	5

2.3.4 Qualification of analytical method

2.3.4.1 Specificity

The specificity of the two methods were assessed to demonstrate whether there is interference caused by excipient and the ability to differentiate the active analyte from other components presenting in the same sample matrix. The assessment was presented by UV detection and peak purity determination.

2.3.4.1.1 Assay procedure for BIC quantitation in drug product

To qualify the specificity of this assay procedure, 0.01% TFA in water as mobile phase, 50% methanol as the blank, castor oil alone being the excipient, 50 µg/mL of BIC in 50% methanol as the drug substance, and the combination of 5:1 BIC: castor oil (CO) in 50% methanol considering to be the drug product were analyzed in order as mentioned.

For the analysis of CO alone, 100 µg/mL of castor oil in 50% methanol was prepared by weighing 5 mg of castor oil in a 50 mL volumetric flask, followed by the addition of 50% methanol to the calibration line. The solution was vortexed and sonicated for 20 minutes, and then filtered with syringe filter PTFE (0.22 µm, 13 mm diameter) by discarding the first 4 drops and collecting the remaining. The sample of 5:1 BIC:CO in 50% methanol was prepared by the combination of 500 µg/mL of BIC in 100% methanol stock solution and 100 µg/mL of castor oil in 100% methanol. The mixture was then produced by 20 minutes of vortex and sonication.

2.3.4.1.2 Method for BIC determination in dissolution study

The specificity of this method for BIC determination in dissolution study was conducted through a set of samples. These included 0.01% TFA in water being the mobile phase, 1x PBS being the blank, castor oil alone in 1x PBS being the excipient, BIC in 1x PBS being the drug substance, and the mixture of BIC and COl in 1x PBS at a 5:1 ratio being the drug product.

To prepare the excipient sample, 10 µg/mL of CO, was diluted from 100 µg/mL of CO in 1x PBS, which involved dissolving 5 mg of castor oil in 50 mL of 1x PBS, followed by vortexing and sonication for 20 minutes, and then filtering with syringe filter PTFE (0.22 µm, 13 mm diameter). The drug product sample was prepared by mixing 50 µg/mL BIC in 1x PBS stock solution with 10 µg/mL CO in 1x PBS at a 5:1 ratio. All samples were analyzed in the following order: mobile phase, blank, excipient, drug substance, and drug product samples, each with two injections.

2.3.4.2 Accuracy

The accuracy of both the assay procedure for BIC quantification and the method for BIC determination in dissolution study was determined as recommended by ICH. This included a minimum of 9 determinations over a minimum of 3 concentration levels, and the acceptance criteria were set to be between 90% and 110% of % recovery, with % RSD < 5%, met for all three levels.

2.3.4.2.1 Assay procedure for BIC quantitation in drug product

To qualify the accuracy of this assay procedure for drug substance, the determination of drug substance, BIC, was assessed with BIC in 50% methanol at three different levels (3 µg/mL, 40 µg/mL, and 80 µg/mL), with triplicate preparation at each level. The samples were serially diluted from the 200 µg/mL BIC in 50% methanol secondary stock solution, as shown in **Table 5**.

Table 5 | Preparation of samples for evaluating the drug substance accuracy of the assay procedure for BIC quantitation.

BIC concentration (µg/mL)	Concentration of BIC stock solution (µg/mL)	BIC stock solution (µL)	Diluent: 50% methanol (µL)	Total volume (µL)
80	200	400	600	1000
40	80	500	500	1000
3	40	75	925	1000

On the other hand, the accuracy of drug product was evaluated by the 5:1 BIC to CO mixture. Samples were prepared from a secondary stock solution of 200 µg/mL of Bictegravir and 40 µg/mL of castor oil in 50% methanol, which was diluted from the combination of 500 µg/mL of BIC and 100 µg/mL of CO in 100% methanol, as presented in **Table 6**. Since the repeatability was already validated, duplicate injections were done for each sample.

Table 6 | Preparation of samples for evaluating the drug product accuracy of the assay procedure for BIC quantitation.

BIC concentration (µg/mL)	Concentration of BIC in stock solution (µg/mL)	Concentration of CO in stock solution (µg/mL)	Stock solution (µL)	Diluent: 50% methanol (µL)	Total volume (µL)

80	200	40	400	600	1000
40	80	16	500	500	1000
3	40	8	75	925	1000

2.3.4.2.2 Method for BIC determination in dissolution study

In terms of the method for BIC determination in dissolution study, the accuracy of drug substance and drug product were qualified with BIC alone or 5:1 BIC:CO mixture in 1x PBS at three levels (2.77 µg/mL, 10.00 µg/mL, 20.00 µg/mL), with triplicate preparation at each level. The drug substance samples were parallel diluted from the 50 µg/mL BIC in 1x PBS stock solution, and the drug product samples were prepared with both 50 µg/mL BIC in 1x PBS and 10 µg/mL CO in 1x PBS stock solutions, as shown in **Table 7** and **8**. Each samples were analyzed by HPLC with duplicate injections being done.

Table 7 | Preparation of samples for evaluating the drug substance accuracy of method for BIC determination in dissolution study.

BIC concentration (µg/mL)	Concentration of BIC stock solution (µg/mL)	Volume of BIC stock solution (µL)	Volume of diluent: 1x PBS(µL)	Total volume (µL)
2.77	50	100	1700	1800
10.00	50	360	1440	1800
20.00	50	720	1080	1800

Table 8 | Preparation of samples for evaluating the drug product accuracy of method for BIC determination in dissolution study.

BIC concentration (µg/mL)	Concentration of BIC in stock solution (µg/mL)	Concentration of CO in stock solution (µg/mL)	Stock solution (µL)	Diluent: 50% methanol (µL)	Total volume (µL)
2.77	50	10	100	100	1600
10.00	50	10	360	360	1080
20.00	50	10	720	720	360

2.3.4.3 Precision: Repeatability

Repeatability indicates the precision under the same operating conditions over a short period of time, which should be evaluated with a minimum of nine determinations covering the specified range for this process or with a minimum of six determinations at 100% of the test concentration. The relative standard deviation (% RSD, coefficient of variation), with the acceptance criterion set at an RSD $\leq$ 2%, was reported to determine the precision of the analytical procedure. In this study, the repeatability of both the assay procedure and the analytical method for dissolution study was determined.

2.3.4.3.1 Assay procedure for BIC quantitation in drug product

For the evaluation of the repeatability of this assay procedure, six different solution samples, which were the extraction from 5:1 BIC:CO formulation paste, considered as drug product, were determined. To prepare the extract of drug product, 24 mg of 5:1 BIC:CO

formulation paste was transferred to a 20 mL scintillation vial, following with the addition of 16 mL of DMSO to dissolve BIC. The final extract was composed of thoroughly dissolved BIC in DMSO by vortex and sonication. The HPLC samples were then prepared from 80 µL of the final mixture diluted with 920 µL of 50% methanol. The theoretical concentrations of the samples should be around 100 µg/mL. After filtration with PTFE syringe filter (0.22 µm, 13 mm diameter), a total of 18 injections with triplicate injections in each sample were conducted during HPLC analysis.

2.3.4.3.2 Method for BIC determination in dissolution study

To qualify the repeatability of the method for dissolution study, six sets of 5:1 BIC:CO sample, with three injections for each sample, were analyzed by HPLC. To prepare the samples, stock solutions of 10 µg/mL CO in 1xPBS and 50 µg/mL BIC in 1xPBS were first prepared. The repeatability samples were then created by combining 180 µL of BIC stock, 180 µL of CO stock, and 1440 µL of 1xPBS diluent. This resulted in samples containing 5 µg/mL of BIC and 1 µg/mL of CO in 1xPBS.

2.3.4.4 Limit of detection (LOD) and limit of quantitation (LOQ)

The determination of signal-to-noise (S/N) ratio was performed by the comparison of measured signals from samples containing known low concentrations of analyte with those of blank samples. Limit of detection (LOD) was generally defined as 3 times of the noise level, and an S/N ratio between 3 or 2 is generally considered acceptable for the estimation of LOD. Limit

of quantitation (LOQ) was normally defined as 10 times of the noise level, with the acceptance criterion set to be S/N ratios ≥ 10.

2.3.4.4.1 Assay procedure for BIC quantitation in drug product

To determine LOD and LOQ of BIC for this assay procedure, decreasing concentration of BIC in 50% methanol were used. The LOD and LOQ were estimated in accordance with the S/N ratios of a level with 6 injections, n = 2 injections with 3 different vials of BIC in 50% methanol sample at the same level.

2.3.4.5 Linearity

Recommended by ICH, the linearity was evaluated by visual inspection of a plot of signal as a function of analyte concentration. To evaluate the linearity, the correlation efficient, y-intercept, and slope of the regression line were submitted, with a minimum of 5 concentrations. Here, the linearity was qualified by linear regression coefficient of determination (R squared), with the acceptance criteria of R squared greater than or equal to 0.995.

2.3.4.5.1 Assay procedure for BIC quantitation in drug product

The linearity of the assay procedure was determined by regression curves in triplicates on three individual days, in which duplicate injections were performed. Of the working range with 1 µg/mL to 100 µg/mL, the mid-point was set at the target level, 50 µg/mL, with the lowest calibrator, 1 µg/mL, lower than one-half of the target, and the highest calibrator, 100 µg/mL, at twice of the target level.

2.3.4.5.2 Method for BIC determination in dissolution study

With regard to the analytical method for BIC determination in dissolution study, the linearity was also qualified by three regression curves on three different days, with duplicate injections conducted. The working range of the regression curves was between 0.417 µg/mL and 50 µg/mL.

2.3.5 Pre-formulation study

2.3.5.1 Filtration validation

Since the formulation was composed of BIC and CO, a syringe filter would be needed for HPLC sample preparation to avoid the damage of CO to the equipment. Therefore, filtration validation was conducted to test the compatibility of BIC with syringe filter PTFE (0.22 µm, 13 mm diameter) as well as the impact of filter to the % recovery rate. To perform filtration validation, the solution of 5:1 BIC:CO in 50% methanol was prepared by the combination of 500 µg/mL of Bictegravir in 100% methanol stock solution and 100 µg/mL of CO in 100% methanol solution and diluted by 50% methanol. 1 mL and 2 mL of the mixed solution was collected by 1 mL syringes and filtered with PTFE (0.22 µm, 13 mm diameter) filter. The solution was first discarded 4 drops and the filtered content was then transferred to HPLC vials for HPLC analysis. The accuracy of the % recovery of sample determination was demonstrated to assess the filtration validation.

2.3.5.2 Stock solution stability

2.3.5.2.1 Assay procedure for BIC quantitation in drug product

The stability of 500 µg/mL BIC in 100% Methanol standard stock solution at refrigeration condition, at the temperature between 2°C to 8°C, was tested. To test the stability of the stock solution for this assay procedure, 100 µL of 500 µg/mL BIC in 100% methanol stock was diluted by 900 µL of 50% methanol for sample preparation, with the theoretical concentration at 50 µg/mL of BIC. On the other hand, to test the stability of drug product solution, the combination of 5:1 BIC:CO in 50% methanol was prepared into 3 different concentration levels (3 µg/mL, 40 µg/mL, and 80 µg/mL) and saved in the HPLC autosampler at the temperature of 4°C. After several periods of time, the samples were then determined by the HPLC assay. The stability was validated by the comparison between the determination of fresh stock solutions and those saved for a period of time, at the acceptance criteria of the % difference of peak area being less than 5.

2.3.5.2.2 Method for BIC determination in dissolution study

To test the stability of the standard stock solution of 50 µg/mL BIC in 1x PBS, the solution was prepared by dissolving 10 mg of BIC API in 200 mL of 1x PBS. The resulting solution was then stored in a glass bottle at refrigeration temperature and analyzed by HPLC at different time points over a period of 2 weeks. On the other hand, the stability of drug product solutions, which were prepared following the procedure of the accuracy samples (refer to **Table 8**), was assessed at two different temperatures (refrigeration and 37°C). One set of the samples was stored in HPLC vials at refrigeration, while the other was stored in scintillation vials at 37°C in an incubator and

shaken at 100 rpm. HPLC analysis was performed at various time points up to 1 week. The acceptance criterion for stability was set as a % difference of peak area less than 5%.

To investigate the stability of the drug product in different containers at 37°C, solutions containing 5:1 BIC:CO in 1xPBS at 2.77 µg/mL, 20 µg/mL, and 50 µg/mL were stored in polypropylene (PCOO) bottles, scintillation vials, and silanized glass vials, with triplicates of each container type. The time points were taken up to 7 days, and the peak area at each time point was compared to the AUC of fresh solution. Moreover, to test the drug product stability in different matrices, a solution of 2.77 µg/mL 5:1 BIC:CO was prepared in both 1xPBS and a 1:1 mixture of 1xPBS and 100% methanol. For the preparation of 2.77 µg/mL drug product in the 1:1 1xPBS:100% methanol mixture, a 5.55 µg/mL 5:1 BIC:CO solution was first prepared by diluting 1 mL of 50 µg/mL of BIC and 10 µg/mL of CO in 1xPBS mixture with 8 mL of 1xPBS. This solution was then further diluted with 9 mL of 100% methanol, as shown in **Table 9**. The resulting solution was aliquoted into PCOO bottles and scintillation vials, with triplicates for each container material.

Table 9 | Preparation of drug product solution in 1:1 1xPBS and 100% methanol mixture for stablity study.

BIC concentration (µg/mL)	Concentration of BIC in stock solution (µg/mL)	Concentration of castor oil in stock solution (µg/mL)	Stock solution (mL)	Diluent: 1xPBS (mL)	Diluent: 100% MeOH (mL)	Total volume (mL)
2.77	50	10	1	8	9	18

2.4 Results

2.4.1 Qualification of analytical method

2.4.1.1 Specificity

The specificity was demonstrated by both UV detection according to the chromatograms at a wavelength of 320 nm and the peak purity assessment determined by the purity angle and the threshold angle. The purity angle is the average value of the angle between each spectrum of the peak and the spectrum at the top of the peak, and the threshold angle is an index value indicating the effect of the noise over the entire peak[93]. If the purity angle is less than the threshold angle, it can be determined that there is no obvious co-elution within the range of the threshold angle that indicates the effect of the noise.

2.4.1.1.1 Assay procedure for BIC quantitation in drug product

The chromatograms of 0.01% TFA in water, 50% methanol, CO alone, 50 µg/mL of BIC in 50% methanol, and 5:1 BIC:COin 50% methanol were detected at a wavelength of 320 nm. As shown in **Figure 4**, no peak other than the BIC main peak at 15-minute retention time in the castor oil sample and the combination of 5:1 BIC to CO, demonstrating that the assay result of BIC was unaffected by the presence of castor oil in the same sample matrix. From the HPLC system equipped with PDA detector, the purity angle and the threshold angle were indicated in every sample analysis. The combination of BIC and CO sample had the purity angle = 0.028, and the threshold angle = 0.223, which further confirmed no apparent co-elution with BIC during the determination of the sample.

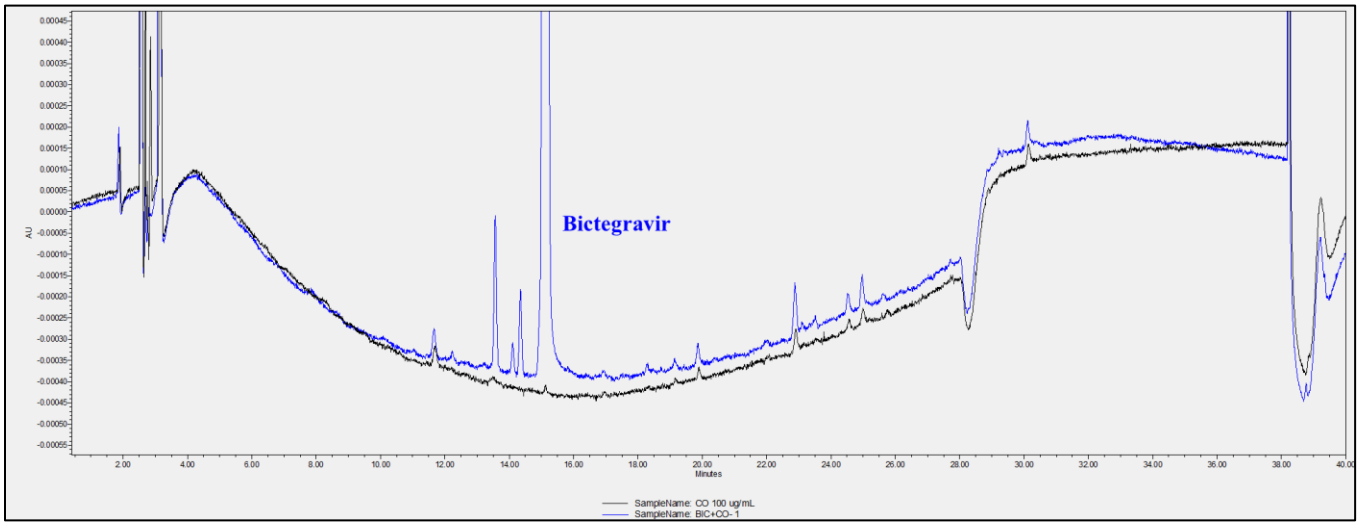


Figure 4 | Chromatogram of the comparison of 100 µg/mL CO in 50% methanol (black) and 5:1 BIC:CO in 50% methanol.

2.4.1.1.2 Method for BIC determination in dissolution study

For the method of BIC determination in dissolution study, the chromatograms of 0.01% TFA in water, 1x PBS, castor oil alone, 50 µg/mL of BIC in 1x PBS, and 5:1 BIC:CO in 1xPBS were detected at a wavelength of 320 nm. As demonstrated in **Figure 5**, the BIC main peak at 15-minute retention time only presented in the mixture of 5:1 BIC to CO but no in the castor oil sample alone. This indicated that the determination of BIC in this method was not impacted by the existence of CO in the same sample matrix. Based on the HPLC system equipped with PDA detector, the mixture of BIC and CO had the purity angle = 0.023, and the threshold angle = 0.220, which further determined that there was no apparent co-elution with BIC during the analysis of the sample.

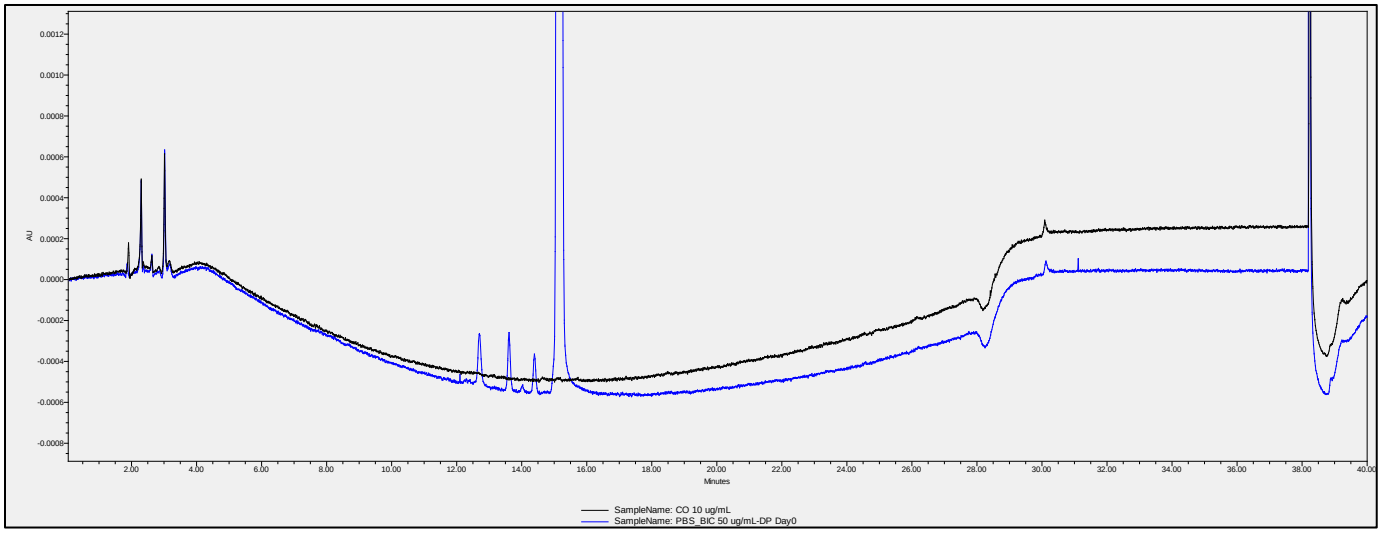


Figure 5 | Chromatogram of the comparison of 10 µg/mL castor oil in 50% methanol (black) and 5:1 BIC:CO in 50% methanol.

2.4.1.2 Accuracy

2.4.1.2.1 Assay procedure for BIC quantitation in drug product

The accuracy of drug substance of the assay procedure was qualified by three concentration levels of 3 µg/mL, 40 µg/mL, and 80 µg/mL of BIC samples in duplicate injections. On the other hand, the accuracy of drug product was also determined by the extracts of 5:1 BIC:CO formulation. The % recovery of all determinations of BIC for both drug substance and drug product fell within 90% to 110%, with %RSD of the three levels less than 5%, confirming the accuracy of this assay method for BIC quantification, as shown in **Table 10** and **11**.

Table 10 | Accuracy of drug substance of the assay procedure for BIC quantitation.

Sample	Theoretical concentration (µg/mL)	Average area	Actual concentration (µg/mL)	% Recovery
Acc L – 1	3.0	35834	3.1	102.6
Acc L – 2	3.0	35352	3.0	101.3
Acc L – 3	3.0	35418	3.0	101.4
Average_L	3.0	35534	3.1	101.8
% RSD_L	0.0	0.73	0.69	0.69
Acc M – 1	40.0	493510	40.0	99.9
Acc M – 2	40.0	488233	39.5	98.9
Acc M – 3	40.0	488791	39.6	99.0
Average_M	40.0	490178	39.7	99.3
% RSD_M	0.0	0.59	0.59	0.59
Acc H – 1	80.0	995367	80.4	100.5
Acc H – 2	80.0	994470	80.4	100.4
Acc H – 3	80.0	1007232	81.4	101.7
Average_H	80.0	999023	80.7	100.9
% RSD_H	0.0	0.71	0.71	0.71
Overall Average				100.6
Overall % RSD				**1.24**

Table 11 | Accuracy of drug product of the assay procedure for BIC quantitation.

Sample	Theoretical concentration (µg/mL)	Average area	Actual concentration (µg/mL)	% Recovery
Acc L – 1	3.0	33302	3.2	106.1
Acc L – 2	3.0	33297	3.2	106.1
Acc L – 3	3.0	32899	3.1	104.9

Average_L	3.0	33166	3.2	105.7
% RSD_L	0.0	0.69	0.64	0.64
Acc M – 1	39.9	454539	40.0	100.2
Acc M – 2	39.9	455759	40.1	100.4
Acc M – 3	39.9	456710	40.1	100.6
Average_M	39.9	455669	40.1	100.4
% RSD_M	0.0	0.24	0.24	0.24
Acc H – 1	79.78	920708	80.7	101.1
Acc H – 2	79.78	916228	80.3	100.6
Acc H – 3	79.78	919197	80.5	100.9
Average_H	80.0	918711	80.5	100.9
% RSD_H	0.0	0.25	0.25	0.25
Overall Average				102.3
Overall % RSD				**2.86**

2.4.1.2.2 Method for BIC determination in dissolution study

The accuracy of method for BIC determination in dissolution study was assessed at three levels (2.77 µg/mL, 10 µg/mL, and 20 µg/mL) of BIC samples performed in duplicate injections and triplicate injections for drug substance and drug product, respectively. The %recovery of BIC for both drug substance and drug product (5:1 BIC:CO) samples fell within 95% to 105%, with a %RSD of the three concentration levels less than 2%, as presented in **Table 12** and **13**. This confirmed the accuracy of this method for BIC quantification in dissolution study.

Table 12 | Accuracy of drug substance of the method for BIC determination in dissolution study.

Sample	Theoretical concentration (µg/mL)	Average area	Actual concentration (µg/mL)	% Recovery
Acc L – 1	3.23	31309	3.19	101.3
Acc L – 2	3.23	31198	3.26	100.9
Acc L – 3	3.23	30488	3.19	99.5
Average_L	3.23	30998	3.24	100.3
% RSD_L	0.0	1.62	1.32	1.33
Acc M – 1	11.62	116074	11.62	100.0
Acc M – 2	11.62	116043	11.45	98.6
Acc M – 3	11.62	117180	11.56	99.5
Average_M	11.62	116987	11.54	99.3
% RSD_M	0.0	0.74	0.72	0.72
Acc H – 1	23.24	240280	23.45	100.9
Acc H – 2	23.24	238403	23.27	100.1
Acc H – 3	23.24	236485	23.09	99.3
Average_H	23.24	238389	23.27	100.1
% RSD_H	0.0	0.80	0.79	0.79
Overall Average				99.9
Overall % RSD				**0.53**

Table 13 | Accuracy of drug product of the method for BIC determination in dissolution study.

Sample	Theoretical concentration (µg/mL)	Average area	Actual concentration (µg/mL)	% Recovery
Acc L – 1	3.23	30538	3.19	99.0

Acc L – 2	3.23	30026	3.15	97.4
Acc L – 3	3.23	31451	3.28	101.7
Average_L	3.23	30671	3.21	99.4
% RSD_L	0.0	2.35	2.17	2.17
Acc M – 1	11.62	116074	11.46	98.6
Acc M – 2	11.62	116362	11.48	98.8
Acc M – 3	11.62	116809	11.53	99.2
Average_M	11.62	116415	11.49	98.9
% RSD_M	0.0	0.32	0.31	0.31
Acc H – 1	23.24	238225	23.25	100.1
Acc H – 2	23.24	238764	23.31	100.3
Acc H – 3	23.24	238541	23.28	100.2
Average_H	23.24	238510	23.28	100.2
% RSD_H	0.0	0.11	0.11	0.11
Overall Average				99.5
Overall % RSD				**1.24**

2.4.1.3 Precision: Repeatability

2.4.1.3.1 Assay procedure for BIC quantitation in drug product

Regarding the qualification of repeatability for the analysis of drug product samples, the relative standard deviation (%RSD) was suggested to be less than 2%. Here, the %RSD of all the six determinations of extracts from 5:1 BIC:CO formulation was less than 2%, as shown in **Table 14**, confirming the repeatability of this assay procedure for BIC determination in drug product.

Table 14 | Repeatability of method of the assay procedure for BIC quantitation.

Drug product Sample	Average Area (µV*Sec)
1	1186689
2	1192473
3	1227801
4	1229220
5	1246143
6	1234409
Average	1219455
SD	24094
% RSD	1.98

2.4.1.3.2 Method for BIC determination in dissolution study

From the 6 determinations of 5:1 BIC:CO in 1xPBS, the %RSD of the areas from the samples was found to be 0.63%, which was less than 2% (**Table 15**). This also indicated the repeatability of the method for BIC determination in in-vitro drug release studies.

Table 15 | Repeatability of method for BIC determination in dissolution study.

Drug product Sample	Average Area (µV*Sec)
1	43514
2	43312
3	43355
4	43581
5	43218

6	43250
Average	43372
SD	274
% RSD	0.63

2.4.1.4 LOD and LOQ

2.4.1.4.1 Assay procedure for BIC quantitation in drug product

Based on the signal-to-noise (S/N) ratios of a concentration level with 6 injections, the LOD was estimated to be 0.2 µg/mL (S/N ratio = 3.38 ± 0.72), and the LOQ was estimated to be 0.5 µg/mL (S/N ratio = 14.15 ± 2.07) for this assay procedure for BIC quantitation (**Table 16**).

Table 16 | LOD and LOQ of assay procedure for BIC quantitation in drug product.

LOQ (0.5 µg/mL)	**Signal to noise ratio (Average ± SD)**	**LOD (0.2 µg/mL)**	**Signal to noise ratio (Average ± SD)**
6 injections	14.15 ± 2.07	**6 injections**	3.38 ± 0.72

2.4.1.5 Linearity

2.4.1.5.1 Assay procedure for BIC quantitation in drug product

To qualify the linearity of the assay procedure, three linear curves were constructed. All three linear curves showed a regression coefficient (R^2) greater than 0.999, indicating a strong linear relationship between the theoretical concentrations of BIC in 50% methanol and the AUC

(**Table 17** and **Figure 6**). The linearity was verified within a concentration range of 1 µg/mL to 100 µg/mL.

Table 17 | Linearity of assay procedure for BIC quantitation in drug product.

Linearity curve	Regression coefficient (R^2)	Regression equation
1	>0.9999	Y = 12021X – 1630.7
2	>0.9999	Y =12405X – 2336.1
3	>0.9999	Y = 12563X – 824.7

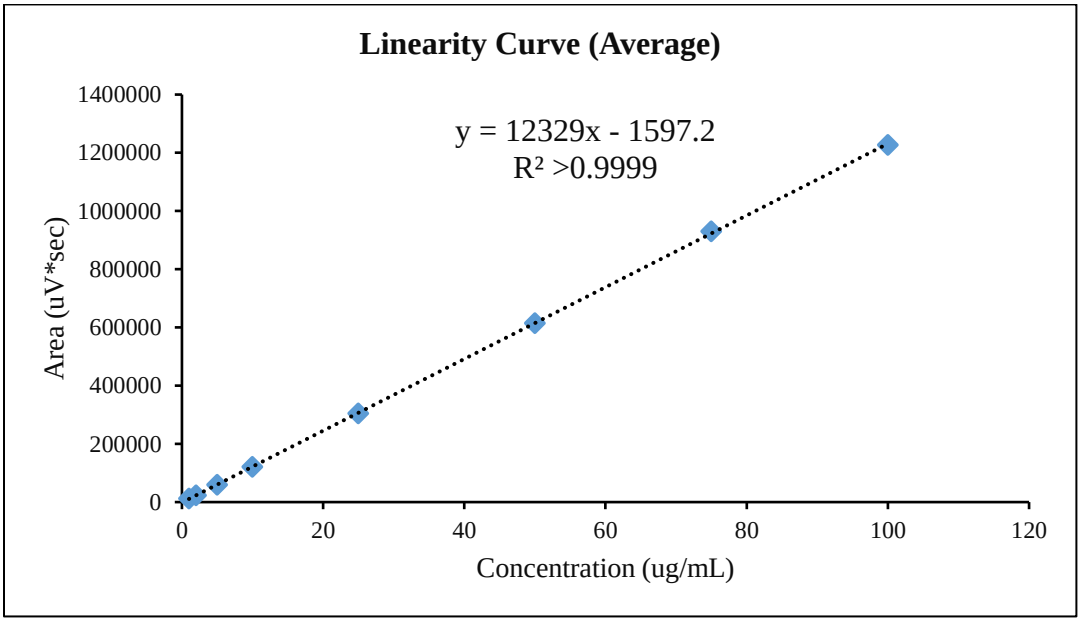


Figure 6 | Linearity of assay procedure for Bictegravir quantitation in drug product.

2.4.1.5.2 Method for BIC determination in dissolution study

In terms of the linearity of the method for BIC determination in dissolution study, all three linear curves demonstrated an R^2 value greater than 0.999, which confirmed a linear correlation between the theoretical concentrations of BIC in 1x PBS and AUC (**Table 18** and **Figure 7**). This relationship was observed within the concentration range of 0.417 µg/mL to 50 µg/mL.

Table 18 | Linearity of method for BIC determination in dissolution study.

Linearity curve	Regression coefficient (R^2)	Regression equation
1	0.9996	Y = 9741.3X – 3326.2
2	0.9997	Y =9561.1X – 4506.1
3	0.9995	Y = 9729.5X – 4127.8

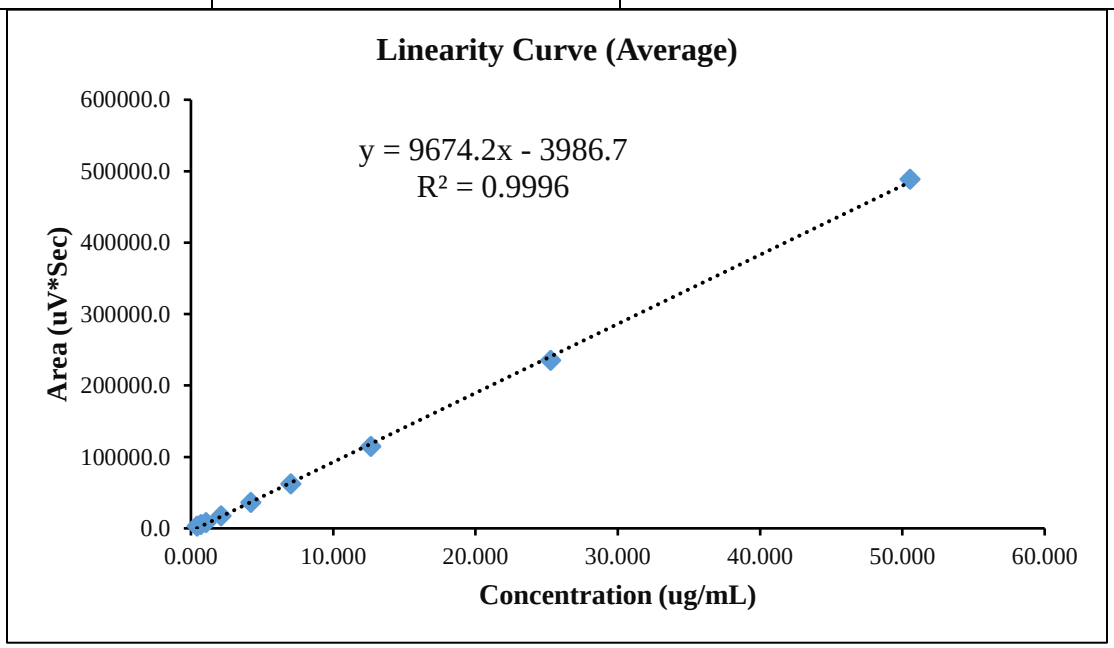


Figure 7 | Linearity of method for BIC determination in dissolution study.

2.4.2 Pre-formulation tests

2.4.2.1 Filtration validation

The % accuracy of the samples with and 5:1 BIC:CO in 50% methanol was within ±2% of theoretical concentration, as presented in **Table 19**, concluding that the syringe filter PTFE (0.22

µm, 13 mm diameter) did not make any impact on samples for the determination of BIC in drug product.

Table 19 | Filtration validation of assay procedure for BIC quantitation.

Volume of filtration	Concentration of BIC in drug product (µg/mL)	% Accuracy
1 mL	50	98.03 ± 0.42
2 mL	50	98.41 ± 0.15

2.4.2.2 Stock solution stability

2.4.2.2.1 Assay procedure for BIC quantitation in drug product

The stability of drug substance stock solution, 500 µg/mL BIC 100% methanol, used in the assay procedure for quantifying BIC in drug products was confirmed for over 2 months, with an accuracy of within ±5% of the nominal concentration, as shown in **Table 20**. In addition, the stability of the drug product solution at three different concentrations was also validated. Analysis of drug product samples after one week showed that the differences in peak areas were all less than 2%, demonstrating the stability of the drug product stock solutions for up to one week (**Table 21**).

Table 20 | Stability of BIC standard stock solution for BIC quantitation in drug product at refrigeration.

Std. concentration (µg/mL)	% Accuracy (After 66 days)
50	103.67 ± 0.35

Table 21 | Stability of BIC drug product solution of assay for BIC quantitation at refrigeration.

Concentration of BIC in drug product (µg/mL)	% Difference of area (After 7 days)

3	-1.4
40	-0.3
80	0.8

2.4.2.2.2 Method for BIC determination in dissolution study

The stability of the BIC drug substance standard stock solution for the method of BIC determination in dissolution study was confirmed for 12 days under refrigeration, with less than 2% of the difference of area, as shown in **Table 22**. However, the stability of the drug product was found to be different at refrigeration temperature and at 37°C. The drug product samples were stable for 5 days at refrigeration temperature, with a % difference of peak area less than 5% compared to fresh solution (**Table 23**), while found to be unstable after just 1 day when stored at 37°C, with the % difference of area more than 10% (**Table 24**).

For the stability of drug product solution in different container materials at 37°C, the % recovery of the peak area at different time points to the peak area of fresh solution drastically decreased in the samples stored in glass and silanized glass vials, while those in PCOO bottles were found to be stable for 7 days, as presented in **Figure 8**. The difference in % recovery might be attributed to nonspecific adsorption of Bictegravir to glass. On the other hand, the drug product in a mixture of 1:1 1xPBS and 100% methanol was confirmed to be stable in both PCOO and glass containers, with almost 100% recovery of the AUC at day 7 compared to fresh solution, as shown in **Figure 9**.

Table 22 | Stability of BIC standard stock solution for BIC determination in dissolution study at refrigeration.

Std. concentration (µg/mL)	% Difference of area (After 12 days)
50	-1.9

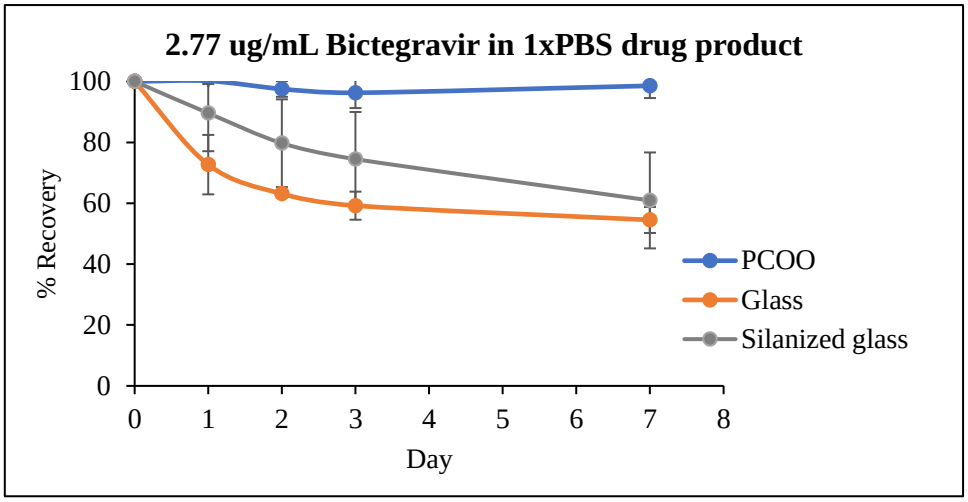
Table 23 | Stability of BIC drug product solution of method for BIC determination in dissolution study at refrigeration.

Concentration of BIC in drug product (µg/mL)	% Difference of area (After 5 days)
2.77	-4.8
10.00	-2.1
20.00	-0.8

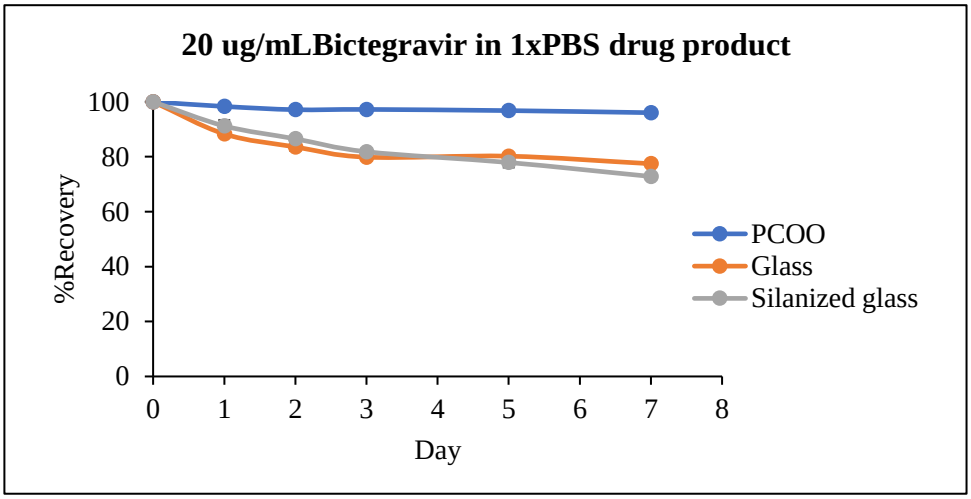
Table 24 | Stability of BIC drug product solution for BIC determination in dissolution study at 37°C.

Concentration of BIC in drug product (µg/mL)	% Difference of area (After 1 day)
2.77	-14.1
10.00	-9.5
20.00	-14.2

(A)


2.77 ug/mL Bictegravir in 1xPBS drug product
% Recovery
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
Day
PCOO
Glass
Silanized glass

(B)


20 ug/mLBictegravir in 1xPBS drug product
%Recovery
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
Day
PCOO
Glass
Silanized glass

(C)

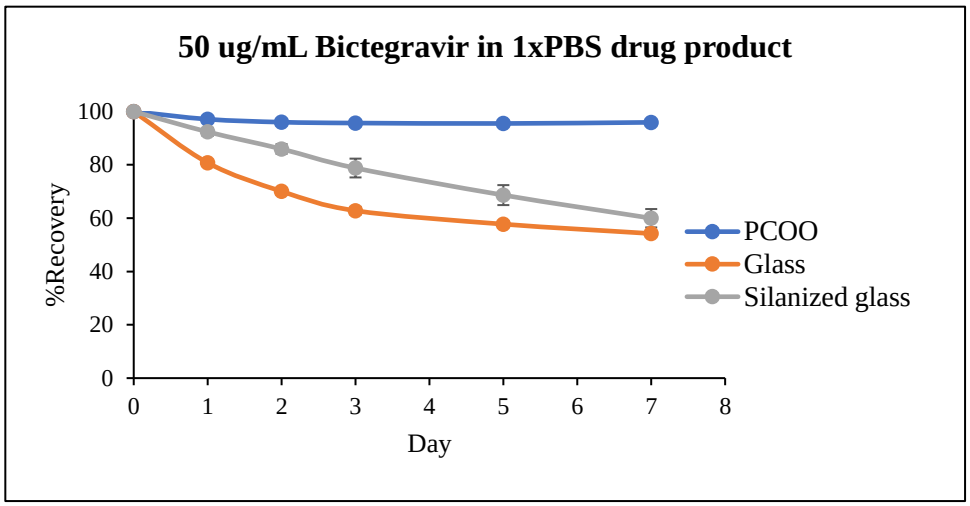


Figure 8 | Stability of different concentrations of drug product solution in 1xPBS for the method for BIC determination in dissolution study

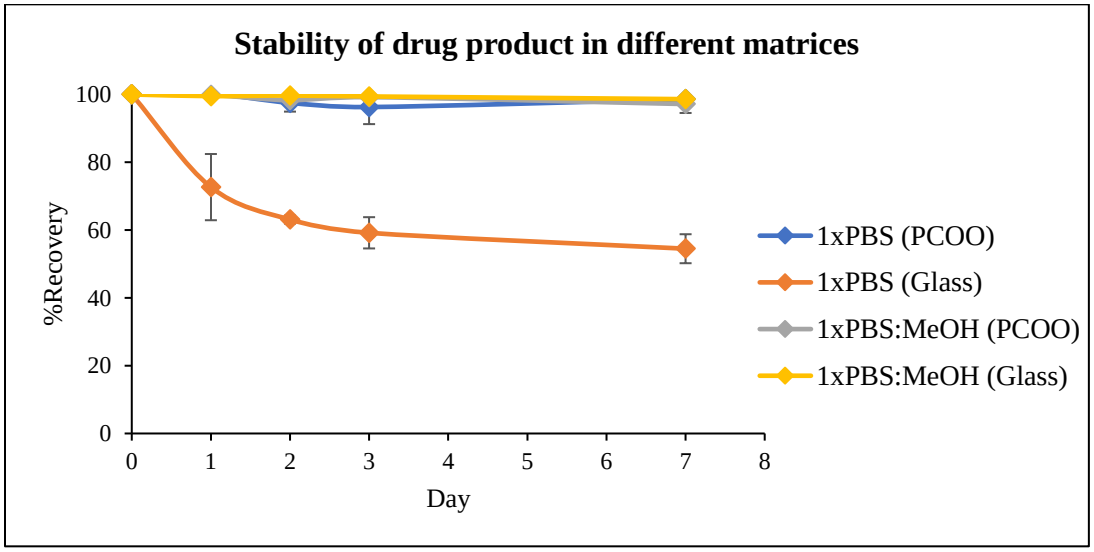


Figure 9 | Stability of drug product solution in different matrices, including 1xPBS and the mixture of 1:1 1xPBS and 100% methanol.

2.5 Discussion

1.1. Qualification of HPLC methods

To conclude, the RP-HPLC methods for the quantitation of BIC in drug products and the determination of BIC in dissolution study have been successfully qualified, which included specificity, accuracy, repeatability, LOD and LOQ, linearity, and concentration range. The assay of BIC quantitation will be used for the characterization of drug product, such as BIC content, content uniformity, and stability assessment. On the other hand, the method for the determination of BIC in dissolution study will be utilized to study the in-vitro drug release profile of BIC-loaded implants. Both HPLC methods showed specificity in analyzing BIC without interference from the presence of castor oil in the drug product. The accuracy and precision of the analysis of drug substance and drug product at the targeted concentrations confirmed the reliability of data generated by the HPLC methods. The methods exhibited linearity within the concentration range of 1 µg/mL to 100 µg/mL BIC in 50% methanol for the quantitation assay, and 0.417 µg/mL to 50 µg/mL BIC in 1x PBS for the determination in dissolution study, which allowed for the quantification of unknown samples by calculating the concentrations based on the established calibration curves. Overall, the qualification of these two HPLC methods ensured that they consistently provide reliable data and established the suitability for analytical purposes, which will be employed in the next chapter.

1.2. Stability of stock solutions

The stock solution for the assay of BIC remained stable at refrigeration temperature for over two months, while the stability of that for BIC determination in dissolution study was found to vary in different conditions. The drug product solution in 1xPBS was stable at refrigeration

temperature for 5 days, while found to be unstable at 37°C. Based on the results obtained, the stability of BIC in 1xPBS drug product solution was compromised at 37°C after just 1 day, with a significant decrease in peak area exceeding a 10% difference. Additionally, a small peak preceded the main BIC peak, which increased with increasing BIC concentrations, indicated the existence of a degradant. Considering the stability study was conducted using scintillation vials, the decrease in peak area could also be caused by the adsorption of BIC in 1xPBS to the glass vials. As BIC is a basic compound with a pKa = 9.81, the adsorption was hypothesized to occur due to the ionic interaction between basic groups and acidic silanol groups and hydrogen bonding[94, 95].

Given that the dissolution study will be performed using PPCO bottles under the condition of 37°C with samples being collected every 3 to 4 days, further investigation for the stability of drug product was conducted in different containers, including PPCO bottles, scintillation vials, silanized glass vials. The drastic decreases in the observed concentrations of samples stored in scintillation vials and silanzied glass vials after 1 day might be attributed to nonspecific adsorption of BIC to glass. Since the dissolution samples would be transferred to glass vials for HPLC analysis, the stability of drug product solutions in an alternative matrix, 1:1 1xPBS and 100% methanol mixture, was further evaluated in glass containers. The results demonstrated that drug product solutions in a matrix containing 100% methanol were stable in glass vials.

In conclusion, to ensure the stability of the dissolution samples during HPLC analysis, it is suggested to dilute them with 100% methanol. The findings from the stock solution stability study provided valuable insights into the suitability of different container materials for maintaining the stability of the drug product solution, thereby facilitating accurate and reliable analysis.

3.0 Cross-Lab Evaluation of Implant Manufacturing Reproducibility

3.1 Introduction

3.1.1 Background

The transfer of pharmaceutical methods and technology between laboratories is a critical aspect of pharmaceutical manufacturing. To ensure consistent performance and effective quality control processes for drug devices, it is important to confirm the reproducibility of manufacturing processes. Therefore, this cross-lab evaluation of implants is conducted to assess the transferability of manufacturing methods from one site to another as well as to ensure that the manufacturing of implants are reproducible, scalable, and can produce products of consistent quality.

In the present study, the objective was to transfer the methods and technology used for the preparation of Bictegravir (BIC)-loaded poly(ε-caprolactone) (PCL) implants from RTI International (Research Triangle Park, NC, USA) to Magee-Womens Research Institute (MWRI, Pittsburgh, PA, USA; our end). In order to scale up the manufacturing of these implants for future animal and clinical studies, a secondary site at MWRI was planned. However, before proceeding with the transfer, a cross-lab comparison was conducted to evaluate the transferability of the techniques used at RTI to MWRI. This cross-lab evaluation of implants involved simulating the conditions during implant preparation in RTI, followed by assessing the drug content, content uniformity, and in-vitro release profile of the implants. These characterizations of the implants

were performed to identify the aspects of the manufacturing process that may be subject to variability of products.

In summary, this study focused on evaluating the transferability of methods and technology for the preparation of BIC-loaded PCL implants from RTI to MWRI. The results of this study would help identify the critical process parameters that need to be monitored to ensure the manufacturing robustness and consistent product quality.

3.1.2 Characterization of 5:1 BIC:CO formulation paste

3.1.2.1 Stability

Stability testing is an essential aspect of the drug development process, particularly in determining the shelf-life of a pharmaceutical formulation. The main purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product changes over time. As per the ICH Guidance for Industry Q1A(R2)- Stability Testing of New Drug Substances and Products, a five percent change in assay from the initial value of a drug product is considered as significant change, indicating the failure of stability. In this study, a stability test of the 5:1 BIC:CO formulation paste was conducted, with a duration based on the period required for implant preparation.

3.1.3 Cross-lab evaluation of implant manufacturing reproducibility

3.1.3.1 Extraction efficiency

An efficient extraction method is critical to assess of drug content and content uniformity of a formulated drug by removing the active pharmaceutical ingredient (API) from the formulation system. The efficiency of an extraction method is referred by the percentage recovery, which represents the known amount of an analyte carried through the sample extraction and processing step of the method[96]. In this study, a criterion of extraction efficiency higher than 95% is established to accurately characterize BIC content and content uniformity of BIC-loaded PCL implants.

3.1.3.2 Drug content and content uniformity

The drug content and content uniformity are critical quality attributes of pharmaceutical formulations that directly impact their safety and efficacy. Content uniformity is an essential test in a therapeutic product specification that assesses the quality of a product within or between batches. The characterization of content uniformity helps ensure that the strength of a product remains within specified acceptance limits[97]. Following the US requirements determined in the USP, the drug content should be within 85% and 115% of label claim, with a %RSD less than 6% for the criteria of content uniformity[98].

In this study, a comparative analysis of the BIC content and content uniformity of BIC-loaded PCL implants produced by both RTI and MWRI was conducted to validate the transfer of the manufacturing technique from RTI to MWRI.

3.1.3.3 In-vitro drug release study

In order to assure the consistent quality and performance of a dosage form, it is important to evaluate the in-vitro release or dissolution of the drug substance. This product performance test evaluates the in-vitro release of a drug substance from a dosage form, which helps assure, or through in-vitro/ in-vivo correlations, document in-vivo performance[99]. According to the FDA Guidance, in-vitro release or dissolution testing can be used to ensure the consistent quality and performance of product or batch.

In this study, an in-vitro release study was conducted to compare the performance of BIC-loaded PCL implants manufactured by both MWRI and RTI. The purpose of this study was to demonstrate the successful transfer of the manufacturing procedure and to conduct a pilot study. The results of this study will help to identify any differences in the in-vitro release profiles of the implants and establish the correlation between different aspects of manufacturing process and product performance.

3.2 Materials

Bictegravir API (Catalog# N16998) with 99.72% purity was manufactured by AstaTech Inc (Bristol, PA, USA), kindly provided by RTI International (Research Triangle Park, NC, USA) and stored in a plastic container sealed at 4°C refrigeration when not in use. Castor oil (Catalog# SR40890) was acquired from Croda International (USA), stored in an Amber glass bottle at room temperature. PC 17 200 µm poly(ε-caprolactone) (PCL) tubes (Catalog# 17481-01) and PCL

pellets (Catalog# 1801002711) were provided by RTI international International (Research Triangle Park, NC, USA) and stored in -20°C freezer when not in use. The heat injection sealer for implant sealing was designed by RTI international and manufactured by the department of engineering at the University of Pittsburgh (Pittsburgh, PA, USA). OPTIMA™ LC-MS grade Acetonitrile and HPLC grade Methanol were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). HPLC grade (≥99.0%) Trifluoroacetic acid was provided by Sigma-Aldrich (Sigma-Aldrich, USA). Phosphate-Buffered Saline (PBS) (10x) pH 7.4 was purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). HPLC grade (≥99.0%) dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (Sigma-Aldrich, USA). 210 mm transparent disposable smartSpatulas® for cage preparation were purchased from Health Care Logistics® (Circleville, OH, USA). 250 mL Lab quality Nalgene™ wide-mouth PPCO bottles with closure were acquired from Thermo Fisher Scientific (Pittsburgh, PA, USA). Milli-Q water was obtained from the Milli-Q® system (Millipore Sigma Advantage 10), installed in the laboratory.

3.3 Methods

3.3.1 HPLC analytical methods

The HPLC analytical methods utilized for the characterization of BIC-loaded PCL implants, as well as the quantitation of BIC in dissolution study, had been qualified and confirmed to be

reliable. Please referred to **Section 2.0 Qualification of HPLC methods** for detailed information of these methods.

3.3.2 Bictegravir and Castor oil paste formulation

3.3.2.1 Preparation of formulation

To prepare the Bictegravir (BIC) and Castor Oil (CO) paste formulation, an accurate amount of BIC base was weighed in a weighing boat. A specific quantity of CO, equivalent to 16.7% w/w of the formulation, was then carefully added to the same weighing boat using a disposable transfer pipette (**Table 25**). The two materials were thoroughly mixed using a disposable spatula until a uniform distribution of the excipient (castor oil) was achieved in the formulation. Then, the entire mixture was transferred into a clean glass mortar and pestle, where it was finely mixed to ensure complete homogeneity of the formulation. This process ensured that the final paste has consistent composition, which was critical for the subsequent manufacturing steps.

Table 25 | 5:1 BIC base : CO formulation.

Ingredient	% w/w	Example (mg)
BIC base	83.3	1000
Castor oil	16.7	200
Total	100.0	1200

3.3.2.2 Characterization of formulation

Two batches of 5:1 BIC:CO formulation paste were prepared for studying the stability of paste. After the drug content and content uniformity of BIC in the formulation paste were confirmed, the two batches were mixed together. The final paste was characterized again and studied for stability.

3.3.2.2.1 Drug content and content uniformity

To analyze the drug content and content uniformity of BIC in the formulation paste, five partitions were taken from different areas of the entire paste. Each partition was weighed in a 20 mL scintillation vial, with a targeted weight of 24 mg, which theoretically contained 20 mg of BIC. For extraction, 16 mL of DMSO was added to the vial to dissolve BIC, with the weight of DMSO recorded. The resulting sample was then vortexed for 5 minutes and sonicated for 15 minutes to, with visual inspection to ensure complete dissolution of BIC in DMSO. The final extract was prepared to achieve 1.25 mg/mL of BIC in DMSO. For HPLC analysis, the extract was diluted with 50% methanol to reach a target concentration of 50 µg/mL of BIC, with the weight of diluent being recorded. After mixing with vortex, the resulting solution was filtered with PTFE (0.22 µm, 13 mm diameter) filter and transferred to HPLC vials for analysis. The HPLC was used to determine the drug content of BIC in formulation, and the %RSD of the five determinations was used to evaluate the content uniformity.

3.3.2.2.2 Stability

To test stability of 5:1 BIC:CO formulation paste, the paste was saved in a 20 mL scintillation vial covered by aluminum foil to prevent light exposure and stored on the bench at

room temperature. The paste was analyzed by HPLC at different time points, from Time 0 to 3 months. The HPLC samples were processed following the sample preparation procedure for drug content and content uniformity. The stability of formulation paste was determined by analyzing the BIC content, which was theoretically 83.3% (w/w) of the formulation.

3.3.3 BIC-loaded PCL subcutaneous implants

3.3.3.1 Preparation of BIC-loaded PCLimplants

3.3.3.1.1 Implant filling

To prepare the PCL implants loaded with a 5:1 BIC:CO formulation paste, the formulation paste, previously prepared and confirmed to be uniform, was filled into the PCL tubes. A stainless steel spatula was utilized to scoop up an amount of the formulation, as shown in **Figure 10**. An empty PCL tube, pre-sealed on one end, was then selected and filled with the formulation. To ensure proper packing, a metal rod was employed to compress the formulation within the PCL tube. This process was repeated until the desired implant length for formulation part or the load of BIC was attained. An implant length of 4 cm for the formulation part was targeted, leaving a headspace of 0.3 cm for the final seal, while a total of 120 mg of BIC loaded to the implant was aimed at, which 144 mg of 5:1 BIC:CO formulation paste might be needed. The headspace was carefully cleaned with a stainless steel rod and a cotton swab before the final sealing.

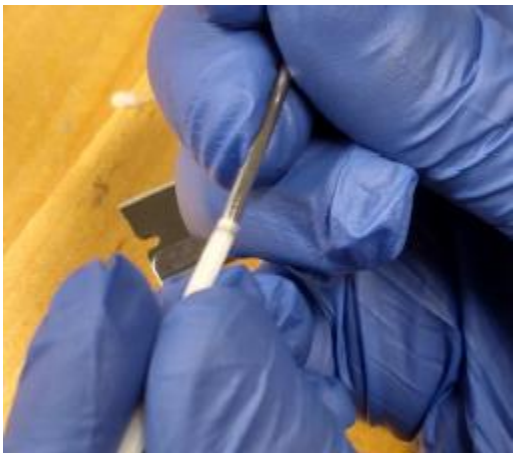


Figure 10 | Procedure of implant filling.

3.3.3.1.2 Implant sealing

To seal the PCL tubes and secure the formulation paste in the implant, a precise and reliable sealing process was employed. First, the PCL tubes were accurately cut to a length of 4.6 cm using a metal jig and a razor blade to remove any excess material. Next, a heat injection sealer was utilized, which was designed by RTI International and manufactured by the Department of Engineering at the University of Pittsburgh. The temperature of the sealer was set at 155°C to ensure effective melting of the PCL pellets. An empty PCL tube was placed onto metal jigs with a 0.3 cm headspace and fitted with a Teflon collar, flush with the top of the tube (**Figure 11 (A)**). PCL pellets were loaded into the heat injection sealer, where they were melted to form the initial sealing material. The Teflon collar on the PCL tube was aligned with the injector nozzle, and the melted PCL in the sealer was then injected into the PCL tube by manually pressing. The PCL was allowed to cool to room temperature and solidify, providing a secure and robust seal. Finally, any

excess PCL around the Teflon collar was carefully trimmed using a razor blade (**Figure 11 (B)**) to ensure a smooth and uniform surface.

To seal the PCL tubes loaded with formulation paste, the headspace of the tube was first cleaned with a stainless steel rod and a thin cotton swab. The same procedure used for sealing the empty PCL tubes was followed. A Teflon collar was fitted, and the tube end was sealed with melted PCL from the heat injection sealer. During the sealing process, the PCL implant itself, instead of the Teflon collar, was held (**Figure 11 (C)**). This method ensured that the formulation paste remained intact and protected inside the PCL implant. Overall, the sealing process plays a crucial role in ensuring the integrity and stability of the PCL implants and prevent the leakage of the formulation paste. The schematic of a final product was shown in **Figure 12**.

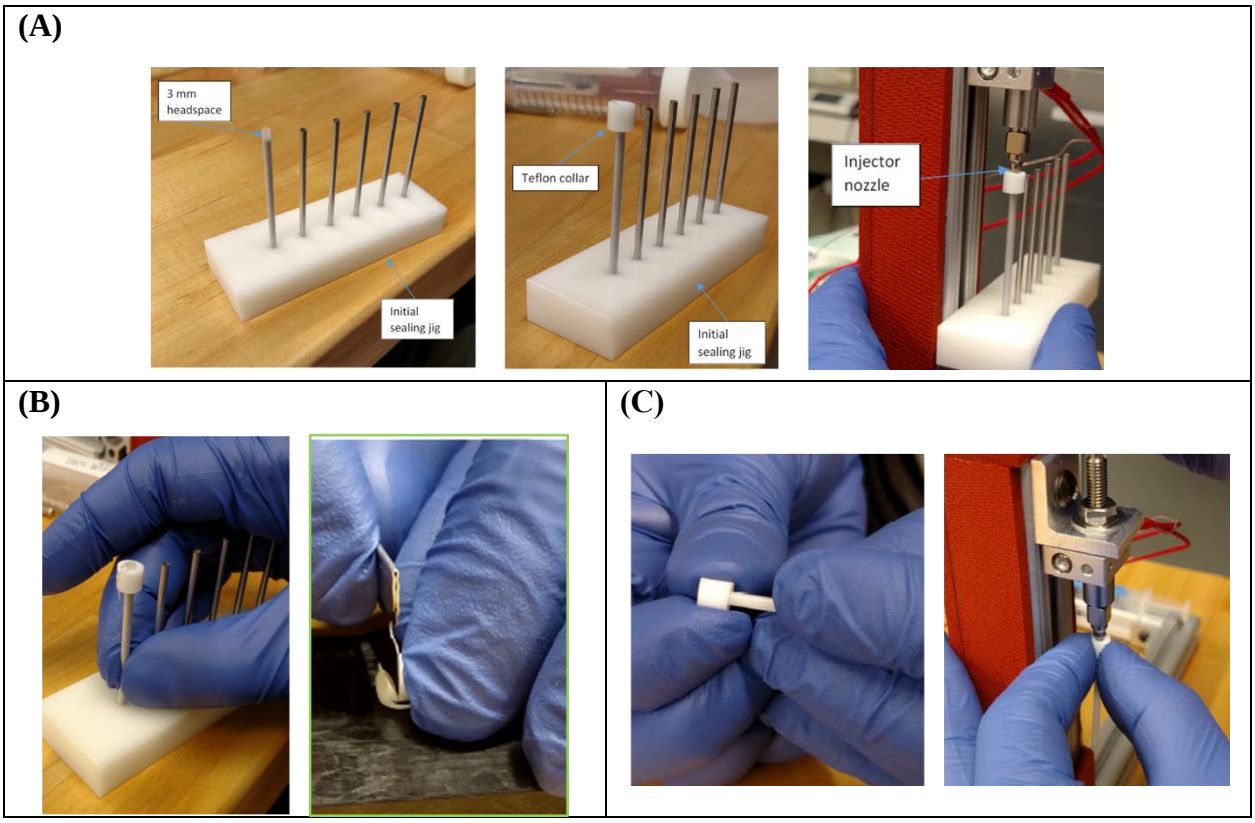


Figure 11 | Procedure of implant sealing.

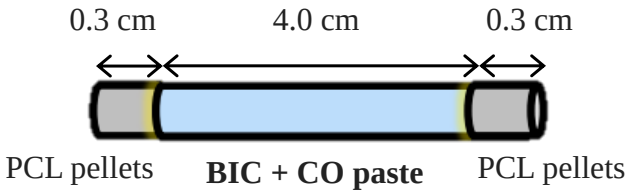


Figure 12 | A schematic of a BIC-loaded PCL implant.

3.3.3.2 Characterization of BIC-loaded PCL implants

3.3.3.2.1 Extraction of BIC from BIC-loaded PCL implants

For the evaluation of the drug content and content uniformity of BIC in PCL implants, an appropriate extraction method was developed. The aim was to extract BIC from BIC-loaded PCL implants to enable its quantification using HPLC.

Two sets of BIC-loaded PCL implants were prepared. Each set consisted of three implants, with each implant being half the length and loaded with half the amount of BIC of the original implants, containing 2 cm of formulation part. One set was loaded with 72 mg of 5:1 BIC:CO formulation paste, which had previously been shown to be uniform. The other set was loaded with a target of 60 mg of BIC API powder and 12 mg of CO separately. The actual weights of loaded formulation paste and BIC API were recorded. Both sets of implants were prepared using the implant filling and sealing procedure.

To extract BIC from the PCL implants, the seals at both ends of the PCL tubes were cut, and the entire implant, including the cut seals, was transferred to a 100 mL glass bottle. Then, 40 mL of DMSO was added, and the solution was vortexed for 5 minutes to ensure that all the formulation paste was removed from the PCL tube. The solution was subsequently sonicated for 15 minutes to dissolve all the contents in DMSO. A targeted 1.5 mg/mL BIC in DMSO extract was finally prepared.

To analyze the extracted BIC using HPLC, 30 µL of the BIC in DMSO solution was diluted with 870 µL of 50% methanol to achieve a 30-fold dilution. The resulting sample was mixed thoroughly by vortex, filtered with a 0.22 µm, 13 mm diameter PTFE filter, and then transferred to HPLC vials for analysis. All the organic solvents were recorded by weights, and the actual

volumes were calculated based on the densities of DMSO being 1.095 g/cm^3 and 50% methanol being 0.9156 g/cm^3.

The percentage recovery of the extracted BIC from the PCL implants was determined by comparing the actual amount of BIC in the implants with the amount of BIC extracted using the developed method. This enabled the evaluation of the effectiveness of the extraction method.

3.3.3.2.2 Drug content (loading capacity) and content uniformity of BIC-loaded PCL implants

Two implants from MWRI and three implants from RTI International were applied for the characterization of drug content and content uniformity. The analytical balance (Mettler Toledo) was used to weigh the whole implants and record their weights. Subsequently, each implant was cut into three approximately equal sections by length using a razor blade, including the seal parts. These sections were then transferred to 100 mL glass vials and weighed individually. To extract BIC from each section, 40 mL of DMSO was added to the glass vial to reach 1 mg/mL BIC in DMSO solution, since there was theoretically 40 mg of BIC in each section (1/3 implant). The procedure for extraction of BIC from BIC-loaded PCL implants was then followed, including vortexing and sonication to completely withdraw formulation paste from PCL tubes and dissolve BIC in DMSO. After extraction, the implant section was removed from the solution and dried under the laboratory hood. Finally, the weights of the dry sections were measured for the mass balance of formulation paste in each section (1/3 implant).

For the determination of the extracted BIC by HPLC assay, 50 µL of the 1 mg/mL BIC in DMSO solution was diluted with 950 µL of 50% methanol to conduct a 20-fold dilution. The resulting sample was mixed thoroughly by vortex, filtered with a 0.22 µm, 13 mm diameter PTFE

filter, and then transferred to HPLC vials for analysis. All the organic solvents were measured by weights, with the actual volumes calculated according to the densities being 1.095 g/cm^3 for DMSO and 0.9156 g/cm^3 for 50% methanol.

3.3.4 In-vitro performance of BIC-loaded PCL implants

3.3.4.1 Dissolution study of implants

The dissolution test of BIC-loaded PCL implants was carried out in 1xPBS buffer (pH = 7.4) at 37°C, with the devices being transferred to fresh buffer at regular intervals to maintain sink conditions. Sink conditions were achieved when the concentration of BIC in 1xPBS did not exceed 1/10 of its solubility, which is about 0.1-0.2 mg/mL. To ensure sink conditions were met, each implant was placed in a 250 mL PCOO bottle containing 200 mL of 1xPBS.

3.3.4.1.1 Preparation of cages

To prevent a BIC-loaded PCL implant from floating during dissolution testing, a cage was used to keep the implant submerged in the buffer. Transparent disposable smartSpatulas® were employed to prepare these cages. One end of the spatula was sealed using an impulse sealer (AIE-100T model), and a 200 µL pipette tip was inserted. The opposite end of the spatula, which was on the end of the pipette tip, was then sealed. Some cuts were made along the length of the spatula to accommodate the implant length, and a BIC-loaded PCL implant was placed inside the spatula

cavity. Lastly, the end of the spatula was sealed, and the remaining part was removed by scissors. The schematic of the design of cage was presented in **Figure 13**.

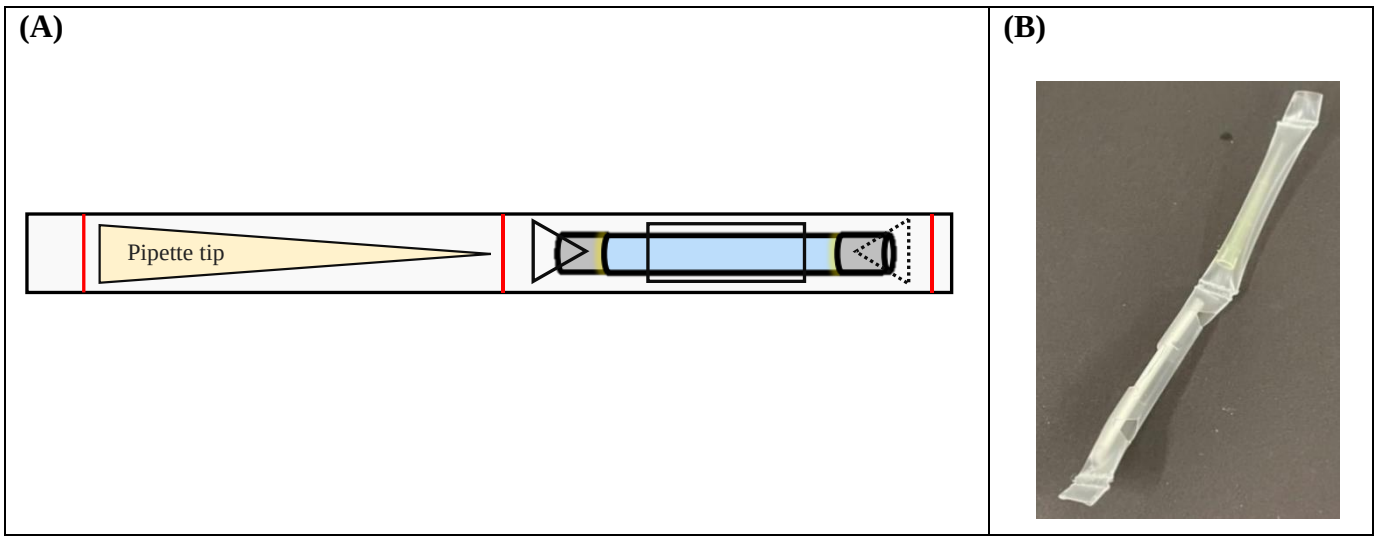


Figure 13 | A schematic of the design of cage for dissolution study (A), and a sample that was actually prepared (B).

3.3.4.1.2 Procedure of release study

To prepare 1xPBS dissolution medium, 10x PBS was diluted with Milli-Q water at a ratio of 1:9 to make a 1x solution. New 250 mL PCOO bottles, with each cap and body being labeled, were filled with 200 mL of the 1xPBS medium and incubated in a VWR® incubating orbital shaker at 37°C for about 30 minutes until the temperature was stabilized. The previously prepared caged implants were submerged in the medium, and the bottles were capped, properly sealed with Parafilm, and placed steadily in the incubating shaker with a rotation speed of 100 rpm.

For dissolution buffer exchange, a new set of 1xPBS buffer-loaded HDPE bottles were prepared, and the implants were transferred to fresh buffer on Monday and Thursday of each week to maintain sink conditions. Clean tweezers, which were rinsed with ethanol and wiped with

Kimwipe before exchanging the next device, were utilized to gently transfer the devices. The bottles were placed back in the incubating shaker to continue the study.

3.3.4.1.3 Analysis of drug release

3.3.4.1.3.1 High performance liquid chromatography (HPLC)

To determine the in-vitro release profile of BIC from BIC-loaded PCL implants, dissolution samples collected on Monday and Thursday of each week were directly analyzed by HPLC. This HPLC system and chromatographic condition were described in the previous chapter, and qualified for specificity, accuracy, precision, LOD and LOQ, linearity, and range. The calibration curve used for the analysis had a BIC concentration range of 0.417 µg/mL to 50 µg/mL. The amount of BIC released was calculated based on this calibration curve.

3.3.4.1.3.2 Ultraviolet-visible (UV-Vis) spectroscopy

To quantify the release of BIC from those BIC-loaded PCL implants, UV-Vis spectroscopy was used in addition to the HPLC method mentioned earlier. The UV-Vis spectroscopy method involved measuring the concentration of BIC in the dissolution samples at a wavelength of 260 nm. A primary stock solution of 50 µg/mL BIC in 1xPBS was prepared by dissolving 10 mg of BIC in 200 mL of 1x PBS. A secondary stock solution of 12.5 µg/mL BIC in 1x PBS was then prepared by diluting 450 µL of the primary stock solution with 1350 µL of 1x PBS. The calibration curve for UV-Vis spectroscopy was established using a range of BIC concentrations between 0.417

μg/mL and 50 μg/mL in 1xPBS. The calibrators were directly diluted from either the primary or secondary stock solution, as shown in **Table 26**.

The calibration curve was plotted with the theoretical concentrations of BIC in 1xPBS versus the UV absorbance, which enabled the quantification of the amount of BIC release by calculating the concentration of BIC in the dissolution samples using the calibration curve. For UV-Vis sample analysis, the dissolution samples were directly analyzed on the same day they were collected, with quality controls (**Table 27**) analyzed before and after to ensure accuracy.

Table 26 | Calibration curve of UV-Vis spectroscopy method for dissolution study of BIC-loaded PCL implants.

Standard solution	BIC concentration (μg/mL)	Concentration of BIC stock solution (μg/mL)	Volume of BIC stock solution (μL)	Volume of diluent: 1x PBS (μL)	Total volume (μL)
1	0.417	12.5	60	1740	1800
2	0.694	12.5	100	1700	1800
3	1.042	12.5	150	1650	1800
4	2.083	50	75	1725	1800
5	4.167	50	150	1650	1800
6	6.944	50	250	1550	1800
7	12.5	50	450	1350	1800
8	25	50	900	900	1800
9	50	50	1800	0	1800

Table 27 | Quality controls of UV-Vis spectroscopy method for dissolution study of BIC-loaded PCL implants.

BIC concentration (µg/mL)	Concentration of BIC stock solution (µg/mL)	Volume of BIC stock solution (µL)	Volume of diluent: 1x PBS (µL)	Total volume (µL)
2.77	50	100	1700	1800
10.00	50	360	1440	1800
20.00	50	720	1080	1800

3.3.4.2 Stability of BIC and CO formulation paste after dissolution study

Following the completion of the 1-month dissolution study of BIC-loaded PCL implants, the drug content and content uniformity of BIC within the implants were analyzed to assess the stability of BIC and CO formulation paste within the PCL tubes. All of the implants were sacrificed in accordance with following the procedure for drug content and content uniformity of BIC-loaded PCL implants. The formulation paste was extracted by DMSO and diluted with 50% methanol for sample preparation, which were then analyzed by the HPLC assay for BIC quantification.

3.3.5 Statistical analysis

Statistical analysis was performed to compare the two groups of BIC-loaded PCL implants prepared by RTI and MWRI. The results were reported as mean ± standard deviation (SD). The unpaired t test was performed using GraphPad Prism version 9.5.0. A p-value <0.05 was considered statistically significant, while ns was used to indicate non-significant differences.

3.4 Results

3.4.1 Characterization of BIC and CO formulation paste

A formulation paste with a 5:1 ratio of BIC to CO was prepared through rigorous mortar and pestle grinding. After being set at room temperature overnight, covered by aluminum foil, the paste's appearance changed from white to a light yellow color (refer to **Figure 14**). In order to assess the quality of the formulation paste, its drug content, content uniformity, and stability were characterized using established methods.

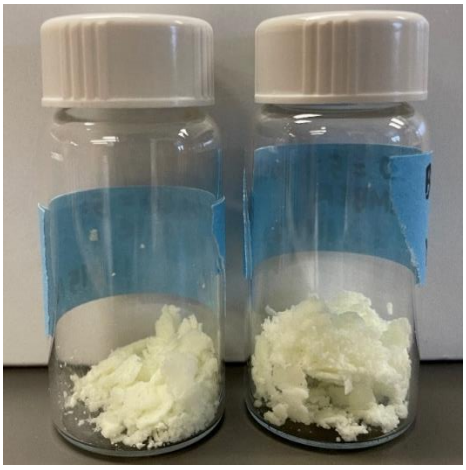


Figure 14 | Appearance of 5:1 BIC:CO formulation paste.

3.4.1.1 Drug content and content uniformity

The drug content and content uniformity of the 5:1 BIC:CO formulation paste was characterized following its preparation. Five partitions were taken from different areas of the

formulation paste to assess the attributes. The average drug content was found to be 86.8%, with a %RSD of 1.6%. This indicates a consistent and uniform distribution of the active pharmaceutical ingredient, BIC, within the formulation paste. Since the targeted label claim of the formulation paste was 83.3% (w/w), the % label claim found to be within 90% to 110% demonstrated that the drug content reached the intended level. In addition, the content uniformity was determined with a %RSD of 1.6%, which was lower than the 2% limit. This confirmed that BIC was uniformly distributed within the formulation paste, indicating a reliable and consistent product (**Table 28**).

Table 28 | Drug content and content uniformity of BIC in 5:1 BIC:CO formulation paste.

Partition	BIC content (%, w/w) in 5:1 BIC:CO formulation paste				Label claim (LC): 83.3% (w/w) BIC			
	Drug content (%, w/w)	Average (%, w/w)	SD	% RSD	% LC	Average (%)	SD	% RSD
1	86.1	86.8	1.4	1.6	103.4	104.2	1.6	1.5
2	85.0				102.0			
3	86.8				104.1			
4	88.2				105.9			
5	87.8				105.4			

3.4.1.2 Stability

The stability study of the 5:1 BIC:CO formulation paste was carried out at room temperature for a period of 84 days, equivalent to 12 weeks. The drug content of the formulation paste was analyzed using HPLC at each time point. The results demonstrated that the formulation

paste consistently met the label claim content of 83.3% (w/w) BIC, with a negligible difference of less than 5% throughout the study period. As a result, the stability of the formulation paste was confirmed for a duration of 12 weeks (**Figure 15**).

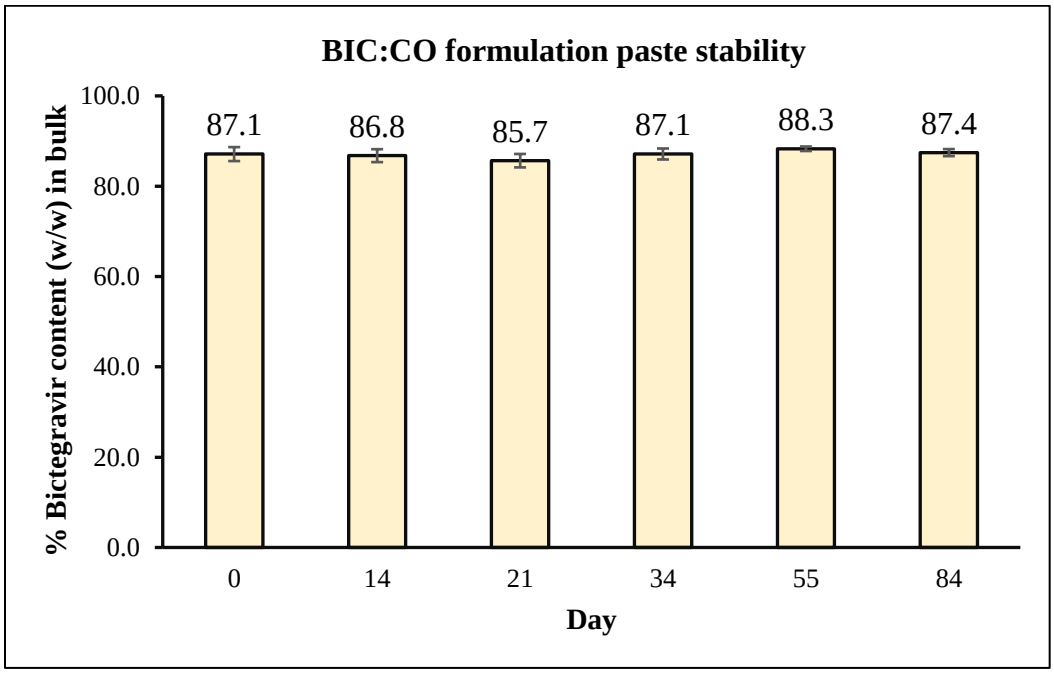


Figure 15 | Stability of the 5:1 BIC:CO formulation paste.

3.4.2 Characterization of BIC-loaded PCL subcutaneous implants

3.4.2.1 Extraction efficiency from BIC-loaded PCL implants

To validate the applicability of our developed extraction method for the characterization of drug content and content uniformity of BIC-loaded PCL implants, the extraction efficiency was analyzed.

For implants loaded with BIC and CO formulation paste, the actual BIC content was determined prior to formulation filling, showing BIC 87.2% (w/w) formulation paste. On the other hand, for implants filled with BIC API and castor oil separately, the actual BIC content was

determined based on the weights of filled API. The %Recovery was calculated as the measured BIC amount by HPLC assay divided by the actual BIC amount in an implant. As shown in **Table 29**, both sets of implants had a %Recovery within the range of 95% to 105%, indicating an extraction efficiency of approximately 100% from BIC-loaded PCL implants using this extraction method. This confirmed that the developed extraction method could be used to accurately determine the drug content and content uniformity of BIC-loaded PCL implants.

Table 29 | Extraction efficiency of BIC-loaded PCL implants using the developed extraction method.

Implant	% Recovery
5:1 BIC:CO formulation paste (n = 3)	102.1 ± 1.5
BIC API + CO (n = 3)	101.1 ± 1.2

3.4.2.2 Drug content (loading capacity) of BIC-loaded PCL implants

In terms of drug content of BIC-loaded PCL implants, the label claim of a BIC-loaded PCL implant was 120 mg of BIC per implant. To determine the accuracy of the label claim, the percentage label claim (%LC) was calculated as the actual BIC amount in a whole implant divided by the label claim. The actual BIC amounts in a whole implant were determined by the HPLC assay, with results being presented in **Table 30**. The average %LC of implants prepared by MWRI was 104.6%, and that of RTI was 100.4%, which indicated the loading capacity of implants produced by both sites achieved the target of 120 mg of BIC in each implant. Besides, the %RSD of the %LC of the three implants produced by RTI was less than 5%, showing a cross-batch content uniformity of BIC in the implants. Overall, the results confirmed that the BIC-loaded PCL implants

prepared by both MWRI and RTI met the targeted loading capacity and demonstrated content uniformity across batches.

Table 30 | Drug content and Cross-batch content uniformity of BIC in BIC-loaded PCL implants from both MWRI and RTI.

Implant		Assay, HPLC	% Label Claim (LC) Label claim: 120 mg BIC/implant			
		Actual BIC amount (mg) in *whole implant*	% LC	Average	SD	% RSD
MWRI	A	124.6	103.8%	104.6%	NA	NA
	B	126.6	105.5%			
RTI	A	117.1	97.6%	100.4%	3.2	3.2
	B	119.8	99.8%			
	C	124.7	103.9%			

3.4.2.3 Content uniformity

To characterize the content uniformity of BIC-loaded PCL implants, the % w/w BIC content of each 1/3 implant section was calculated by dividing the measured BIC amount by the weight of BIC and CO formulation, which was theoretically 83.3% w/w. The measured BIC amount in 1/3 implant was determined by the HPLC assay after the extraction of BIC from each section. The weight of formulation within 1/3 implant was determined by a mass balance method, wherein the weight of the 1/3 implant section including the PCL tube and the formulation paste was measured, and the weight of the 1/3 PCL tube alone, from which the formulation paste had been extracted, was then subtracted.

The %RSD of BIC content % w/w within each section of an implant was found to be less than 2% in most of the implants prepared by MWRI and RTI, demonstrating the content uniformity

of BIC within a single implant. Besides, the BIC content within the implants met the target of 83.3% w/w (**Table 31**). However, one implant from RTI showed only about 74% w/w, which was outside the acceptable range. This discrepancy might be due to the deterioration of the PCL tube caused by DMSO after extraction, as presented in **Figure 16**. The damage to the PCL tube could have led to the loss of PCL tube weight, thereby increasing the weight of formulation and resulting in the lower %w/w of BIC in formulation.

Table 31 | Content uniformity within a single implant, prepared by both MWRI and RTI.

Implant		Section	Assay, HPLC	Mass balance	BIC content %w/w			
			Measured BIC amount (mg) in *1/3 implant*	Weight of formulation (mg) in *1/3 implant* = Section weight – PCL tube weight	% w/w	Avg (% w/w)	SD	**%RSD**
MWRI	13	I	43.4	49.8	87.1%	87.9%	0.7	**0.8**
		II	40.3	45.6	88.4%			
		III	40.9	46.3	88.3%			
	17	I	41.6	47.5	87.5%	87.8%	1.6	**1.9**
		II	41.4	47.9	86.4%			
		III	43.6	48.7	89.6%			
RTI	11	I	37.9	44.3	85.4%	85.9%	0.4	**0.4**
		II	37.2	43.3	86.0%			
		III	42.0	48.7	86.1%			
	12	I	38.2	44.0	86.8%	86.3%	0.4	**0.5**
		II	38.3	44.4	86.4%			
		III	43.2	50.4	85.9%			
	13	I	38.9	53.0	73.3%	73.8%	1.8	**2.5**
		II	39.6	54.7	72.3%			
		III	46.3	61.1	75.8%			

(A)

(B)

Figure 16 | PCL tubes after extraction by DMSO.

3.4.3 In-vitro performance of BIC-loaded PCL implants

3.4.3.1 Analysis of drug release

3.4.3.1.1 HPLC analysis

To determine the in-vitro release of BIC from BIC-loaded PCL implants, the HPLC method for BIC quantification in the dissolution study was used. The standard curves with good linearity used for sample analysis were shown in **Figure 17** and **Table 32**. Based on the HPLC results, the cumulative drug release and daily drug release profiles for the implants produced by RTI and MWRI were plotted, as presented in **Figure 18** and **Table 33**. While there was no significant difference in cumulative drug release, the P-value of the daily release profile was less than 0.0001, demonstrating a significant difference in the in-vitro release of the implants manufactured by the two labs. This difference might be because of the elevating release of BIC from implants by MWRI after 2 weeks of dissolution study.

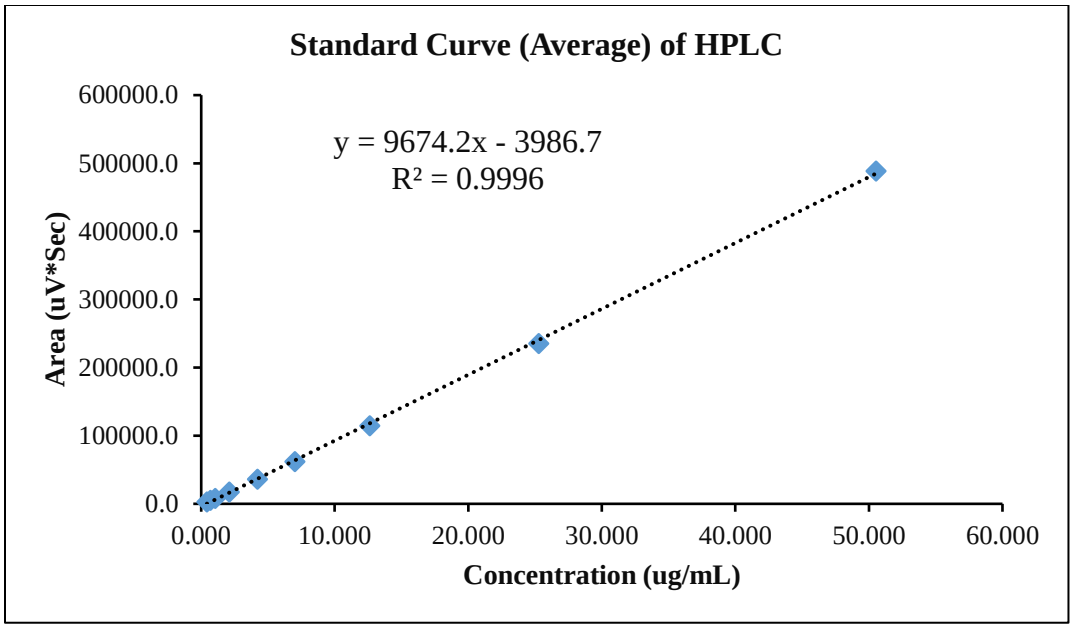


Figure 17 | Average standard curve of HPLC method for BIC determination in dissolution study.

Table 32 | Standard curves prepared for the HPLC analysis of in-vitro release study of BIC-loaded PCL implants.

Time point (Day)	Standard curve
3	Y = 9741.3X – 3326.2 R^2 = 0.9996
7	Y = 9561.1X – 4506.1 R^2 = 0.9997
10	
14	Y = 9729.5X – 4127.8 R^2 = 0.9995
17	
21	Y = 9386.3X – 3459.6 R^2 = 0.9999
24	
28	
31	

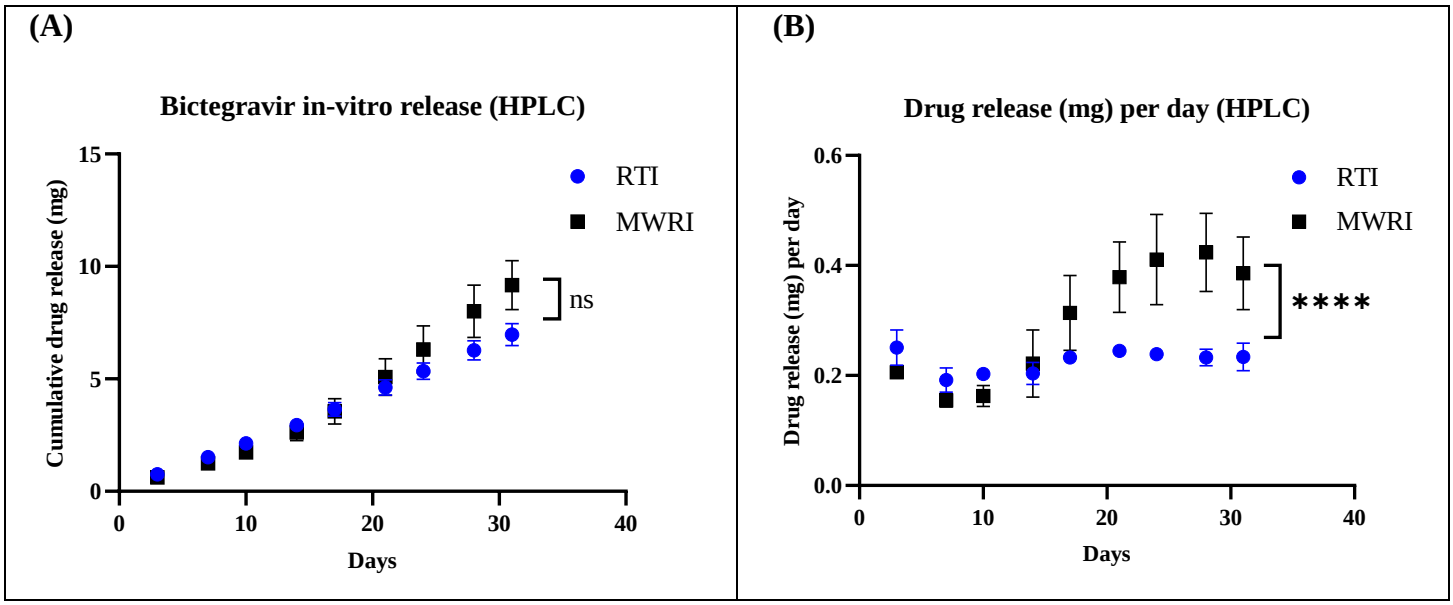


Figure 18 | Cumulative drug release (A) and daily drug release (B) of BIC from BIC-loaded PCL implants analyzed by HPLC.

Table 33 | Cumulative drug release data of BIC-loaded PCL implants by HPLC analysis.

Day	RTI (n = 5)			MWRI (n = 3)		
	Average cumulative drug release (mg)	SD	%RSD	Average cumulative drug release (mg)	SD	%RSD
3	0.753	0.097	12.8	0.619	0.034	5.5
7	1.521	0.185	12.2	1.239	0.082	6.6
10	2.129	0.201	9.5	1.727	0.130	7.5
14	2.943	0.278	9.5	2.616	0.372	14.2
17	3.642	0.299	8.2	3.559	0.564	15.9
21	4.623	0.341	7.4	5.077	0.817	16.1
24	5.339	0.367	6.9	6.309	1.054	16.7
28	6.271	0.426	6.8	8.006	1.161	14.5
31	6.972	0.491	7.0	9.163	1.091	11.9

3.4.3.1.2 UV-Vis analysis

Other than HPLC study, the dissolution samples were immediately analyzed by UV-Vis spectroscopy upon collection. The calibration curve was plotted by the theoretical concentration of BIC versus the corresponding UV absorbance values, and the linearity of the curve was confirmed within the concentration range of 0.417 µg/mL to 50 µg/mL, with a regression coefficient (R^2) greater than 0.999, as presented in **Figure 19** and **Table 34**.

Using the calibration curve, the concentration of the dissolution samples was determined based on their respective absorbance values. The amount of BIC released at different time points was then calculated by multiplying the measured concentration with the volume of the dissolution medium, which was 200 mL of 1xPBS.

Both cumulative and daily drug release profiles were generated, as presented in **Figure 20** and **Table 35**. There was no significant difference observed in cumulative drug release between the implants prepared by RTI and MWRI. However, as the daily release of implants manufactured by MWRI started to increase after 2 weeks, the daily drug release exhibited a significant difference between the two groups, with a P-value of 0.0003, which was less than 0.05.

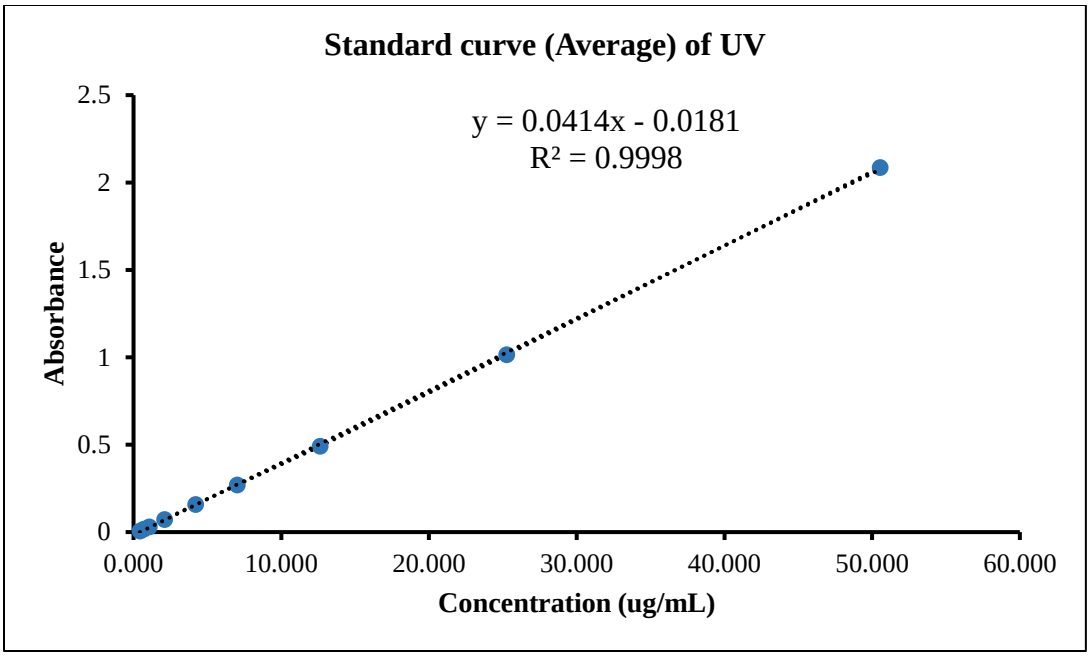


Figure 19 | Average standard curve of UV-Vis analysis for BIC quantification in dissolution study.

Table 34 | Standard curves prepared for the UV-Vis analysis of in-vitro release study of BIC-loaded PCL implants.

Time point (Day)	Standard curve
3	Y = 0.0415X – 0.0301 R² = 0.9999
7	Y = 0.0412X – 0.0109 R² = 0.9998
10	
14	Y = 0.0417X – 0.0154 R² = 0.9998
17	
21	
24	
28	
31	

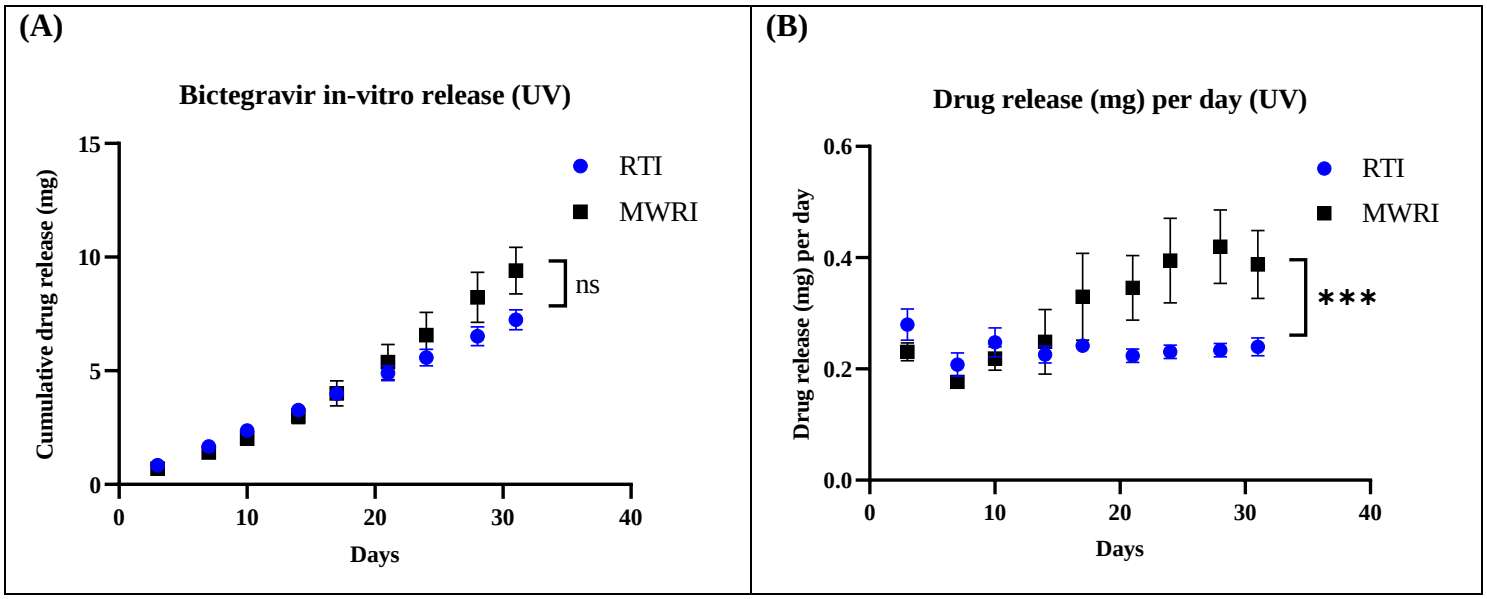


Figure 20 | Cumulative drug release (A) and daily drug release (B) of BIC from BIC-loaded PCL implants analyzed by UV-Vis.

Table 35 | Cumulative drug release data of BIC-loaded PCL implants by UV-Vis analysis.

Day	RTI (n = 5)			MWRI (n = 3)		
	Average cumulative drug release (mg)	SD	%RSD	Average cumulative drug release (mg)	SD	%RSD
3	0.839	0.085	10.2	0.694	0.047	6.8
7	1.671	0.163	9.8	1.402	0.050	3.6
10	2.368	0.205	8.6	2.015	0.094	4.7
14	3.270	0.259	7.9	3.011	0.326	10.8
17	3.996	0.288	7.2	4.000	0.553	13.8
21	4.893	0.328	6.7	5.385	0.781	14.5
24	5.585	0.364	6.5	6.569	1.005	15.3
28	6.520	0.408	6.3	8.236	1.107	13.4
31	7.241	0.444	6.1	9.401	1.025	10.9

3.4.3.1.3 HPLC vs. UV-Vis

While HPLC analysis might be more powerful and sensitive, the 40-minute retention time of the HPLC method for BIC determination in dissolution study could be time-consuming, causing the stability of the dissolution samples to be a concern. In contrast, UV-Vis was faster and straightforward, making it a more cost-effective option than HPLC. To investigate whether UV-Vis could be used as a substitute for HPLC, the cumulative and daily drug release data obtained from HPLC and UV-Vis analysis were compared as the same set of implants was analyzed by both methods. As shown in **Figure 21**, no significant difference was observed between the two methods, indicating the results obtained by HPLC and UV-Vis analysis were comparable. Therefore, UV-Vis could be a valid alternative for HPLC in the analysis of BIC release from BIC-loaded PLC implants.

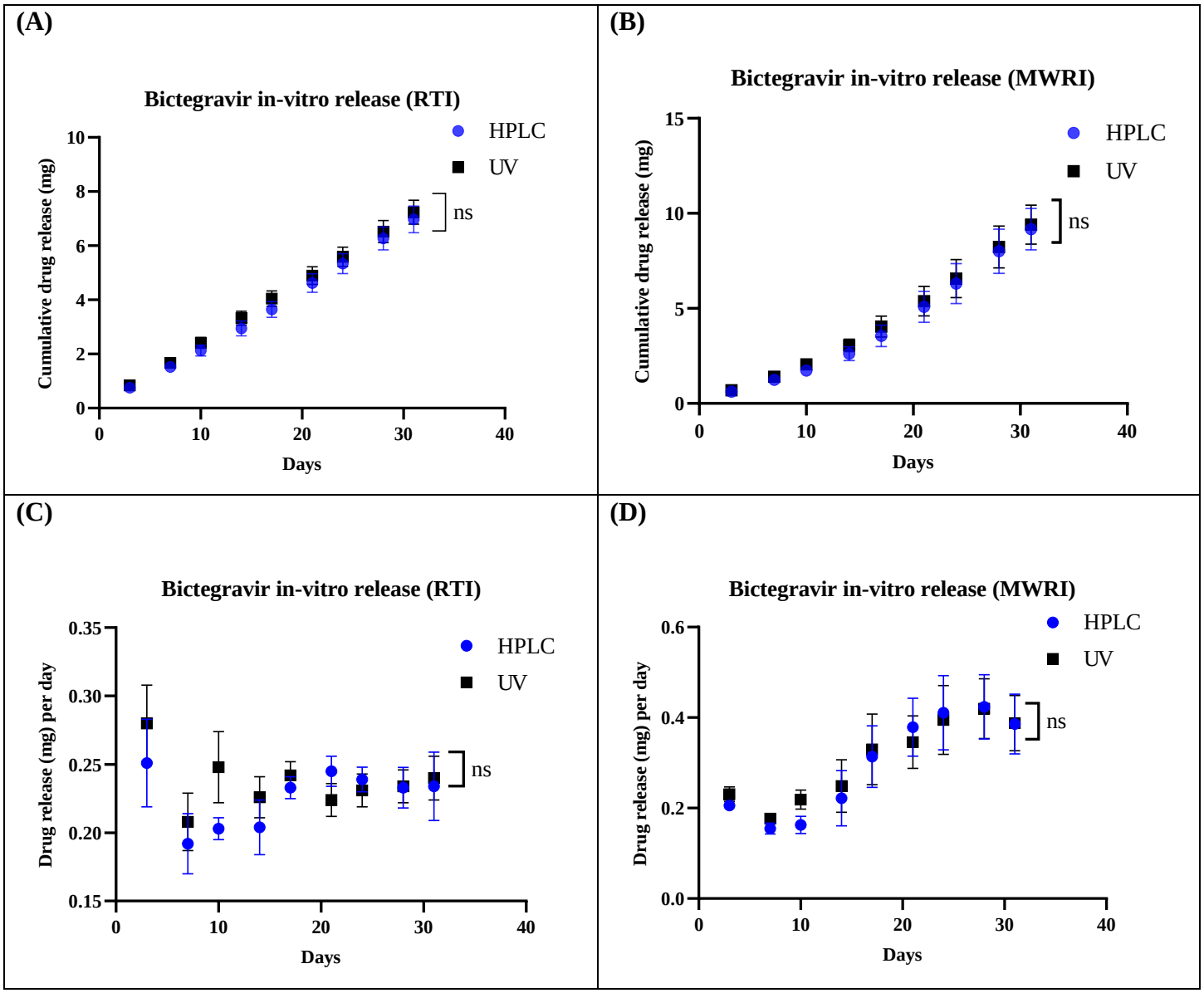


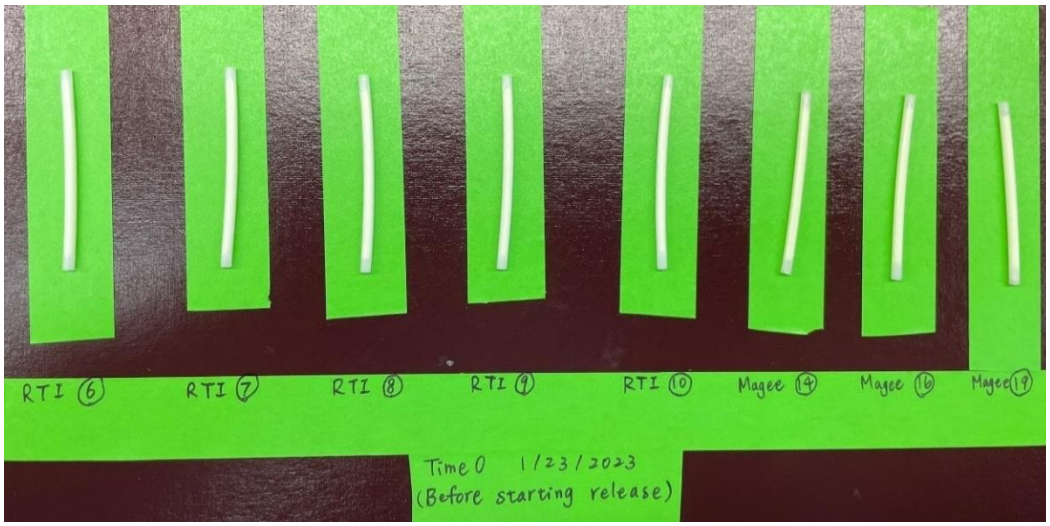
Figure 21 | Comparison of HPLC and UV-Vis analytical methods for determination of BIC release profiles in the dissolution study.

3.4.4 Stability of BIC and CO paste formulation after dissolution study

After the completion of the 31-day dissolution study, visual inspection revealed that the formulation portion of the BIC-loaded PCL implants manufactured by both sites had become yellowish in color, with the implants by MWRI displaying more dark yellow, as shown in **Figure 22**. Therefore, it would be necessary to investigate the stability of the BIC and CO formulation paste within the PCL tubes after dissolution study.

The actual amount of BIC in a whole implant was determined by the addition of the remaining BIC in the PCL implants after dissolution study and the cumulative BIC released during dissolution study. Although the label claim of these implants was 120 mg of BIC per implant, the BIC content within a whole implant was previously determined, with an average of 120.48 mg of BIC in RTI implants and 125.52 mg of BIC in MWRI implants. The %recovery of the amount of BIC to both label claim and the analyzed BIC content was presented in **Table 36**. The average %recovery of BIC from both sets of implants were close to 100%, suggesting that the formulation paste was stable, and there was no significant loss of BIC during the duration of the dissolution study. Moreover, the HPLC analysis of the formulation paste extracted by DMSO and diluted with 50% methanol revealed only the main BIC peak, with no detectable degradant peak, which further confirmed the stability of the formulation paste.

(A)



(B)

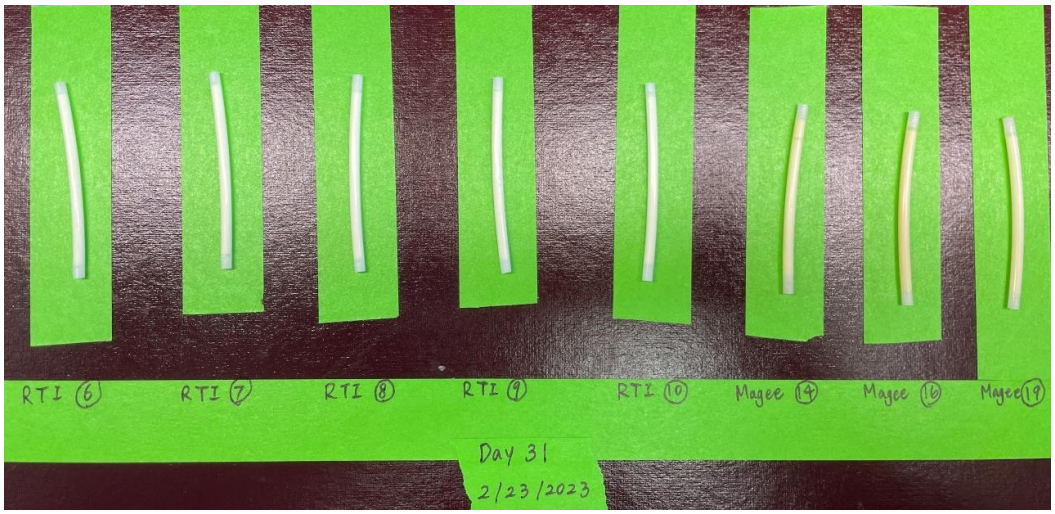


Figure 22 | BIC-loaded PCL implants before (A) and after (B) dissolution study.

Table 36 | Stability of BIC and CO paste formulation after dissolution study.

Implant		Assay, HPLC			Label claim: 120 mg BIC/implant				BIC content			
									RTI implants: 120.48 mg MWRI implants: 125.52 mg			
		Remained BIC (mg)	Total BIC released (mg)	Actual BIC amount in whole implant (mg)	% Recovery	Avg	SD	% RSD	% Recovery	Avg	SD	% RSD
RTI	6	113.0	7.5	120.4	100.4%	100.7%	2.2	2.2	100.0%	100.2%	2.2	2.2
	7	115.9	7.4	123.3	102.8%				102.4%			
	8	110.5	6.7	117.2	97.7%				97.3%			
	9	116.3	6.9	123.3	102.8%				102.3%			
	10	113.3	6.3	119.6	99.7%				99.3%			
MWRI	14	116.2	9.6	125.7	104.8%	103.8%	1.0	1.0	100.2%	99.2%	1.0	1.0
	16	114.5	10.0	124.5	103.8%				99.2%			
	19	115.4	7.9	123.3	102.8%				98.3%			

3.5 Discussion

3.5.1 Cross-lab evaluation of implant manufacturing

3.5.1.1 Characterization of implants

Prior to the implant preparation, 5:1 BIC:CO formulation paste was prepared, within which BIC was found to be uniformly distributed and its stability under room temperature was established for three months using the HPLC assay for BIC determination. The PCL tubes were filled with the formulation paste and sealed with melted PCL pellets to prepare BIC-loaded PCL implants with a length of 4.6 cm implant with 0.3 cm seal ends. To characterize the implants, an extraction method using DMSO was developed to extract BIC from PCL tubes, which was used for to assess drug content and content uniformity of the implants. The label claim (LC) was 120 mg BIC per implant. The average %LC of MWRI and RTI implants demonstrated that the drug content within a whole implant both met the targeted BIC loading capacity. The cross-batch content uniformity of implants was also confirmed based on the %RSD of the %LC, which was less than 5%. Besides, the content uniformity within a single implant was confirmed by the %RSD of BIC content % w/w within each 1/3 implant section. While most implants had about 83.3% w/w BIC in formulation, one implant from RTI showed only about 74% w/w. This might be attributed to PCL tube damage induced by DMSO extraction. To further investigate, the damaged PCL tube will be analyzed with microscopy to evaluate its structural integrity and differential scanning calorimetry (DSC) to assess whether the material structure has been damaged.

3.5.1.2 Difference in drug release of implants from MWRI and RTI

According to the dissolution study, the in-vitro drug release profile of BIC-loaded PCL implants differed between those produced by MWRI and RTI. While the daily drug release from RTI implants showed consistent behavior, the release profile of BIC from MWRI implants demonstrated increasing drug release around 17 to 20 days during the dissolution study, which led to a significant difference between the two sets of implants. Despite the fact that the manufacturing processes conducted at MWRI and RTI were identical, including formulation preparation, implant filling, and implant sealing, the observed difference in the drug release profiles suggested that the sealing integrity of the ends of the PCL implants might be the underlying cause.

The mechanism of drug release of BIC from PCL implants should have been diffusion through the PCL membrane. Our hypothesis was that the sealing technique might not have been executed appropriately, resulting in a possible breach of the sealing. Therefore, extra drug leaked from the defects of the implant sealing, leading to the observed increase in the drug release profile of MWRI implants. It is worth noting that the implants produced by the two laboratories, MWRI and RTI, were visually similar. Besides, the same amount of formulation was placed in a PCL tube measuring 4 cm in length, with sealing ends measuring 0.3 cm. These suggest that the differences in the drug release profiles were merely related to the amount of formulation or physical dimensions of the implants.

3.5.1.3 Formulation discoloration

A time-dependent color change was observed in the BIC and CO formulation paste. The freshly prepared paste exhibited a white color, which turned light yellow overnight. Although this discoloration suggested possible chemical instability, no degradation of BIC was observed.

Moreover, during the dissolution study, the implants produced at both MWRI and RTI displayed a yellowish hue that intensified over time. Notably, the formulation paste prepared by MWRI appeared darker yellow compared to that of RTI in the implants. This discrepancy could be attributed to the longer hold time after manufacturing for MWRI implants, which exceeded over one month, while the RTI implants were freshly prepared. Besides, different storage conditions were employed, with MWRI implants being stored on the bench at room temperature and RTI implants being refrigerated at 4°C.

The observed discoloration is hypothesized to be linked to the oxidation of castor oil in the formulation paste. Previous research has indicated that castor oil is susceptible to oxidation due to the presence of double bonds and hydroxyl groups on its main fatty acid, which are easy to oxidize[100]. The deterioration of castor oil can lead to darkening of its color, which may be caused by the formation of high-molecular-weight oxidant products that contribute to increased light absorption and scattering. Although the formulation paste appeared stable during the dissolution study, a more comprehensive stability study of BIC-loaded PCL implants is necessary to ensure the safety and quality of products.

3.5.2 Conclusion

In this chapter, BIC-loaded PCL implants were prepared at MWRI and then compared with those manufactured at RTI. Various aspects of the implants, including drug content, content uniformity, and in-vitro drug release profiles, were characterized to conduct a cross-lab comparison. This comparison was successfully conducted, demonstrating that both sets of implants

exhibited similar appearance, comparable loading capacity, and uniform distribution of BIC within the implants. However, a notable difference was observed in the in-vitro drug release profiles of the MWRI implants, which showed significantly higher release after a certain period of time. This disparity could potentially be caused by drug leakage from defective sealing of the implants. Therefore, the sealing integrity was identified as a critical processing parameter that may introduce variability of implant manufacturing.

In light of these findings, it can be concluded that strict control and qualification of sealing integrity of implants are essential to ensure reproducibility, scalability, and consistent quality of implant products, if transferring to the next stages.

3.5.3 Limitations of the work

There are some limitations in this work that should be considered during the process of the cross-lab comparison. One limitation is related to the heat injection sealer used in the manufacturing process. The sealer was designed by RTI and manufactured at the Department of Engineering, University of Pittsburgh. While the design specifications were followed, the materials used in the sealer may not be exactly from the same resource or the same supplier, particularly those that were in shortage, as manufacturing occurred at different locations. Therefore, there might have been some differences between the heat injection sealers utilized by MWRI and RTI. Additionally, variations in temperature control of the sealer between the two sites might have occurred, which could impact the sealing integrity of implants based on RTI's previous

experiences. Moreover, a lack of experience for sealing of implant could lead to incomplete sealing integrity.

Another limitation of the study concerns the hold time after manufacturing and storage condition of those BIC-loaded PCL implants for dissolution study, which varied between the two laboratories. The MWRI implants were prepared and held for over one month, while the RTI implants were freshly produced just before the dissolution study. Furthermore, the storage conditions differed, with the MWRI implants being stored on the bench at room temperature, while those at RTI being saved in refrigeration at 4°C. These variations in time duration and storage condition between the two laboratories could have influenced the stability of the implants and consequently affected their drug release profile. In future studies, it is advisable to fill freshly prepared formulation paste to PCL implants, which should then be stored in refrigeration at 2°C to 8°C prior to characterization and dissolution studies.

3.5.4 Future direction: Test for sealing integrity

Based on the observed increasing daily drug release after 2 weeks in MWRI implants during the dissolution study, we have hypothesized that sealing integrity is an important aspect that could impact the release rate of BIC from PCL implants. This may be attributed to potential drug leakage from the PCL tubes. Therefore, it is essential for future studies to develop a method for assessing the sealing integrity of the PCL implants.

Previous research has demonstrated that the sealing integrity of implants can be characterized by incorporating a dry pellet containing 70 kDa isothiocyanate-Dextran (RITC-Dx),

a high molecular weight water-soluble polymer conjugate, into the implant core[101]. The size of RITC-Dx is too large to diffuse through the polymer membrane, but it can be easily detected and quantified through fluorescence. Therefore, the detection of RITC-Dx in the surrounding media indicates the content of implants have passed through defects in the seal, demonstrating compromised sealing integrity. Therefore, it is hypothesized that a gel formulation containing RITC-Dx can be injected to PCL tubes to test sealing integrity. To expedite the study, a mechanical stress test for the full length of implant can also be conducted, which would cause immediate release of the drug and, if included, fluorescent dye to the surrounding fluid.

4.0 Conclusion and next steps

In this thesis, analytical methods were assessed, technology transfer was facilitated, and potential aspects that could impact the reproducibility of the implants were identified. Two RP-HPLC methods for bictegravir (BIC) quantification in drug product and in dissolution studies were qualified. These analytical procedures were deemed reliable and suitable for further assessment of the implants. The stability of the 50% methanol BIC stock solution was confirmed for over one month for assay purposes in refrigeration. The stock solution of 1xPBS with BIC was established as stable for over one week under the same conditions. Because BIC was found to be more stable in 1:1 1xPBS and 100% methanol mixture when stored in glass containers at 37°C, it is suggested to dilute the dissolution samples with 100% methanol during HPLC analysis.

In terms of technology transfer, the long-acting BIC-loaded PCL implants for HIV PrEP were prepared at MWRI following the procedure developed by RTI International. This involved the preparation and characterization of the BIC and castor oil formulation paste, followed by filling the formulation paste into PCL implants and sealing them with melted PCL pellets using a heat injection sealer. Various parameters of the implants from MWRI and RTI were characterized and compared. An efficient extraction method was developed to assess the drug load of a single implant and evaluate the content uniformity within an individual implant and between implants. The in-vitro release profiles were determined through a 1-month dissolution study, in which the implants were caged and placed in PPCO bottles loaded with 1xPBS (pH = 7.4) at 37°C on an orbital shaker with 100 rpm. The observed differences in drug release profiles between the two sets of implants

in the cross-lab comparison highlighted the importance of sealing integrity as a critical processing parameter for ensuring the reproducibility, scalability, and quality of implants.

4.0.1 Next Steps

Based on previous studies conducted by RTI, the insights regarding the administration of a biodegradable implant from end users in African countries demonstrated people's high desire for long-acting PrEP implants, particularly if the dosing could be extended and implant placement could be minimally detected[102, 103]. Additionally, the safety and efficacy of a subcutaneous biodegradable PCL implant were confirmed in macaques[104]. These indicated the potential and feasibility of applying BIC-loaded PCL implant for HIV prevention.

However, the current drug content of the BIC-loaded PCL implants is predicted as insufficient for long-term efficacy required for HIV prevention. Therefore, the next step of this study aims to increase the dosage of BIC in PCL implants. One potential approach to achieve this is through the utilization of hot melt extrusion (HME). HME is a continuous pharmaceutical process that can improve the solubility and bioavailability of poorly water-soluble drugs, such as BIC (BCS class II), by producing amorphous solid dispersions.

The HME technique involves mixing the active pharmaceutical ingredients (API) and polymer by pumping them with a rotating screw at temperatures above their glass transition temperature (Tg), and sometimes above the melting temperature (Tm), to achieve molecular-level mixing[105]. This process converts the components into an amorphous state with uniform shape and density, thereby increasing the solubility and dissolution profile of drugs.

Previous studies have successfully employed HME to manufacture drug-loaded implants, such as praziquantel loaded PEG/PCL implants and raloxifene hydrochloride-loaded subdermal implants[106]. The Tm and Tg of PCL are 60°C and -60°C, respectively, while the Tm of BIC is

higher than 130°C. Therefore, it is feasible to use HME technique for the preparation of BIC-loaded PCL implants. Other physicochemical properties of the polymer, such as melt viscosity, solubilization capacity, and mechanical properties, may need to be screened before the application of HME[107].

It is hypothesized the HME technique can be utilized to increase the loading of Bictegravir in implants for effective HIV prevention. Two potential methods can be conducted: Firstly, a matrix (monolithic)-style drug delivery system could be produced by extruding a mixture BIC and PCL. Alternatively, BIC extruded pellets could be filled into a PCL tube to manufacture a as a reservoir-style BIC-loaded PCL implant.

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